Molecular Epidemiology of Cutaneous Leishmaniasis from Azad Jammu and Kashmir, Pakistan and Plants Based Control

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بسم الله الرحمن الرحيم

DECLARATION

I hereby declare that the work presented in the following thesis "Molecular Epidemiology of Cutaneous Leishmaniasis from Azad Jammu and Kashmir, Pakistan and Plants Based Control" is my own effort, except where otherwise acknowledged, and that the thesis is my own composition and no part has been copied from any other source. Also, it is declared that the present work has not been submitted to any other university.

Nargis Shaheen

Dedicated

To

My Grand Father (Late)

Captain Muhammad Said Khan

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Nargis Shaheen

Contents

List of Abbreviations

ABER	Annual blood examination rate
API	Annual parasitic rate
CL	Cutaneous leishmanaisis
FTIR	Fourier transform infrared spectroscopy
RFLP	Restriction fragment length polymorphism
SPR	Slide positivity rate
GC-MS	Gas chromatography mass spectroscopy
Hrs	Hours
IC	Inhibitory concentration
ITS	Internal transcribed spacer
LC	Lethal concentration
µg	Microgram
Min.	Minutes
Ml	Milli litter
N	Number of samples
Nm	Nano meter
PCR	Polymerase chain reaction
Sec	Seconds
SI	Selective index
TFC	Total flavonoid content
TPC	Total phenolic content
FRP	Ferric reducing power

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GENERAL ABSTRACT

Cutaneous leishmaniasis (CL) is a neglected tropical disease worldwide especially in the various rural areas of Pakistan including Azad Jammu and Kashmir (AJK). The leishmaniasis is prevalent in 102 countries/areas worldwide. Approximately, it effected 350 million people worldwide. *Leishmania* identification on species level is imperative to determine the clinical prognosis and to choose the most suitable therapeutic regimen. The chemotherapy has been used for *Leishmania* infection including amphotericin B, miltefosine, liposomal amphotericin, paromomycin, and pentavalent antimonials. However, several reports have been published regarding the limit of these drugs due to side effects, low efficacy, toxicity, increasing frequency of drug resistance, high cost, and the long period of treatments. The Leishmanial vaccines may not become available in the near future; the search for better drugs should be continued. To identify new treatments, natural products can provide an infinite source of chemical diversity. New drugs should be non-toxic, cheap, more efficient, and easily available especially for the low-income population. Now a day, a special focus is on medicinal plants used against CL. The bioactive phytocompounds present in the plant derivatives can be a good source for discovering new antileishmanial medicines. In the current study, we conducted one active surveys in 2018 to investigate the molecular epidemiology of CL infection and *Leishmania* spp. identification in Azad Jammu and Kashmir. We have conducted another epidemological survey in continue study, to see trend in two years 2018 and 2019. The genetic diversity of two *Leishmania* species (*Leishmania major* and *Leishmania tropica*) were also determined in 2019 under studied population. The most prevalent species of (*L. tropica*) was cultured for evaluation of antileishmanial activity. For this purpose, we have conducted a survey for the leaves collection of medicinal important plants (*Pyrus pashia* (*Rosacea*), *Malus pumila (Rosacea), Prunus persica (Rosacea), Pyrus communis (Rosacea), Prunus armeniaca (Rosacea*), *Ficus glomerata (Moraceae),* and *Diospyros lotus* (*Ebenacea*) from AJK. We subjected the powder form of collected leaves to soxhelet apparatus for preparation of ethanolic crude extract. The ethanolic crude extract was subjected for phytochemical screening, cytotoxicity bioassays, and *in-vitro* antileishmanail activity against *L. tropica*. The most bioactive plant extract was subjected to column chromatography and thin layer chromatography for fraction isolation. All isolated fraction extracts were subjected for

antileishmanial activity of promastigotes and amastigotes of *L. tropica* and cytotoxicity. The most effective fraction was characterized by UV-Vis spectroscopy, FTIF, and GC-MS analysis. Subsequently, all identified compounds in feaction (F7) were analyzed for their bioactivity by *in-silico* molecular docking study. The current study revealed that more cases of CL in 2018 and 2019 were observed from Mirpur followed by Kotli and Muzaffarabad. The findings of 2018 and 2019 revealed that the highest monthly number of CL isolates in the summer were observed followed by spring, autumn, and winter respectively. Individual with age between 1-20 years of age were more likely infected with CL than others (21-40 and 41-60 years old) in both years. Most of the inhabitants were not getting any treatment nor used bed nets in 2018. Among all *Leishmania* spp. only one spp, (*L. tropica*) was observed in 2018 while three species (*L. tropica, L. major,* and *L. infantum*) were identified in 2019 survey. The inter-genetic diversity of *L. tropica* showed significant value (p=0.02) when comparing with *L. tropica* strains obtained from different distrcits of AJK. The *L. major* showed significant value (p=0.05) when comparing *L. major* starins with other *L. major* strains in 2019 survey. The leaves extract of *Pyrus pashia* (*Rosacea*), *Malus pumila (Rosacea), Prunus persica (Rosacea), Pyrus communis (Rosacea), Prunus armeniaca (Rosacea*), *Ficus glomerata (Moraceae),* and *Diospyros lotus* (*Ebenacea*) showed the presence of various phytochemical compounds (saponins, terpenoids, flavonoids, alkaloids phenols, tannins, and coumarins)by phytochemical analysis. Among all plant ethanolic extracts *P. armeniaca* showed highest Total Phenolic Content (279.62±5.40µgGAE/mgDW) and Total Flavonoid Content (205.70±2.41µgQA/mg DW), along with highest antioxidant activities (120.37±4.90µgAAE/mgDW) and Ferric Reducing Power values (278.71±1.03µgAAE/mgDW). All plant extracts showed cytotoxicity in the safety range >1000µg/ml except *F. glomerata* having LC_{50} values of 454.34µg/ml. The ethanolic leaves extract of *P. armeniaca* showed significant antileishmanial activity (IC_{50} 16.18µg/ml) against promastigotes of *L. tropica*. Being the most effective crude extract, 142 fractions of *P. armeniaca* were isolated chromatography and then further 12 fractions were obtained by TLC. Out of all fraction extracts (F2-F12), fraction (F7) showed significantly the highest antipromastigotes activity with IC_{50}11.48±0.82µg/ml compared with standard drug (amphoterecine B) used as positive control (i.e. 11.60±0.70. The amastigotes of *L. tropica*

showed IC_{50} 21.03±0.98µg/ml compared with control 22.03±1.02µg/ml. The UV-Vis spectroscopic analysis of bioactive fraction (F7) revealed the presence of six peaks at 665.00, 607.00, 536.00, 505.50, 359.00, and 321.00nm with the absorption of 0.276, 0.085, 0.097, 0.109, 0.897, and 1.369 respectively. The FTIR spectra revealed the presence of alkane, aldehyde, carboxylic acid, thiols, alkynes, and carbonyls compounds from fraction F7. The GC-MS analysis of ethanolic fraction (F7) showed the presence of 9 compounds i.e (1) benzeneethanol, alpha, beta dimethyl, (2) carbazic acid, 3-(1 propylbutylidene)-, ethyl ester, (3) 1, 2-benzenedicarboxylic acid, diisooctyl ester, (4) benzeneethanamine a-methyl, (5) 2aminononadecane, (6) 2-heptanamine-5-methyl, (7) cyclobutanol, (8) cyclopropyl carbine, and (9) nitric acid, nonyl ester. Subsequently, all (n=9) identified compounds were analyzed for their bioactivity by *in-silico* molecular docking study. Among all compounds, the 1, 2-benzenedicarboxylic acid, diisooctyl ester bound well to the PTR1 receptor. The fraction (F7) showed acceptable results with no cytotoxicity. It can be used for curing skin lesions of CL caused by *L. tropica*. However, *in-vivo* and *in-vitro* studies of the compound i.e. 1, 2-benzenedicarboxylic acid, diisooctyl ester required in the future. The antibacterial activity of 1, 2-benzenedicarboxylic acid, diisooctyl ester compound reported previously (Ajoke *et al*., 2014). Moreover, *P. armeniaca* is effortlessly accessible in different areas of Pakistan along with AJK, and inexpensive. Current findings can aid to constitute an effective alternative to side free drugs. The compound 1, 2-benzenedicarboxylic acid, diisooctyl ester is commercially available could be used in the treatment of skin lesions of CL caused by *L. tropica.*

GENERAL INTRODUCTION

1.1.Leishmaniaisis

Leishmaniasis is one of the neglected tropical diseases, (NTDs) out of seventeen known parasitic infections. The NTDs are prevalent in tropical and subtropical underdeveloped countries (WHO, 2010) and Leishmaniasis ranked 4th among NTDs. (Hakkour *et al*., 2016). Over the last decade, *Leishmania* infection is the second main cause of illness and deaths (Bern *et al.,* 2008). Worldwide it is prevalent in 102 countries/areas (Uzun *et al.,* 2018) and affects approximately 1.5-2 million pesople, while 350 million are at risk the of *Leishmania* pathogenesis (Souza *et al.,* 2018; WHO, 2018; Torres Guerrero *et al*., 2017). The causative agent of leishmaniasis are parasite protozoan of the genus *Leishmania* and are transferred via vector sandfly belongs to two genera *Lutzomyia* and *Phlebotomus* (Tiuman *et al*., 2011; Kamhawi, 2000).

Three clinical forms of leishmaniasis have been reported concerning parasite location in the infected tissues i.e. Visceral leishmaniasis (VL) which is a less common and it causes spleen and liver destruction and is lethal, if does not receive timely treatment; Cutaneous leishmaniasis (CL), which affects only localized skin parts; and mucocutaneous leishmaniasis (MCL), which has the ability of mucus tissue destruction (Bifeld and Cols, 2015; Akhoundi *et al*., 2016). About 0.7-1.2 million CL and 0.2-0.4 million VL cases are reported annually. Approximately CL cases (90%) are spread across 3 main areas i.e. (a) Syria, Afghanistan, Saudia Arabia, and Iran (b) Tunisia and Algeria (c) Peru and Brazil (Alvar *et al*., 2012; Alvar *et al.,* and WHO, 2012). CL is characterized by severe lesions on the skin and long-lasting scars on the infected part (WHO, 2014). Currently, 54 *Leishmania* spp. are known and twenty-one are humans pathogenic (Akhoundi *et al.,* 2016) (Table. 1.1)

Table 1.1: *Leishmania* spp. vector, reservoir host, and causing human disease

Sr.#	***Leishmania* spp.**	**Infection**	**Reservoir host**	**Vector**
1	*L. tropica*	CL, VL, MCL	Humans	*P. sergenti*
2	*L. major*	CL, ML	Gerbils, rodent	*P. papatasi, P. duboscqi*
3	*L. aethiopica*	CL, DCL, MCL	Hyraxes	*L. lomgipes, P. pedifer*
4	*L. donovani*	VL, PKDL	Rodent, humans	*P. martini, P. orinntalis, P. argetenipes*
5	*L. infantum*	CL,VL, ML	Foxes, dogs, jackals, rodent	*P.arias, p. pcrmiciosufi, .longipalpis*
6	*L. chagasi*	VL	Oposums, dogs, foxes	*L.longipalpis*
7	*L. mexicana*	CL, DCL, MCL	Rodent	*L. olmeca*
8	*L. amazonensis*	CL, DCL,	Rodent	*L. flaviscutellata*
9	*L. braziliensis*	CL, MCL	Rodent, anteaters	*Lutzomyiaspp.*
10	*L. guyanensis*	CL, MCL	Sloths, anteaters	*L. umbratilis*
11	*L. panamensis*	CL, MCL	Sloths	*L. tropidoictal*
12	*L. peruviana*	MCL	Dogs	*L. verucarum, L. pvmenis*
13	*L. pifanoi*	CL	Rodents	*Lu. Olmeca bicolor, Lu. flaviscutellata*
14	*L. venezuelensis*	CL	Rodents	*Lu. Rangeliana, Lu. Olmeca bicolor*
15	*L. garnhami*	CL	Marsupials	*Lu. Youngi*
16	*L. naiffi*	CL	Edentates	*Lu. Ayrozai, Lu. Paraensis*
17	*L. shawi*	CL	Mokkeys, edentates	*Lu. Whitmani*
18	*L. colombiensis*	CL	Edentates	*Lu. Hartmanni, Lu. Gomezi*

19	*L. lainsoni*	CL	Rodent	*Lu. Velascoi, Lu. Ubiquitalis*
20	*L. lindenbergi*	CL	Wild animals	*Lu. Antunesi*
21	*L. killicki*	CL	Rodents	*P. sergenti*

DCL: Diffuse Cutaneous Ceishmaniasis; L: *Lutzomyia*; PKDL: Post Kala-azar Dermal Leishmaniasis; ML: Mucosal Leishmaniasis; P: *Phlebotomus* (Oryan, 2015).

Out of twenty-one *Leishmania* spp., two species have been recognized in Pakistan for CL. *L. tropica* has the widest distribution and is highly prevalent in urban areas (Qureshi *et al*., 2016). In the Southern dry region of countries, *L. major* was seen more often (Katakura, 2009). It is stated by several studies that, the unclear association was found among the skin lesions and leishmaniasis type, generally refereeing to the factors of host and the immunoinflammatory response is important in the type of lesion and severity assessment (Katakura, 2009; Marco *et al*., 2006; Rahman and Bari, 2006; Murray *et al*., 2005). Similarly, no correlation between CL lesion type and *Leishmania* parasites were observed during sequencing of gene investigation on isolates from Baluchistan and Sindh (Myint *et al*., 2008). *L. major* is a main type of parasite in lowland regions and *L. tropica* is in high land areas. *L. tropica* is most commonly found in Multan and other investigations also with a small sample size from different country parts (Ayub *et al.,* 2003; Rab *et al.,* 1997). The clinical presentations of CL vary in different prevalent areas (Murray *et al*., 2005). Devastating epidemics of the CL have also been described from all provinces of Pakistan Sindh, Punjab, Khyber Pakhtunkhawa (KP), and Baluchistan along with Azad Jammu and Kashmir (AJK) (Noor & Hussain, 2017; Kakarsulemankhel, 2004; Talat *et al.,* 2014). In 1935, the first case of leishmaniasis was reported from Baluchistan following an earthquake. The geographical zones of AJK are hit by leishmaniasis such as KP (Qureshi *et al.,* 2016; Noor & Hussain, 2017), Punjab (Iftikhar *et al.,* 2003; Kakarsulemankhel, 2004), Gilgit Baltistan (Jaffarany and Haroon, 1992; Ayub *et al.,* 2003) and India (Aara *et al.,* 2013). Previously, the Mirpur district of AJK revealed an unusual increase in the CL cases from 2011-2014 (Mughal *et al.,* 2014).

The geographic distribution of sandfly vector habitat is disturbing by various activities e.g. deforestation and agricultural activities (Firdous *et al*., 2009; Kassi *et al*., 2008). Animal breeding also plays a vital role along with ground cracks and dark niches, in providing appropriate habitat for the sand flies (Ayub *et al*., 2001; Katakura, 2009). Most patients reported for CL are from Uthal, Quetta, and Ormara areas of Baluchistan (Shakila *et al*., 2006). The highly increasing cases of CL are reported infection are due to people belonging to refugees from the neighboring country torn war of Afghanistan

migrated (1980s and 1990s) (Kassi and Kasi, 2005). The increasing CL cases are reported in some areas of the country. In rural areas, CL is currently unknown and possibly underestimated (Kassi *et al*., 2004). One example is that the study based on survey highest cases of school-going children. Due to socio-culture factors, the proportion of affected females may not be entirely known (Kakarsulemankhel, 2004).

1.2.*Leishmania* Life Cycle

Despite variations in the geographical distribution caused by various *Leishmania* spp*.,* all species have the same life cycle between a mammal and an infected vector of genus *Phlebotomus.* The spectrum and outcome of diseases are directly linked with host and parasite. The cycle starts when the infective vector takes a blood meal and injects the infective stage of the parasite (promastigote) from their proboscis. The motile flagellated promastigotes released in the blood get phagocytosed reticuloendothelial cells (macrophages or mononuclear). The promastigotes internalized and lost their flagellum and then modify into non-motile intracellular amastigotes. It is replicated by binary fission, macrophages ruptured and spreading to uninfected cells (Miller *et al*., 1999; Bonecchi *et al*., 1998). After taking the blood meal, the sandfly becomes infected via ingesting amastigote infected reticuloendothelial cells. The amastigotes turn into infected procyclical promastigotes in the vector (sandfly). Within vectors, the developmental stages of the parasite are strongly affected by increasing pH and decreasing temperature (Sallusto *et al*., 1998; Liew *et al*., 1990).

The CL causes skin sores that develop within a few weeks or months after infection and can changes appearance and size over time (Hundson *et al*., 2003). The key CL causative agent in Kenya and Africa are *L. aethiopica, L. major*, and *L. tropica* (James *et al*., 2006). The etiological agents of CL include *L. tropica, L. major* in central Asia, Africa, and the Middle East while *L. mexicana* and *L. braziliensis* complex cause CL in South America.

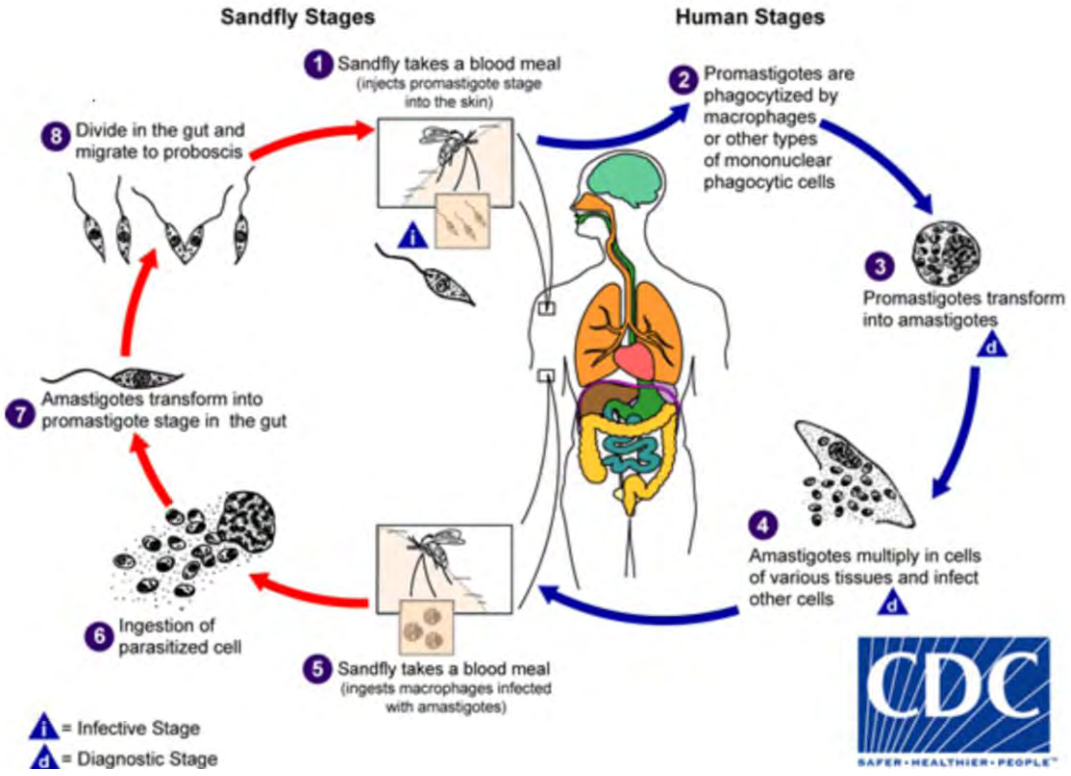


Fig.1.1: The *Leishmania* parasite life cycle comprising *Phlebotomus* fly and mammal (Stenger *et al*., 1994; Sacks and Noben Trauth, 2002).

After an incubation period (2-6 weeks), the CL lesion initiates as a papul**e.** After papule enlargement crust develops in it. A shallow ulcer is exposed when the crust falls. The nodular ulcer remains for 6-12 months before experiencing spontaneous resolution; if the ulcer is left untreated then a depressed scar is left. The *Leishmania* infection due *to L. tropica* or *L. major* is impossible to differentiate on the clinical level because both species erupt similarly. The size of the lesion ranges from a few mm to 4cm. The *L. major* usually present more than 3 lesions (Klaus and Frankenburg, 1999; Al Jawabreh *et al*., 2004). The irregular manifestation in the case of *L. major* are lymph nodes enlargement and the infection spread deeply into muscles and subcutaneous tissues (Al-Gindan *et al*., 1989; Vardy *et al*., 1993). One more clinical form of *L. major* is a sporotrichosis pattern and hyperkeratotic type (Kubba *et al*., 1987). The CL due to *L. tropica* (called *Leishmania*

recidivans) showed wet papules around or in the scars of the primary lesion which slowly spread into the *Leishmania recidivans* (Klaus and Frankenburg, 1999 : Sacks *et al.,* 1999).

1.3.Diagnosis

The methods for CL diagnosis have ranged from clinical imaging, data based on epidemiology, detection of amastigotes in stained smear by using microscopy, or *in-vitro* parasite culturing (Herwalt, 1999; Reed, 1996). These conventional methods have shown many disadvantages such as less sensitivity, need high expertise, and being unable to identify *Leishmania* spp. The *Leishmania* spp. vary in pathogenicity and required various therapeutic regimes and control strategies. To overcome these limitations, focused on polymerase chain reaction (PCR) based techniques were introduced and proved excellent in specificity and sensitivity (Osman *et al.,* 1998). The protocols based on PCR were considered best than conventional methods, even though some of the investigations report contradictory findings (Sharquie *et al*., 2002; Sundar and Chakravarty, 2012). These discrepancies might be due to sampling methods and different standards being used to define CL cases. Various methods used for the classification, identification, and characterization of *Leishmania* are geographical classification such as new world versus old world and clinical and epidemiological criteria. Then advanced criteria began starting with stereotyping (Schnur *et al*., 1972), isoenzyme analysis, and monoclonal antibodies. Then molecular-based techniques were introduced e.g. Southern hybridization/kDNA and nuclear DNA. Fingerprinting and PCR-based fingerprinting with non-specific primers (El-Tai *et al*., 2001). These approaches are unable to discriminate the parasite on the species level. When these techniques are performed in different laboratories, these methods are not easy to compare. The MEE (Multilocus Enzyme Electrophoresis) is a reference method for species differentiation and categorization. The disadvantage of these techniques is that it is expensive, laborious, and slow. Further need for culture (*in-vitro*) makes it inappropriate for large-scale analysis at different laboratories (Jamjoom *et al*., 2002).

For large-scale research, DBS (dried blood spots) are supposed to be a preferable option to traditional vein punctures for blood collection (Fajardo *et al.,* 2014; Ostler *et al.,*

2014; McDade, 2014; Hijjawi *et al.,* 2016). The DBS is a simple, low-cost method for archiving, collecting, and transporting has encouraged the researchers to investigate better DNA extraction and PCR based techniques (Makowski *et al*., 1995 1995; Catsburg *et al.,* 2007; Sorensen *et al.,* 2007; Hollegaard *et al.,* 2013; Bensoussan *et al.,* 2006). The PCR techniques i.e ITS-PCR was specific to identify the *Leishmania* parasites at the species level. The ITS1 (internal transcribed spacer) exists between 5.8S rRNA and 18S rRNA genes (Schonian *et al*., 2003). To be a *Leishmania* target, this gene has high conservation, and its polymorphism allows the typing of species. Sequencing is more extensively used to discriminate and identify species variation of parasites (Cantacessi *et al*., 2015). The PCR-RFLP (restriction fragment length polymorphism) is a preferred technique that identifies the DNA fragment patterns by digestion using a restriction enzyme and the band sizes visualized by gel electrophoresis. At the sites of restriction, the nucleotide option caused DNA fragments and was applied as markers on the linage map of genetics. For *Leishmania* spp., the length difference in the patterns and number of fragments may be used (Marfurt *et al*., 2003; Akhoundi *et al*., 2013), mainly useful for ITS1 (Schonian *et al*., 2003).

1.4.Genetic Diversity of *Leishmania* Parasite

The pathogens formed by a range of genetic variants are more prone to transfer infection into many hosts (Woolhouse *et al*., 2001). Several studies have been conducted previously that explained the polymorphism in the natural population of different *Leishmania* spp. (Schonian *et al*., 2001; Mauricio *et al*., 2001; Cupolillo *et al*., 1998). There is no enough information available on parasitic genetic diversity and the connection with ecoepidemiological disease appearance (Guerbouj *et al*., 2001). Several DNA markers such as rRNA genes, microsatellites, the gp63 gene locus, mini-exon, antigen gene, extracellular DNA e.g., minicircles of kinetoplast DNA, ribosomal internal transcribed spacer (ITS) regions have been previously used (Yurchenko *et al*., 2000 to differentiate the parasite on the species level, heterogeneity of the variable regions was exploited (Noyes *et al*., 1998). *L. major* has been reported by using numerous DNA markers for genetic variation investigations in Iran, e.g., ITS PCR-RFLP, PCR-RFLP based on 3 regions ITS1, PCR-

RFLP of the largest subunit RNA polymerase 11, single-strand conformation polymorphism, RPOIILLS & DPOLB (mitochondrial DNA polymerase beta gene), and sequencing of rDNA ITS. The 18S, ITS1, and 5.8S rDNA of ribosomal DNA has been previously used for the identification of *L. major* heterogeneity in various regions.

1.5.Therapeutic Approaches and their Limitations

Antileishmanial drugs play a vital role in curing leishmaniasis (Alvar *et al*., 2006). The 1st molecule used against leishmaniasis was trivalent antimony. Since 1940, the 1st line drugs are pentavalent antimony, sodium stibogluconate, and N methyl glucantime. The various side effects of these drugs at early anaphylactic treatment have been reported e.g. rash, muscle pain, hyperthermia, bleeding, and tachycardia. At the end of treatment many other side effects like cardiac, hepatic, renal, hematological, and pancreatic, disorders are reported (Dedet, 1999; Roberts *et al*., 2009, Matoussi *et al*., 2007; Masmoudi *et al*., 2006). The treatment duration ranged from twenty to twenty-eight days by intramuscular or intravenous administration (Berman *et al.,* 2003). The problems arise when resistance to this drug has been established by leishmanial strain (Lira *et al*., 1999; Sunder, 2001; Grogl *et al*., 1992). The appearance of parasitic resistance depends on various factors such as elimination of the pharmacokinetic drug, biochemical differences, patient's immune system, *Leishmania* spp. the structural level that is responsible for drug selective response (Croft *et al*., 2006). Lastly, treatment access is difficult due to poverty that affects the population of developing countries. This condition significantly facilitates the disease progression. The drugs like pentamidine and amphotericin B come as the 2nd line of treatment. The amphotericin B inhibits the demethylation of lanosterol by disrupting the membrane permeability. It is generally used against all types of leishmaniasis, effective with high cure rates administered intravenous infusion, has a toxic effect on hematology composition, amp B isome are less toxic and are highly effective only in VL patients, but it is too much expensive (Dedet, 1999; Berman *et al.,* 2003; Thakur *et al*., 1996; Guerin *et al*., 2002). The pentamidine inhibits the parasite DNA synthesis via blocking the thymidine synthetase and tRNA binding. Slow infusion and the inherent toxic effect of the dose reaching the pancreas, kidney, and blood lineages are used for administration (Dedet,

1999). Paromomycin is widely used as an antibiotic with no severe side effects. The results of the phase iv investigation in India showed that paromomycin leads to side effects like edema, itching, tenderness, and erythema. The reports of effectiveness are confused by the natural healing of CL, due to mixed results. The successful treatment shows a slight advantage for placebo and is less effective than pentavalent (Robert *et al*., 2009). The imiquimod drugs in combination with antimony showed to reduce the healing period for patients with CL infection (Croft *et al*., 2006; Arevalo *et al*., 2001). In Pakistan, glucantime is widely used for the treatment of CL and available as a registered drug from April 2018 up to now in the government hospitals. Glucantime is provided by WHO on an irregular basis. As a result, antimonial products are available in local markets with unknown quality and high cost and are regularly administered by unqualified staff (Kamink *et al*., 2019). Glucantime reported various side effects such as abdominal pain, fever, nausea, muscle pain, cardiomyopathy, pancreatitis, nephropathy, and hepatic complications (Ayub *et al*., 2003; Bailey and Lockwood, 2007; WHO, 2014; Afghan *et al*., 2011; Bhutoo *et al*., 2003; Kassi *et al*., 2008; Bailey and Mahaffee, 2017; Karami *et al.*, 2013).

1.6. Natural Methods

However, the exploration for novel drugs is not gaining interest because it required 12-15 years and cost more than $1.0 billion to discover, distinguished, and develop novel agents against *Leishmania* parasites (Hughes *et al*., 2011). In this aspect, the development of new, cost-effective, less toxic therapeutics and more accessible compounds is urgently needed as those recognized in natural plants (Lage *et al*., 2013). As most of the effects of leishmaniasis belong to the low economic level so the use of expensive drugs is far from such people (Oryan *et al*., 2014). Until now, no successful vaccine has been reported against *Leishmania* parasites and no safe chemical eradication for vector control is provided in developing countries (Lahlou *et al.,* 2019; Bouyahya *et al*., 2017). Therefore, people seek cheaper and readily available herbal therapy. The natural product is a precious source of novel products with the highest biological activity (Cheuk *et al*., 2016). Plant pharmacological assessment provides an attractive and good source for safe and novel medical plant growth. It is necessary to check the cytotoxicity of selected plants to improve

safety. For the assessment of toxicity determination, brine toxicity and MTT assay are considered as rapid, reliable, and low cost (Olila and Opuda-Asibo*,* 2001).

Various investigations on antileishmanial activities on medicinal plants have been summarized in Table 1.2. For the evaluation of antileishmanial activities, different criteria are considered i.e. minimum inhibitory concentration (MIC), drug concentration that causes 50% growth inhibition (IC_{50}) in addition to selective index (Mahmoudvand *et al*., 2014; Monzote *et al.,* 2014). The selective index greater than 10 showed the safety of antileishmanial compounds or drugs (Mahmoudvand *et al*., 2014).

Although several techniques e.g. phytochemical analysis have been used to estimate and determine the presence of bioactive substances in plant extracts, for example, flavonoids, alkaloids, tannins, steroids, phenolic, and terpenoids compounds, but chromatographic, and spectroscopic techniques are the most popular and useful tools used for this purpose. The Fourier Transformed Infrared (FTIR) spectroscopy has become a quick method to recognize and characterized functional groups (Grube *et al*., 2008) allowing the analysis of structural and compositional information in plants. For qualitative research and distinguishing certain compound groups, ultraviolet-visible spectroscopy can be used. The natural compounds can be identified by UV-Vis spectroscopy (Kemp, 1991). GC-MS technique is widely used to identify the bioactive constituents of hydrocarbons, acids, alkaloids, alcohols, steroids, nitro, and amino compounds. The GC and MS associated with specific detection techniques have become a sophisticated means for various compound analyses (Vinodh *et al*., 2013; Jabir *et al*., 2010).

The process starts with the collection of the desired plants which is subjected to the extraction process e.g. using soxhlet apparatus. The complex crude extracts are analysed to various phytochemicals and biological assays. The bioactive plant extracts are fractionated using Column chromatography. The fractions are then subjected to various bioassays to find active fractions. The active fraction is subjected to several techniques for the identification of Phytochemicals. This is a low cost and simple way to increase the chance to discover bioactive compounds. Due to poor pharmacokinetic properties, many

drugs are failed to enter markets which increases huge losses to pharmaceutical companies (Fang *et al*., 2018). The latest drug discovery tool is based on a computer-aided tool. This can be extended to the screening of phytochemical drugs present in different medicinal plants (Sliwoski *et al*., 2014). The most known predictive tools are computational prediction models, had a critical role in the selection of pharmacological research methodologies, and have also been applied *in-silico* pharmacological output prediction (Loza-Mejia *et al*., 2018). Now a days, molecular docking is reliable process for drug designing and research. This approach offers knowledge on drug-receptor interaction that is very helpful in predicting the binding orientation of drug candidates on their target protein (Lindoso *et al.,* 2012).

Table 1.2: Plants used against various *Leishmania* spp. reported from previous studies.

Scientific name	Part used	*Leishmania* spp. Tested	Reference
Croton pullei var. glabrior	Stem, bark	*L. amazonensis*	Guimaraes *et al.,* 2010
Valcriana wallichii Dc	Root	*L. major, L. donovani*	Ghosh *et al*., 2011
Cymbopogon citrates and citral	Aerial	*L. infantum*, *L.major*	Machado *et al*., 2012
Calophyllum brasiliense	Leaf	*L. amazonensis*	Tiuman *et al*., 2012
Curcuma longa Linn.	Turmeric	*L. major*	Fouladvand *et al.,* 2013
Strychnos pseudoquina	Stem,	*L. amazonensis*	Lage *et al*., 2013
Physalis angulate	Stem	*L. amazonensis*, *L. braziliensis*	Nogueira *et al*., 2012
Senna spectabilis	Flower	*L. major*	de Albuquerque Melo *et al.,* 2014
Olea europeaea	Leaf	*L. amazonensis, L. donovani*, *L. major*	Sifaoui *et al*., 2014
Bixa Orellana	Seed	*L. amazonensis*	Monzote *et al.,* 2014
Berberis vulgaris L.	Bark, root	*L. majorL. major*	Salehabadi *et al*., 2014
Lippia sidoides Cham	Aerial parts	*L. amazonensis*	de Medeiros *et al.,* 2011
Galipea longiflora	Bark	*L. amazonensis*	Calla-Magarinos, 2012
Moringa stenopetala	Root	*L. aethiopica*	Bekele *et al*., 2013

Azad Jammu and Kashmir is known as a global center of plant diversity (Shinwari, 2010). Approximately, eighty percent of angiosperms species are confined to Kashmir and Western mountains (Nasir *et al.,* 1972; Ali *et al*., 1978; Ali, 2008). Various studies have been conducted on phytosociological, ethnobotanical studies of Pakistan and Kashmir but due to inaccessibility because of climate variations and vastness, several areas are still unexplored. Trar Khel is one of the areas which received the least attention of researchers. Topographically it is a mountainous area and is a moist temperate Himalaya. It needs special attention for environmental conservation sustainable development of natural sources (Haq *et al*., 2010). No study has been conducted in AJK against the *Leishmania* parasite. A wide range of pharmacological effects of *Pyrus pashia* (*Rosacea*), *Malus pumila* (*Rosacea*), *Prunus persica (Rosacea), Pyrus communis* (*Rosacea*), *Prunus armeniaca* (*Rosacea)*, *Ficus glomerata (Moraceae,)* and *Diospyros lotus* (*Ebenacea*) has been reported previously i.e. *P. armeniaca* has been reported such as antioxidant (Guclu *et al.,* 2006), antimicrobial activities (Schor and White*,* 2010), against inflammation (Minaiyan *et al.,* 2014; Madrar *et al.,* 2009), and anti asthamic (Erdogan and Kartal, 2011). Minaiyan *et al.,* in (2014) reported that *P. armeniaca* has been used in many parasitic diseases. Biological activates of *P. pashia* were also previously described by Guven *et al.,* (2006). Murugan *et al.,* (2014) reported that *P. communis* has a strong role in wound healing and anti-inflammatory. The *M. pumila* and *P. persica* have anti-inflammatory (Hyson, 2011; Edrah *et al*., 2015; Kashyap *et al.,* 2015)*, D. lotus* used as antiseptic, antitumor, and antidiabetic (Uddin *et al.,* 2011, 2014) activities. The *F. glomerata* showed antidiabetic, anti-inflammatory, antiulcer, antidiuritic, antidiarrheal, antiasthmatic, and antipyretic (Jagtapsupriya *et al*., 2012; Kambali *et al*., 2014). *P. armeniaca* (family rosaceae) is an important medical plant born in North Western Himalayas (valleys of Kashmir, Shimla, Uttarakhand, and Kulu in India at 3000m altitudes (Baytop, 1999). The reported pharmacological features of *P. armeniaca* are antiparasitic, antiaging, antiangina, renoprotective, anticancer, antitherosclerating, antioxidant, and hepatoprotective (Erdogan *et al*., 2011). By this encouragement, an extensive study on these medicinal important plants from AJK is desired. The potential of medicinal plants and identification of compounds is valued for successful cheap drug trials.

Objectives

- To study the epidemiology of *Leishmania* parasite by using conventional and molecular techniques and sequencing of *Leishmania* isolates in the different areas of Azad Jammu and Kashmir, Pakistan.
- Screening of plants for the antileishmanial activity.
- Isolation and purification of fractions with antileishmanial activity and characterization
- To find the active site of pteridine reductase 1 gene by *in-silico* docking

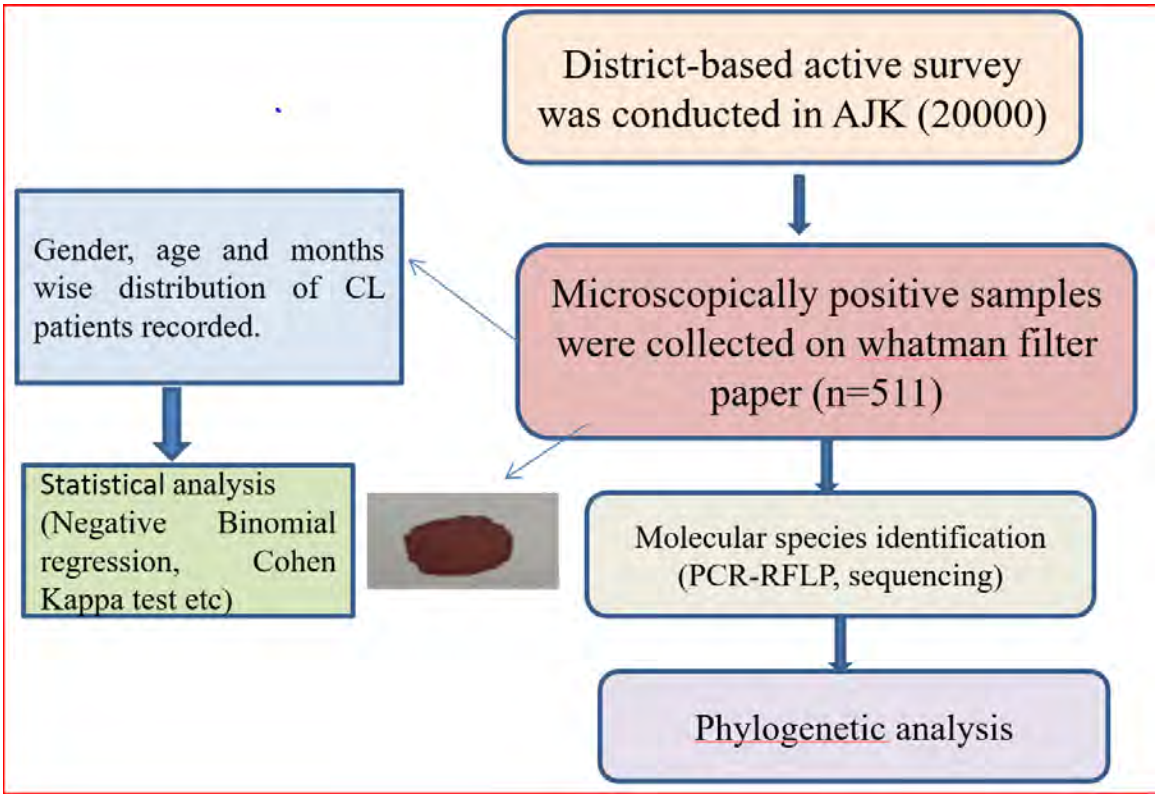
Molecular Epidemiological Survey of Cutaneous Leishmaniasis from Azad Jammu and Kashmir, Pakistan

ABSTRACT

Cutaneous leishmaniasis (CL) in Azad Jammu and Kashmir is an emerging and neglected tropical disease, which is an underdeveloped area. Prevalence and parasite species identification are the key factors to control disease in a particular population. The microscopic technique is used for the identification of amastigote in skin scrapings, biopsies, or impressions on smears. But these are not sufficient for the detection of *Leishmania* parasite on species level because *Leishmania* spp. have identical morphological exhibitions in slide microscopy. Molecular techniques i.e. PCR-RFLP is best methos for *Leishmania* species identification. Due to a lack of previous data, we performed a district-based active CL surveillance in 2018. The data of CL, suspected (n=20,000) cases were analyzed statistically. The blood samples were collected from *Leishmania*-positive patients for *Leishmania* spp. identification by ITS1-PCR RFLP and also obtained accession numbers MN891719-28 from the gene bank. The phylogenetic tree was constructed using MEGA6 software. Out of total suspected cases (n=20000) in the study area, Mirpur was found to be most affected with 4.02% (135/3360) of CL cases in Mirpur, whereas Nelum was reported with less than 1.58% (8/505) number of cases. The slide positivity rate, annual parasitic incidence, and annual blood examination rate were 2.27 per 1000 population, 0.08 and 0.34%. The males were more infected 58.12% (297/511) than females 41.88% (214/511) and 1-20 years of age group were found highly infected 82.78% (423/511) than 21-40 years 13.89% (71/511) and 41-60 years 3.33% (17/511) in the studied population. The patients 56.36% (288/511) patients had a single lesion whereas 29.35% (150/511) had two, only 10.76% (31/288) and 8% (12/150) were using bed nets. The patients 14.29% (73/511) had three or more lesions that were not using bed nets. Only 27.98% (143/511) patients had received treatment, while 72.02% (368/511) didn't. Microscopically positive cases were found to be 2.56% (511/20000) and ITS1-PCR positive cases were found to be 91.39% (467/511). The RFLP assay confirmed the presence

of *L. tropica* in 467 samples. A district based extensive study is needed for the determination of new foci in AJK.

GRAPHICAL ABSTRACT



INTRODUCTION

Currently, a high number of *Leishmania* infection cases observed among travelers, migrants, military personal, and ecotourists. Even, this unexpected travel like migration due to conflicts and violence (e.g. in Syria) leads to dissemination of *Leishmania* spp. in other uninfected regions. (Dawit and Girma, 2013; Salam *et al*., 2014). Also, due to these migrations, various *Leishmania* spp. appears with varied susceptibility to drugs and resistance (Desjeux, 2004; Blum *et al*., 2004). The clinical syndromes of leishmaniasis are associated with the host immune response and by the balance between parasitic factors (i.e. virulence, resistance, and tropism) (Copeland and Aronson, 2015; WHO, 2016). The factors which influence the clinical features of CL (polymorphic disease) are poorly understood. In 1935, the first case of leishmaniasis was reported from Baluchistan following an earthquake. The zones of AJK are hit by leishmaniasis such as Khyber Pakhtunkhawa (Qureshi *et al.,* 2016; Noor & Hussain, 2017), Punjab (Iftikhar *et al.,* 2003; Kakarsulemankhel, 2004), Gilgit Baltistan (Jaffarany and Haroon, 1992; Ayub *et al.,* 2003) and India (Ara *et al.,* 2013). Previously, Mirpur district of AJK revealed an unusual increase in the CL cases from 2011-2014 (Mughal *et al.,* 2014).

For the elimination, no specific operations were carried out by the program between 1999 and 2012 (Chowdhury *et al.,* 2014; Mondal *et al.,* 2008). For the registration of required insecticides, it took a long time. Deltamethrin 5WP (April / May 2012) sprying activity was introduced in 8 highly Upazilas (sub-districts) (Chowdhury *et al.,* 2018). Up to now, no one country was able to completely implement the IVM strategy in the area. The IRS, which can decrease vector density, remains operationally difficult and costly, and its acceptance by the community is not optimal Many research highlights its limitations, insecticides resistance, implementation quality, cost, occupational hazard, sand fly adaptation, size of holes (>3mm), etc (Ostyn *et al*., 2008; Das *et al*., 2008; Chowdhury *et al*., 2011; Coleman *et al.,* 2015).

Cutaneous lesions may be multiple and different in size and are highly affected by one or two lesions. The clinical perspectives and responses to treatment efficacy also vary according to *Leishmania* spp. (Al-Jaser, 2005). In Pakistan, *L. tropica* has been related to

ACL as the causative agent (Rowland *et al.,* 1999; Noyes *et al.,* 1998) while *L. major* contributed to ZCL form (Afghan *et al.,* 2011; Bhutto *et al.,* 2003). The CL is more prevalent in rural and remote areas but unfortunately, its incidence rate is underestimated due to poor diagnosis (McGwire and Satoskar, 2014). Over the previous decades, various treatment options on species dependent have been studied such as thermotherapy (TT) and chemotherapy. In chemotherapy, meglumine antimoniate (glucantime) is recommended by WHO while resistance has been reported against *L. tropica* (Oliveira *et al.,* 2011; Hadighi *et al.,* 2006). In another study, glucantime has shown significantly lower efficacy in curing acute CL in children than in adults (Layegh *et al.,* 2011). Hence, *Leishmania's* identification on species level is imperative to determine the clinical prognosis and to choose the most suitable therapeutic regimen (Morizot *et al.,* 2013).

Leishmania spp. identification is vital for proper treatment of CL infection. Presently, there is no single reference test available for the diagnosis of CL in the ruler areas Diagnosis of CL in non-endemic regions is intriguing, but manifestations of the lesions seen are used in endemic areas to diagnose due to time consumption and high costs (Arfan and Rahman, 2006 ; Hosseinzadeh *et al.,* 2012; Ramezany *et al.,* 2018). The mistreatment or reinjection can cause severity in infection and hurdle in diagnosis (Alsamarai and Alobaidi, 2009).

Leishmaniasis is important to diagnose properly before the infection leads to severity (Kobets *et al*., 2012). Clinical characteristics (supported by epidemiological data) and laboratory tests are the basis for the diagnosis of CL. Numerous diagnostic methods, including direct parasitological analysis (microscopy, histopathology, and parasite culture) and/or indirect serology and molecular diagnostic testing, have been identified with a huge variation in diagnostic accuracy. An occasional test known as Montenegro skin test is used in the CL diagnosis (e.g. in epidemiological surveys), due to its specificity, simplicity, and high sensitivity. Choosing the diagnostic test used also depends on the diagnostic facility's available facilities and services and not on diagnostic accuracy. Due to time consumption, high cost, non-feasible techniques, most of the laboratories follow microscopy only, which is affordable (Rotureau *et al.,* 2006). The examination of skin scratching, the impression

of smears can easily reveal the presence of etiological agents, as well as microscopy of cultured species, which have higher sensitivity (Weina *et al*., 2004). The immunological tests are unable to differentiate newly occurred infection from the old one and are not very trustworthy in patients with low immunity levels (Silvapandiyan *et al.,* 2009). The DNA extraction and PCR made more accurate parasite species diagnosis and phylogenetic studies (Boggild *et al.,* 2007).

For large scale research, dried blood spots (DBS) are a safer alternative to standard vein puncture for blood collection (Fajardo *et al.,* 2014; Ostler *et al.,* 2014; McDade, 2014; Hijjawi *et al.,* 2016); Makowski *et al.,* 1995; Catsburg *et al.,* 2007; Sorensen *et al.,* 2007; Hollegaard *et al.,* 2013; Bensoussan *et al.,* 2006). Several molecular targets for PCR diagnosis were tested in the *Leishmania* parasite including the miniexon gene (Fernandes *et al.,* 1994), minicircle kinetoplast DNA (Qureshi *et al.,* 2016), gp63 gene, (Victoir *et al.,* 2003) and internal transcribed spacer among others. Samples spotted on filter paper collected from the study region were examined by PCR amplification of ITS1 in the current investigation and RFLP for parasite diagnosis at species level as reported by Cupolillo *et al.,* (1995) and Schonian *et al.,* (2003). The recent research is a partial attempt toward observing the epidemiology of CL infection and species identification in AJK by microscopy and molecular analysis.

MATERIALS AND METHODS

2.1. Study Area and Collection of Samples

A district-based active CL surveillance was performed by the members of the research team of Quaid-i-Azam University Islamabad in AJK, Pakistan. The study was done in 2018 with the approval of the District Health Officer. The bioethical committee of Quaid-i-Azam University approved the protocol (#BEC-FBS-QAU-152). The data was collected from the local population included in the study area was based on visual inspection of the ulcerated skin and skin lesions. Two data sets were used for sample collection i.e. (i) a detailed questionnaire was filled by interviewing each patient. The questionnaire encompassed data on gender, age, the number of lesions, bed nets, and therapy used. (ii) The skin scraps were collected from suspected patients (n=20000) of CL infection for *Leishmania* diagnosis through microscopy. The blood drops at the margin of lesions that were spotted on 3MM filter paper were collected from microscopically positive patients (n=511) for species identification by molecular analysis. Data were collected from 10 districts of AJK i.e. Sudhnoti, Poonch, Haveli, Muzaffarabad, Hattian, Bagh, Bimber, Neelum, Kotli, and Mirpur shown in Fig.2.1. The written and oral consent was also recorded from the respondent or their elders.

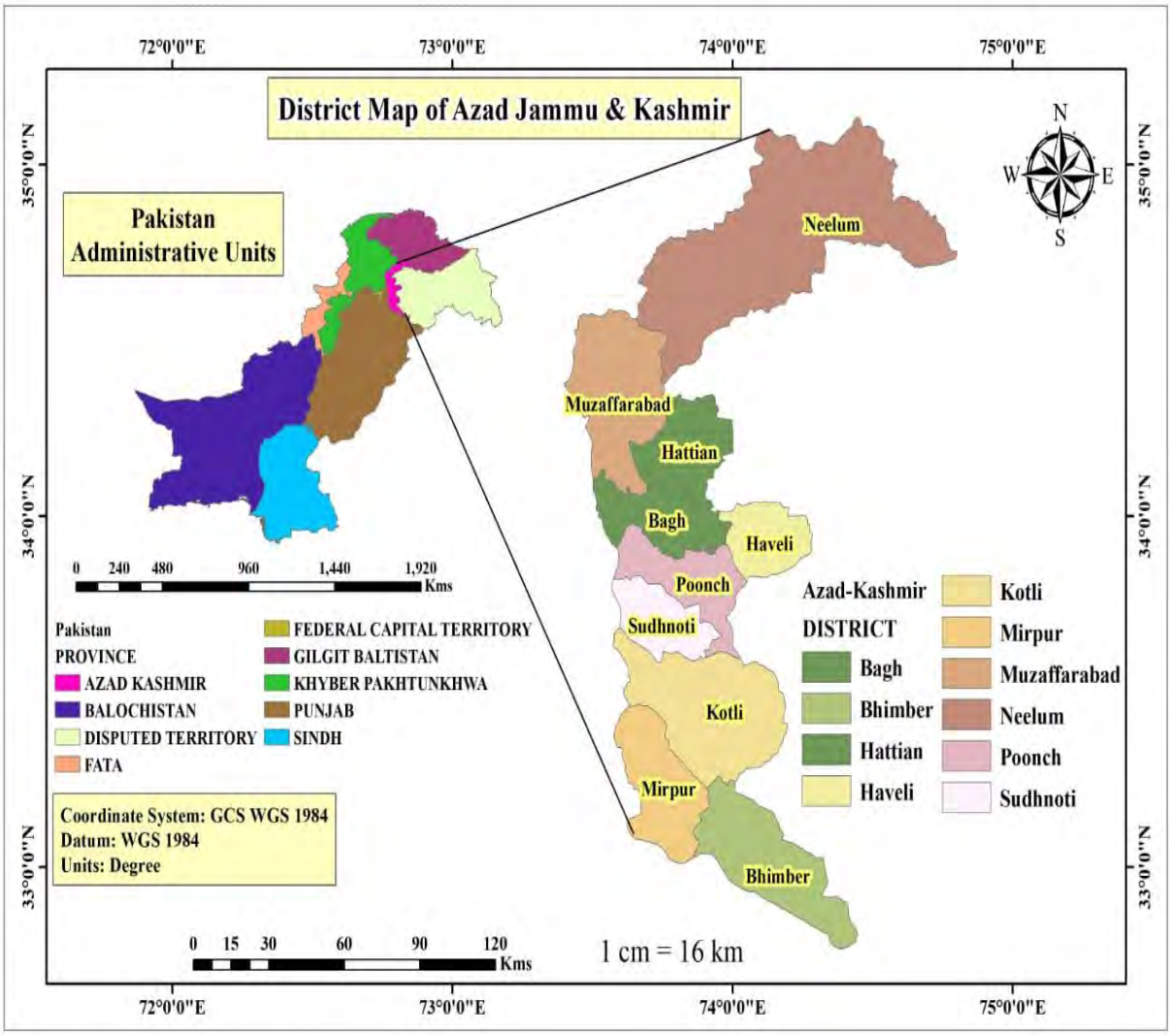


Fig. 2.1: Map shows the study area of cutaneous leishmaniasis in Azad Jammu and Kashmir. **A:** Pakistan Administrative Units surrounding AJK. **B:** showing ten districts of AJK included in the present study.

2.2. Laboratory Diagnostic Procedure

2.2.1. Sample Collection for Microscopy

The samples for microscopy were collected from patients in the presence of medical representatives. The infected tissues were taken using a disposable scalpel blade (no.11) and smeared on a glass microscopic slide. Dried the smear, fixed in methanol (100%), Giemsa stained and then binocular microscope (optic, 500 series) was used for observation.

2.2.2. Sample Collection for DNA Extraction

The microscopically positive samples (n=511) of CL patients were collected for species identification. Briefly, sterile Whatman filter paper was touched at the site of lesion cut, allowed to air dry carefully, and then individually wrapped in the aluminum foil (Fig. 2.2). The samples were transferred to the Parasitology laboratory, Quaid-i-Azam University, Islamabad, and were stored at room temperature with silica gel until further analysis.

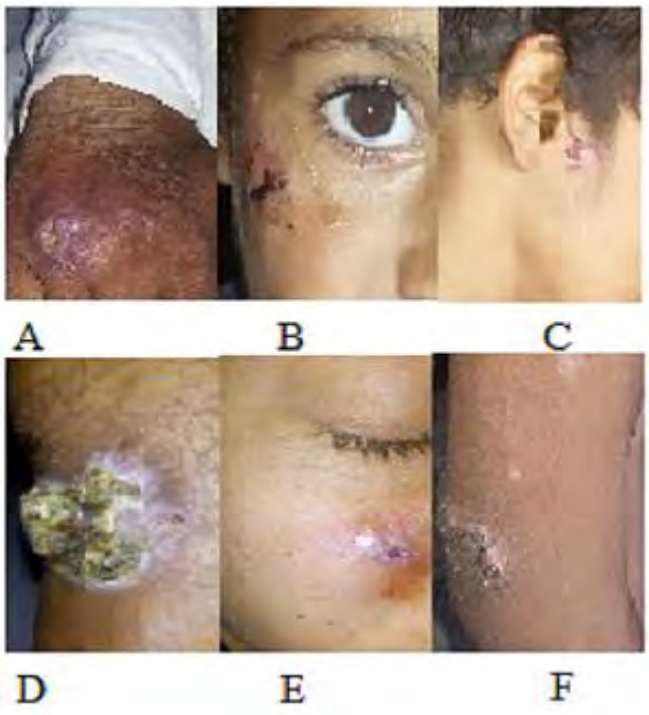


Fig.2.2: Shows *Leishmania* lesions of infected patients from A-F

2.2.3. DNA Extraction

The spotted filter paper (n=511) was punched with a paper puncher and placed into lysis buffer (250µl), Triton X 100 (2.5µl), proteinase kinase (-20mg/ml) (1.26µl) in (1.5ml) Eppendorf tube and incubated at 37°C for overnight. After incubation, the filter paper was pressed several times with a sterile micropipette tip and removed from the Eppendorf tube. The specimen was centrifuged at 8050g for 10 minutes and the supernatant was discarded. The pallet was subjected to phenol-chloroform, ethanol (1:10:1), and Na acetate for precipitation. The pelleted DNA was dried for 5 minutes by a vacuum dryer and redissolved in (50µl) TE (pH 8) buffer. The sample was kept at -80°C until use (Boggild *et al.,* 2010).

2.2.4. PCR Amplification of Internal Transcribed Spacer 1

The microscopically positive samples (n=511) were subjected to PCR amplification. The *Leishmania* specific primers: LITSR forward 5'CTGGATCATTTTCCGATG3' and reverse L5.8S 5'TGATACCACTTATCGCACTT3' were used for the amplification of ITS1 of the ribosomal DNA (Schonian *et al.,* 2003). The amplification was done with the PCR master mix (2X tag green) (Thermo Scientific, Lot: 00514943). Amplification was done in a 50µL final volume of a reaction containing 25µL dream green PCR master mixture, forward primer 1µM, reverse primer 1µM, DNA template 1µl, and nuclease-free water (Thermo Scientific, Lot# 00496023) to 50µl. DNA sample amplification was performed in a thermal cycler (Kyratec Australia, Sc300) under the cyclic conditions: initial at 94°C for 3min followed by 35 cycles at 95 °C for 30sec\, 50°C for 30sec, 72°C for 60sec followed by a final extension at 72°C for 5min. The MN891719-28 *Leishmania* confirmed samples were used as positive (+) whereas uninfected blood as negative (-) control. The amplified product was later analyzed by electrophoresis on ethidium bromide (0.5µg/ml) agarose gel (1.7%) and visualized under gel doc system (Extra Gene, USA) and the picture was captured using a gel documentation system. For DNA molecular weight standard, a 100bp ladder (Thermo Scientific) was used in each run.

2.2.5. Restriction Fragment Length Polymorphism Analysis of Amplified ITS1

The amplified products 5µl were digested with BsuR1 (HaeIII) enzyme, following the instructions of the manufacturer. Briefly, the reaction mixture contained 5µl of the amplified product, 1µl of BsuR1 (HaeIII) (Thermo scientific), 7.5µl of dH_2O and 1.5µl of 10X buffer (Thermo Scientific) in a microtube and was incubated for 2hrs at 37°C. Around 10µl of the reaction solution was analyzed by electrophoresis at 100V in1X tris-acetate EDTA buffer r (1mM EDTA, 0.04M tris-acetate, pH 8) on agarose gel (4%) and UV-illuminator r (Extra Gene, USA) was used for visualization. The 100bp gene ruler DNA ladder of 100 and 20bp (Thermo Scientific) was used as the DNA molecular marker.

2.2.6. DNA Sequencing and Phylogenetic Analysis of ITS1 Region

The gene jet purification kit (Cat # K0701) was used for purification of amplified samples and was sent for sequencing to Korea (Macrogen Inc.). The sequences under the accession numbers MN891719-28 were deposited to the gene bank. The multiple alignment program MEGA 6 was used to align the sequences. The neighbor-joining method was used to inferred the evolutionary history and analysis involved twenty-three nucleotides sequences. The MEGA6 software was used to construct the phylogenetic tree (Tamura *et al.,* 2013).

2.3. Statistical Analysis

The CL incidence count was analyzed to age, season, and gender. The initial data examination confirmed that the data count is"0" inflated and overdispersed having a mean (21.29167) was lesser than the variance (774.1286). Therefore, we used three models in software R (Development core team 2014) i.e. m1; zero-inflated negative binomial regression with random season effect.

Zero-inflated (incidence~ age+ gender, season, data= disease, dist= "negbin").

m 2; zero-inflated negative binomial regression with no accidental result.

Zero- infl (incidences ~ age + gender, data=disease, dis"negbin").

m 3; simple negative binomial regression

mlg . bn(incidences ~ age + mebneg+ aesaeb, data=disease).

Vuong test was used for the model's comparison, this recommends that the negative binomial model had significant improvement p=1.1470e_06. Over zero-inflated negative binomial model. Therefore the negative binomial model was used in the present study. The package ggplot2 (in R language) was performed for data visualization. The statistical analysis for other data parameters was conducted by SPSS V. 21. For the comparison of 2 diagnostic methods, i.e. microscopy, and PCR Cohen Kappa was calculated as excellent (0.81-1.00), fair (0.21-0.40), moderate (0.41-0.60), slight (0-0.20), poor (0), and substantial (0.61-0.80) agreement (Cunningham *et al*., 2012). Various formulas were used for the calculation of annual parasitic rate, slide positivity rate, and annual blood examination rate from suspected cases.

RESULTS

2.4. District-Wise Distribution of Cutaneous Leishmaniasis

Out of 20000 CL suspected inhabitants based on visual inspection of the ulcerative skin and skin lesions, 511 samples were microscopically positive and 19489 were negative. The highest positive cases of CL were observed in Mirpur 4.02% (135/3360) whereas Muzaffarabad was the second highly affected district having 2.84% (97/3421) of CL infection. The Kotli ranked at third number having 2.66% (78/2937) CL positive cases. The rest of the districts as Sudhnoti, Bimber, Bagh, Poonch, Haveli, and Hattian had 1.81(50/2756), 2.92 (51/1745), 1.83(35/1913), 2.12(23/1084), 1.53(20/1308) and 1.44% (14/971) CL positive cases respectively, whereas Neelum showed the lowest 1.58% (8/505) CL positive cases. The occurrence of CL infection in the local population of Azad Jammu and Kashmir was 2.56% of the total population studied.

Table. 2.1: Epidemiology of cutaneous leishmaniasis in Azad Jammu and Kashmir.

District name	Population sizes	Suspected cases	Microscopic positive slides	Negative cases	SPR (%)	API / 1000	BER (%)
Bagh	459,840	1913	35	1878	1.82	0.07	0.41
Bimber	520,059	1745	51	1694	2.92	0.09	0.14
Hattian	285,026	971	14	957	1.44	0.04	0.34
Haveli	188,086	1308	20	1288	1.52	0.10	0.69
Kotli	957,157	2937	78	2859	2.65	0.08	0.30
Mirpur	564,045	3360	135	3225	4.01	0.23	0.59
Muzaffarabad	665,744	3421	97	3324	2.83	0.14	0.51
Neelum	236,462	505	8	497	1.58	0.03	0.21
Poonch	618905	1084	23	1061	2.12	0.03	0.17
Sudhonti	3667,932	2756	50	2706	1.81	0.01	0.07
Total		**20000**	**511**	**19489**	**2.27**	**0.08**	**0.34**

The overall SPR (slide positivity rate) in the current study (2018) was 2.27% in all districts of Azad Jammu and Kashmir. The SPR was highest in Mirpur (4.01%) while the lowest (1.44%) in Hattian as compared to other districts. The SPR of Bimber, Muzaffarabad, Kotli, Poonch, Bagh, Sudhnoti, and Haveli were 2.92, 2.83, 2.65, 2.12, 1.82, 1.81, and 1.52% respectively. The district Neelum showed 1.58 SPR. The API (annual parasitic rate) was 0.08 per thousand populations in all cases under study. The API was highest (0.23%) in Mirpur while the lowest (0.01%) in Sudhnoti. The API in Muzaffarabad, Haveli, Bimber, Kotli, Bagh, and Hattian were 0.14, 0.10, 0.09, 0.08, 0.07, and 0.04% respectively. The Neelum and Poonch showed the same (0.03%) API. The ABER was 0.34% per 1000 population. The highest (0.69%) ABER was recorded in Haveli and the lowest (0.07) in Sudhnoti followed by Mirpur, Muzaffarabad, Bagh, Hattian, Kotli, Neelum, Poonch, and Bagh had 0.59, 0.51, 0.41, 0.33, 0.30, 0.21, 0.17, and 0.14% respectively (Table.2.1).

Table 2.2: Analysis of negative binomial regression showing the effect of age, gender, and seasons on *Leishmania* incidence (n=511).

Co-efficient	**Categories**	**Estimate**	**St.error**	**Z value**	**Pr(>\z/)**
Intercept		3.5575	0.1387	25.654	< 2e-16 ***
Age in years	1-20	0.3103	0.1178	12.992	< 2e-16 ***
	21-40	-1.8331	0.1447	-12.670	< 2e-16 ***
	41-60	-3.2472	0.2565	-12.662	< 2e-16 ***
Gender	Male	0.3671	0.1147	3.199	0.00138 **
Seasons	Spring	0.4287	0.1581	2.712	0.00670 **
	Summer	0.7906	0.1515	5.217	1.82e-07 ***
	Winter	-1.0975	0.2195	-5.000	5.74e-07 ***
	Autumn	3.4357	-0.3904	22.75	0.005***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.005' ***' 0.1 '' 1

The predictor's sex, age and season predicting number of incidences are significant predictors

2.5. Seasonal Variation of Cutaneous Leishmaniasis

The findings revealed that the highest monthly number of CL isolates in the summer 41.49% (212/511) (June to September) were observed followed by spring 30.72% (157/5110 (March to May), autumn 20.94% (107/511) (October to November) and winter 6.85% (35/511) (December to February) respectively. Minimum cases of CL were found in winter. However, the expected log recorded for CL cases was highest in summer i.e. 0.7906 while winter showed the lowest expected log i.e.-1.0975. The expected log for spring was 0.4287 while autumn showed the highest incidences than winter (-1.0975) (Table 2.2 and Fig. 2.3).

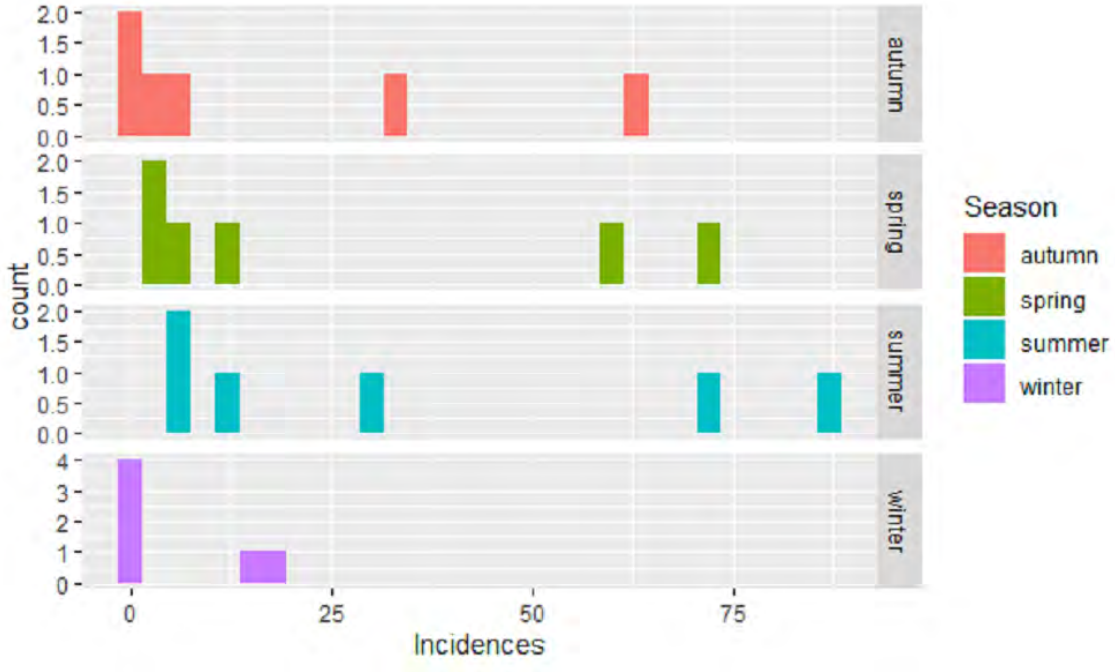


Fig. 2.3: Seasonal distribution of cutaneous leishmaniasis in Azad Jammu Kashmir, X-axis represents CL incidences and Y-axis the count of CL based on microscopy.

2.6. Gender and Age-wise Occurance of Cutaneous Leishmaniasis

In the current finding, the proportion of the affected population under study in 2018 revealed that a higher rate of infection was observed in males 58.12% (297/511) than

females 41.88% (214/511). The male population had a higher expected log (incidence) value (0.3671) than females that had been used as constant for other variables (Table. 2.2).

The highest proportion of 82.78% (423/511) was observed at the 1-20 years old. Only 13.89% (71/511) of patients were observed in 21-40 years followed by 3.33% (17/511) in 41-60 years old. The ratio of males to females was different based on age. Males 46.97% (240/511) were more frequently affected than females 35.81% (183/511) at the age of 1-20 years. However, among aged 21-40 years, the infection was highest in females 9.39% (48/511) than males 4.50% (23/511) while in aged 41-60 showed only 1.76% (9/511) males and 1.57% (8/511) females. The CL incidence in the age group 1-20 years old was 0.3103 on the log scale which was the highest among the observed age groups that of 21-40 (-1.8331) and 41-60 (-3.2472) (Table.2.2, Fig.2. 4).

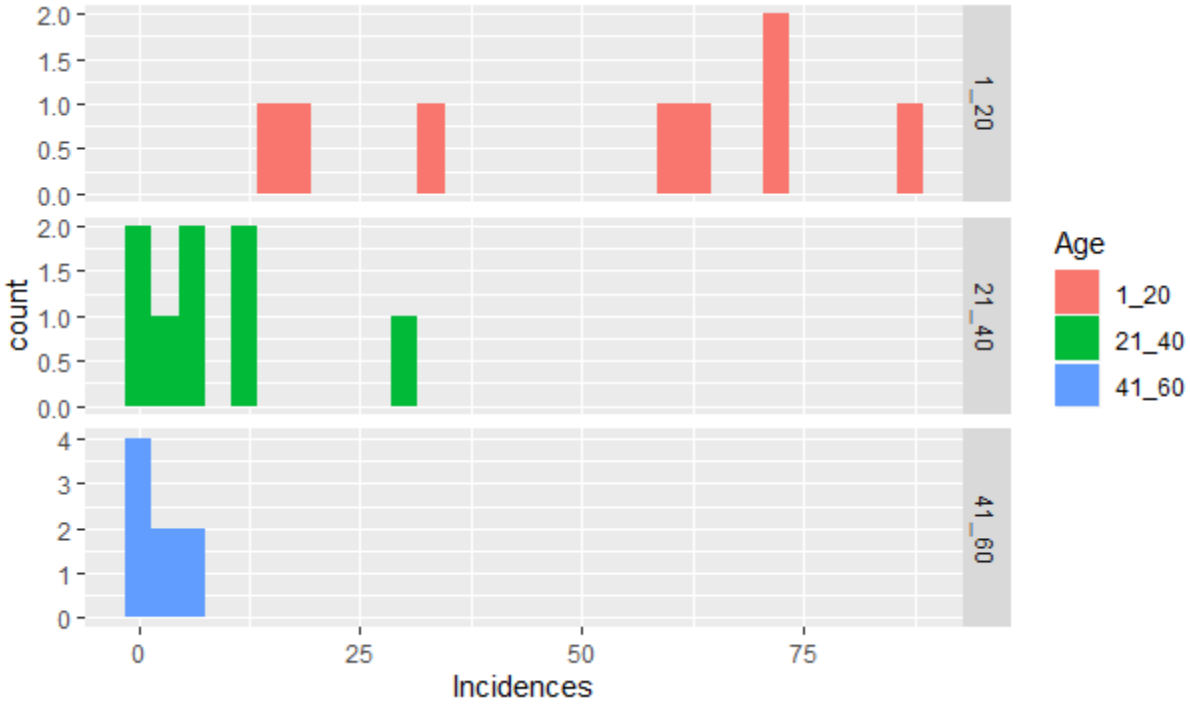


Fig.2.4: Age-wise distribution of CL infected population-based on microscopy

2.7. Distribution of Bed Nets, Number of Lesions, and Treatment

Overall, the single infected lesion was highest in the studied population as it was 56.36% (288/511) whereas two lesions infection was 29.35% (150/511) and three or more was 14.29% (73/511) and showed significant value (p=0.000). Among the whole infected

patients with CL, only 8.41% (43/511) were using bed nets whereas 91.58% (468/511) were not using bed net having significant value (p=0.000). Only 31 patients were using bed nets having a single lesion and infected with two lesions only 12 were using bed nets while no one was using bed nets having three or more lesions. All 18.98% (97/511) patients were treated with chemotherapy with meglumine antimoniate by intramuscular injection. The 9.00% (46/511) CL infected patients taking treatment by thermotherapy. About 72.02% (368/511) of patients were not visiting any health centers. The value of p=0.001 (Fig. 2.5).

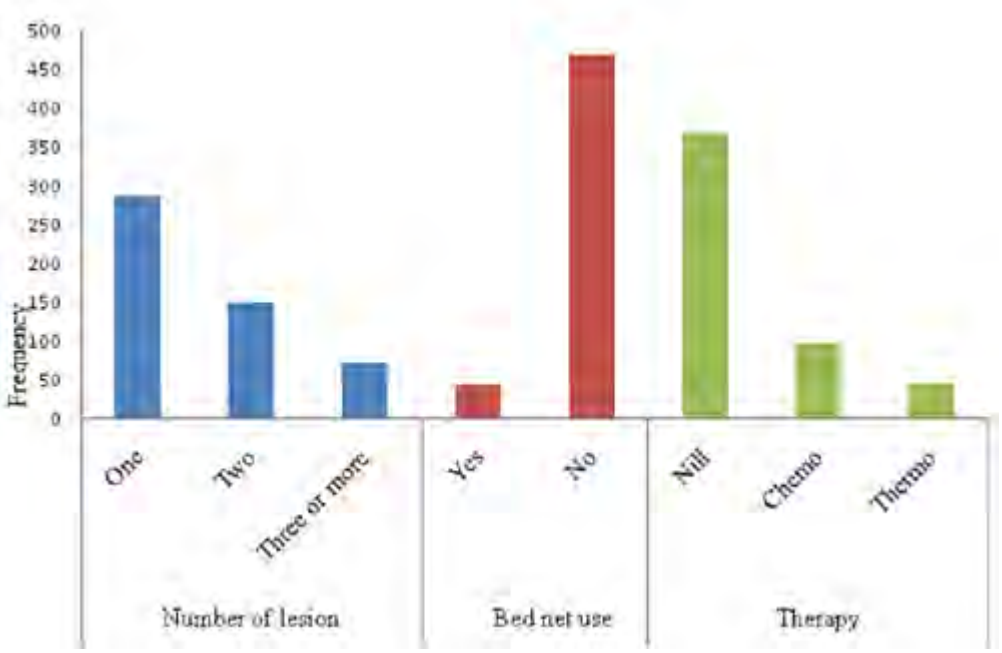


Fig.2.5: The frequencies of the number of lesions, bed net, and history of treatment among infected patients**.**

2.8. ITS1-PCR of Cutaneous *Leishmania* Cases

The microscopically positive CL samples (n=511) were subjected to ITS1-PCR for amplification of the ITS1 region (Fig. 2.6). The PCR diagnosis showed that 91.39% (467/511) cases were found to be positive and 8.61% (44/511) negative. The district-wise distribution of PCR positive cases were found to be in Mirpur 23.29% (119/511), Muzaffarabad 16.24% (83/511), Kotli 14.48% (74/511), Sudhnoti 9.39% (48/511), Bimber 9.20 (47/511), Bagh 6.46% (33/511), Poonch 4.11% (21/511), Haveli 3.91% (20/511), Hattian 2.74% (14/511), and Neelum 1.57% (8/511). The two diagnostic methods i.e.

microscopy and ITS-PCR assay showed 0.960 value of Kappa that showed excellent kappa agreement with a significant value of 0.000 while 0.006 was a standard error.

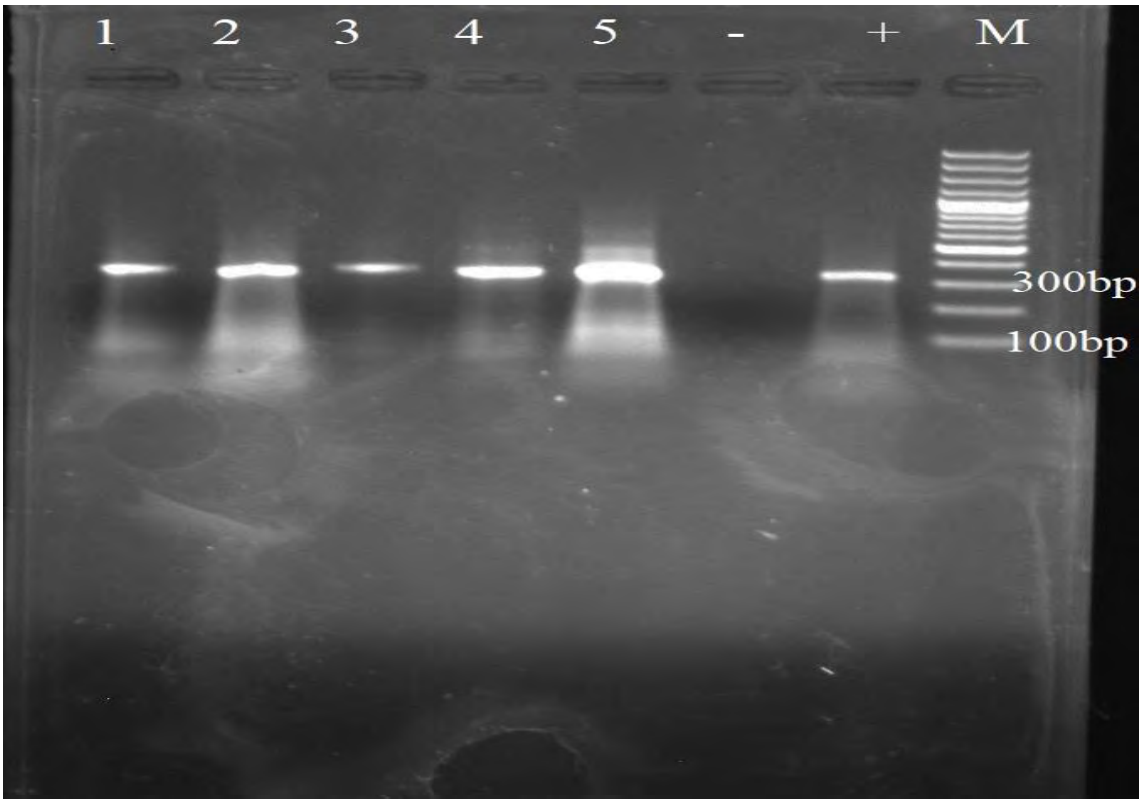


Fig.2.6: The PCR amplification with specific ITS1 primers of CL specimens giving approximately 300-350bp amplification band size when visualized under UV transilluminator. Lane M: 100bp DNA ladder. +ve standard MN891719 *L. tropica*, Lane -ve: blood from uninfected people and Lane 1-5: samples from infected patients.

2.9. Analysis of RFLP

Restriction of the ITS1 gene amplicons of amplified product with the endonucleases HaeIII generated 2 band patterns at 200 and 60bp. Among 511 microscopically positive samples observed from AJK, 91.39% (467/511) were identified as *L. tropica* by ITS1 PCR-RFLP and 8.61% (44/511) remained negative (Fig. 2.7).

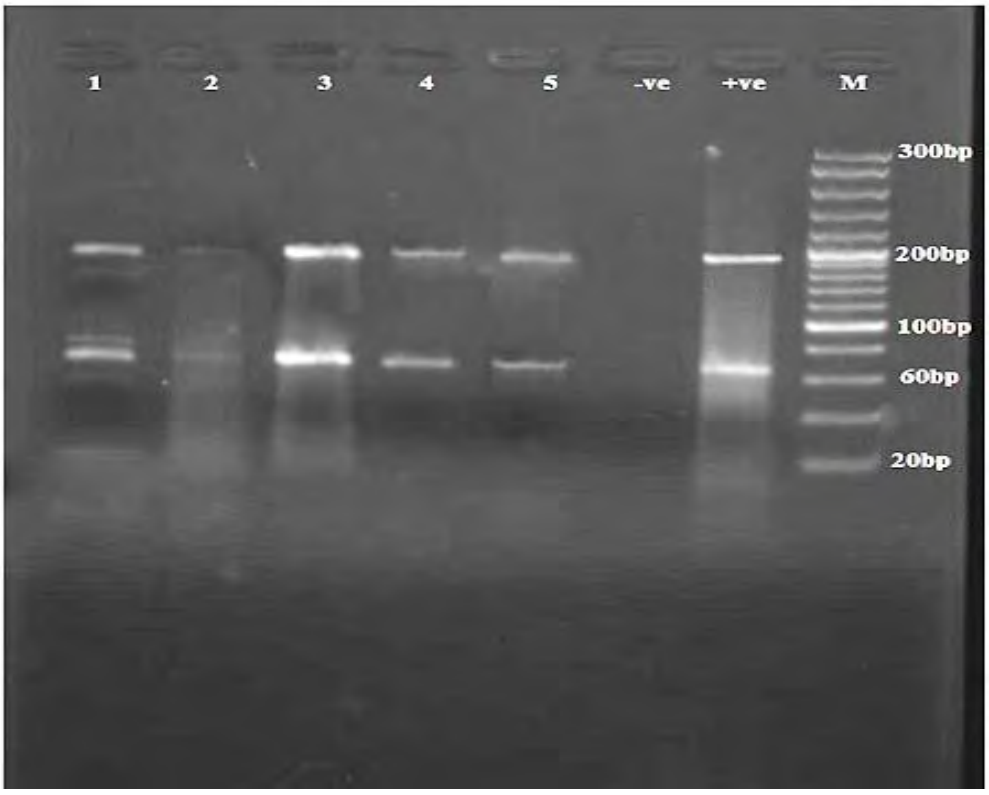


Fig.2.7. Showing the digestion of amplified ITS1 region with a restriction enzyme of Hae III. Lane M: 20bp DNA ladder, Lane -ve: negative control, Lane +ve: positive control of *L. tropica* showing two bands (60 and 200bp), Lane 1,2,3,4, and 5 samples for *L. tropica* detected in infected samples.

2.10. Sequence and Phylogenetic Analysis

According to the BLAST similarity research, the ITS1 sequencing results in the current study confirmed the presence of *L. tropica*, the causative agent of ACL. The nucleotide sequences were submitted into Gene bank with the accession numbers of MN891719-28. The constructed phylogenetic tree in Fig.2.8 involved twenty-two nucleotides and the sum of branch length was 1.00979406 with 1000 bootstrap replicates (Felsenstein, 1985). The phylogenetic tree was drawn to scale, with the same branch length unit, while the maximum composite probability approach measured evolutionary distances (Tamura *et al.,* 2004). The included position containing missing data and gaps were eliminated and the codons were 1^{st} +2^{nd} +3^{rd} +noncoding. In the final dataset, there were about 140 positions. However, in the phylogenetic tree, the isolates of *L. tropica* from

Mirpur (MN891719), Kotli (MN891720), Muzaffarabad (MN891725) showed homology to the *L. tropica* isolates of Iran. The Bimber (MN891724) and Bagh (MN891727) showed a relationship to Syrian and (MN891722) Sudhnoti and Haveli (MN891723) sequences belonged to Spain while Neelum (MN891721) belongs to India. The sequence of districts Poonch (MN891726) and Hattian (MN891728) belong to Iran and Syrian.

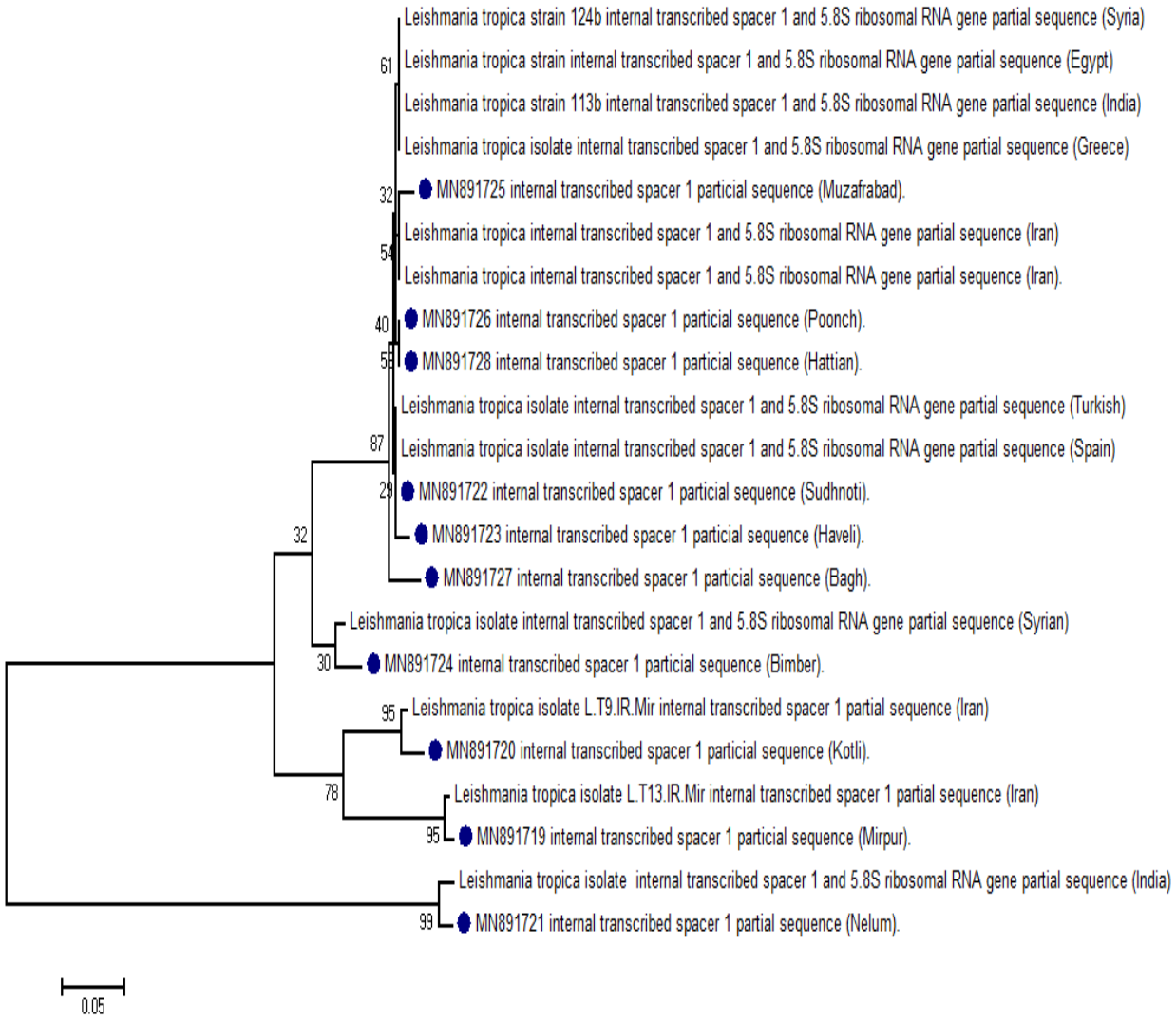


Fig. 2.8. The neighbor-joining tree was constructed based on the ITS1 gene sequence of the *L. tropica*. The 511 microscopically positive samples were identified on species level by PCR-RFLP technique. Out of 511 PCR amplified samples, 10 samples were randomly selected for *L. tropica* reconfirmation by sequencing.

DISCUSSION

The distribution of CL shows wide patterns in Pakistan and is continuously disperse in adjacent areas. Recently, several outbreaks were reported from the Northern and Southern regions of Pakistan. It is suspected that the disease-free areas that adjoin borders with endemic regions are also at risk. The present study was carried out in AJK, geographically in contact with Gilgit Baltistan (Ayub *et al.,* 2003), Punjab (Kakarsulemankhel, 2004), KPK (Qureshi *et al.,* 2016; Noor & Hussain, 2017), and Indian administered Kashmir (Ara *et al*., 2013), which are prone for CL infection. In the respective study area, an unusual increase in the CL cases from 2011-2014 was reported from Mirpur (Mughal *et al.,* 2014). Unfortunately in most of the areas in AJK people are unaware of CL infection. Hence, an active survey was conducted to find the epidemiology of CL in local inhabitants. In present investigations, Mirpur showed the highest CL infection and lowest in Neelum. The seasonal elements play a vital role in the spread of CL because, compared to winter, the sand fly is more involved during the warmer months of the year. In the current analysis, CL cases were observed throughout the year (2018), whereas the maximum cases were found in warm months and gradually decreased with temperature changed from warm to winter. Our study was also supported by Durrani *et al.,* (2011), where CL-positive cases were reported throughout the year from Northern Pakistan. The highest CL cases in summer might be as a result of the occurrence of the highest number of gravid sand flies as they need to consume a greater blood quantity for their egg development. The lowest rate of CL infection in autumn and winter was due to the flushing effect of rainfall or snow on immature emerging sand flies in AJK.

The CL infection was more prevalent in males than females (Table. 2), that are similar to results observed by Galgamuwa *et al.,* (2017) and Sandanayaka *et al.,* (2014) in Sri Lanka, Aara *et al.,* (2013) in India, and Enami *et al.,* and Alavinia *et al.,* in (2009) in Iran. The highest prevalence of CL in males is because they are more involved in outdoor activities and they commonly do not cover their skins, which maximizes their exposure to vector bites (Gadisa *et al.,* 2015). Also, females are restricted to household activities, obligatory for them to be at home before dusk, the onset of the period of sand flies activity

as observed by Al Jawabreh *et al.,* (2003). Current investigation revealed, most infected people were of 1-20 years of age due to malnutrition and weak immune system. This was also supported by other researchers (Qureshi *et al.,* 2016; Layegh *et al.,* 2011; Karami *et al.,* 2013; Zijlstra, 2016). The lowest occurrence in 41-60 years of age was 3.33% observed in current results. This finding was related to the observations conducted by Nawaz *et al.,* (2010). We also agreed with Ullah *et al.,* (2009) who determine the lowest prevalence rate in the 46-60 years group.

In our investigation, patients presented single (56.36%), two (29.35%), and three or more (14.29%) lesions. Our results are in agreement with Talari *et al.,* (2006), who reported the occurrence of the single lesion with a rate of 58.4%, two with 29.2% and more than two 12.38% in CL patients. Another study conducted by Qureshi *et al.,* (2016) also showed the highest occurrence of the single lesion as compared to other numbers of lesions in CL patients. However, from the current analysis, it seems that multiple lesions are also not uncommon; these lesions could be due to long periods of exposure to sand flies. The vector bite might control by the use of bed nets when people are sleeping outside the rooms at night. However, the 3mm size mesh bed net gave additional protection than the larger because the sandflies are about 2-3mm wing size long. Bern *et al.,* in (2000) suggested that the bed nets (pyrethroids treated) minimize the vector biting from 64-100%. The bed nets (insecticide-treated) have been permitted by the Pesticide Evaluation Scheme of WHO as these bed nets are very effective for a long time (WHO, 2015) but the people of Azad Jammu and Kashmir are generally unwilling for bed nets paying due to high financial burden in villages. In the current study, 72.02% of patients did not visit any health center because of unawareness; people use several harmful applications of lighted matches, gasoline, and acids for the treatment of CL infection. The same results were also reported in Ecuador (Weigel *et al.,* 1994). In AJK, drug sellers have been linked to the choice of treatment and therapy. Various studies also clarified this in Pakistan (Jamal *et al.,* 2013). Hence, it would be better to argue that local population awareness about CL and its vector might be a successful tool to decrease the upcoming scenario of this infection (Rahman and Rehman, 2018).

The parasite species identification is very important in disease prognosis and in deciding appropriate therapy. Molecular techniques have been proved to be a powerful tool for detecting parasites as well as parasite species identification by PCR. In the current study, the ITS1 sequence (300-350bp, species-dependent) was chosen as the target for our PCR assay because several recent studies have shown that ITS-PCR followed by FRLP is a suitable tool to classify *Leishmania spp*. (Schonian *et al.,* 2003; Bensoussan *et al.,* 2006). The key benefit of ITS-PCR is that the identification of species can be accomplished by digesting a single restriction enzyme (HaeIII) PCR product and this is sufficient to differentiate almost all medically relevant *Leishmania spp*. (Schonian *et al.,* 2003). In our analysis, of the 511 microscopies confirmed cases of CL, 91.39% (467/511) were successfully amplified by ITS-PCR. However, the 8.61% (44/511) (untreated) microscopy confirmed cases were not amplified by ITS-PCR. The PCR negative results might involve many factors i.e. (i) false microscopy identification (ii) less quantity of sample on filter paper (<5ng/µl) (iii) poor handling resulted in contamination because microscopy needs expertise and is time-consuming (Boggild *et al*., 2010). Current results showed excellent concordance between microscopy and ITS-PCR i.e. (0.960±0.006). A similar level of agreement was also reported by Gao *et al.,* (2015) between two diagnostic tests. The patients under treatment were CL positive because intra-lesional treatment showed poor response which may be due to the acquisition of partial treatment. The reason might be an incomplete treatment that needs to develop resistance in parasites against the drug. Further, the lesions are normally painless and remained neglected for a long time providing more chances for the spread of infection (Jamal *et al.,* 2013). The cost of treatment is also beyond many patients. Scio-economical conditions and malnutrition are also other important factors responsible for weak immunological responses against this infection in the poor population of AJK. The present study suggested that there is a need to control *Leishmania* infection by vector control using insecticide repellent and impregnated clothes, bed sheets, curtains, and bed nets with insecticide and exploring new species-specific drugs for parasites (Reyburn *et al.,* 2000; Yaghoobi-Ershadi *et al.,* 2006).

CONCLUSION

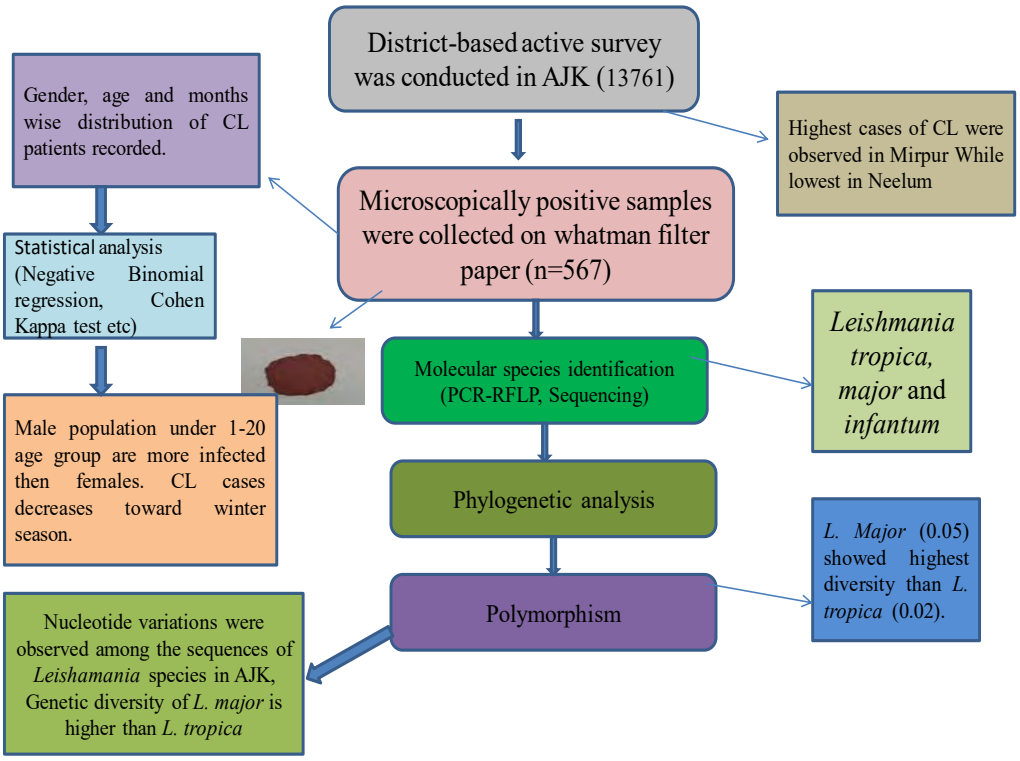
The finding of our study concluded that CL-infected inhabitants were not aware of CL infection and its preventive measures. There is a need for more health education to create awareness among the local population of AJK. The PCR-RFLP assay identified the presence of *L. tropica* in samples under study, which has an anthroponotic transmission (Rowland *et al.,* 1999; Noyes *et al.,* 1998). The scars of negative inhabiatntas may be due to bacterial infection (S*taphylococcus aureus* and S*taphylococcus epidermidis*) and low parasitic load because of home remedies (Doudi *et al.,* 2010). For proper treatment and making controlling strategies more epidemiological studies are recommended in AJK. An extensive study is also needed to find the unkown etology of scars which showed negative results for CL infection.

Genetic Diversity of Cutaneous Leishmaniasis from Azad Jammu and Kashmir, Pakistan

ABSTRACT

The genetic diversity of *Leishmania* spp. in North Eastern Pakistan remains undetermined despite increased cases of cutaneous leishmaniasis (CL). This study was designed to decipher the molecular characterization and genetic diversity of *Leishmania* spp. in North Eastern Pakistan. Out of 13761, CL suspected cases, 567 cases were microscopically positive and confirmed as *Leishmania* spp. by internal transcribed spacer (ITS) gene amplification through the PCR- RFLP technique. Further, isolates were directly sequenced to conduct phylogenetic analysis for genetic diversity. Among suspected CL cases, Mirpur showed the highest proportion of CL infection with 4.85% (118/2431) of the cases, while the Neelum district showed the lowest percentage at 3.29% (9/273). The slide positivity rate, annual blood examination rate, and annual parasitic incidence rate were 3.84, 0.27, and 0.01% respectively, and the incidence of CL in the age group 1-20 years old was higher in males (50.92%) than females (25.75%). The RFLP analysis and sequencing confirmed the occurrence of *L. tropica*, *L. major,* and *L. infantum*. *Leishmania tropica* (p=0.02) confirmed significantly higher nucleotides variation than *L. major* (p=0.05). Current findings confirmed the prior assumption that anthroponotic CL is the primary CL form present in North Eastern Pakistan. Moreover, this is the first report based on molecular identification of *L. major*, and *L. infantum* from North Eastern Pakistan. This remarkable heterogeneity in the *Leishmania* spp. is the leading cause of treatment failure and emergence of new haplotypes. Therefore more extensive investigations are recommended from all geographical regions of North Eastern Pakistan, especially those using a large sample size.

GRAPHICAL ABSTRACT



INTRODUCTION

The *Leishmania* parasite is identified by epidemiological, clinical and ecological complexity. The presence of *Leishmania* worldwide (except Antarctica) and the ability to infect sand fly species and mammalian hosts show that *Leishmania* parasite is capable to survive and adapt different environments. The biotic interaction and changes in the environment can inflict selection pressure on different life-history traits and manipulate their genetic diversity. The genome comparison exhibited a high level of heterogeneity in the *Leishmania*. At the phylogenetic divergence level, markers e.g. ribosomal ribonucleic acid is necessary to form an image of various phylogenetic subdivisions. The genetic divergence observed at the subgenus level is very high and decreases in species level (Beverley *et al.,* 1987; Rioux *et al*., 1990). Moreover, the phylogenetic and genetic data showed that various taxa can show the variable level of genetic heterogenecity. For example subgenus of *Leishmania* showed more heterogeneity than the *Viannia* subgenus (A.l.ban uls *et al*., unpublished observations). All species also exhibited different genetic diversity e.g. *L. guyanensis* and *L. donovani* is less polymorphic than *L. mexicana* (Cupollilo *et al*., 1995; 1998; A.l.ban uls *et al.,* unpublished observations). Many other studies showed old-world as following: *L. tropica> L. aethipica> L. major> L. donovani* (Schonian *et al.,* 2001).

The most debated factor and the controversial subject is the genetic diversity of the parasites (Baghaei, 2005). The genetic variability provides information on traits and is linked to the ancient species emergency in an area or country (e.g. vector and host diversity, the existence of various *Leishmania* spp.). Even though numerous investigations have presents the polymorphism in a natural population of various *Leishmania* spp. (Cupolillo *et al.,* 1998, 2003; Mauricio *et al.,* 2001; Schonian *et al.,* 2001). No information is available so far on the genetic diversity of the *Leishmania* parasites and the connection with eco-epidemiological features of the disease (Cupolillo *et al*., 2003; Zemanova *et al*., 2004) and with parasite evolution (Ibrahim and Barker 2001; Victoir *et al.,* 2005).

Various factors such as genetic makeup of the host, immune status, parasite features, and vector-associated factors are used to determining clinical manifestations of *Leishmania* infection. The *Leishmania* infection may show that the parasite is modified to itshost (Lainson and Shaw, 1987). Various investigations on epidemiology stated that infected human beings stay asymptomatic or show fewer or more serious processes related to pathology (Ho *et al*., 1982; Zijlstra *et al*., 1994; Sharma *et al*., 2000; Bucheton *et al*., 2003; Riera et al., 2004). However, overlap in clinical manifestations is due to dissimilar *Leishmania spp*. For example, *L. amazonensis* and *L. tropica* are reported for VL (Alexio *et al*., 2006; Alborzi *et al.,* 2006). Similarly, *L. donovani* or *L. major* reported for mucosal leishmaniasis in Tunisia and Sudan. Also, in Guyana disseminated leishmaniasis is observed due to *Leishmania guyanensis* (Couppie *et al*., 2004).

The gene order is much conserved between thirty species of *Leishmania* even if interloci distances may vary (Ravel *et al*., 1999). However, various studies reported a high level of nucleotide sequences polymorphism (Beverlet *et al.,* 1987; Ravel *et al*., 1999). Based on restriction patterns between *L. major*, the key divergence in the DNA sequence ranged from 13-25 percent. This is comparable with that observed among species of animals that diverged ten to eighty million years ago (Britten, 1986; Beverley *et al*., 1987). One another study also confirmed this level of divergence among genome comparisons in *L. major* and *L. infantum* (Ravel *et al.,* 1999). The whole sequencing of *L. infantum* and *L. braziliensis* genome will give details regarding the genetic divergence among these species. The genetic diversity of an organism can be detected by sequencing different complete genomes from various isolates of the natural population. It's not feasible to complete sequence of *Leishmania* population. Various other molecular-based methods are accessible to investigate the genetic diversity of *Leishmania* spp. PCR based assays are valuable tools for the investigation of genetic diversity within parasite species. The DNA amplification from the low number of parasites in low quantity sample volume can defeat the limitation of culturing. Various PCR-based methods have been used for *Leishmania* typing. Multilocus Enzyme Electrophoresis (MLEE) is sometimes used for *Leishmania* detection (Banuls *et al*., 2007). Multilocus Sequence Typing (MLST), RFLP, and sequencing have

been conducted for this purpose (Yehia *et al*., 2012; de Silva *et al*., 2010). The RFLP method is a simple and fast tool currently used in molecular epidemiological studies. They give useful detail on the genetic diversity and nature of the unique geographical location of the population. The PCR-RFLP method identifies small variations in a specific gene where a single deletion or addition exists by an enzyme. This method has been used for various DNA based markers such as gene loci gp63 (Victoir *et al*., 2003), antigen genes (Quispe Tintaya *et al*., 2004), microsatellites (Bulle *et al*., 2002), small subunit rRNA (van Eys *et al*., 1992), mini-exon & ribosomal internal transcribed spacer regions (Cupolillo *et al*., 2003) and extranuclear DNA such as minicircle of kDNA (kinetoplast DNA) (Yurchenko *et al.,* 2000) have been already used. In terms of validation and sensitivity, the ITS1 region offers the best resolution for the discrimination of most medically relevant *Leishmania* spp. (Odiwuor *et al*., 2011). ITS1 is the sequence between 18S ribosomal RNA and 5.8S ribosomal RNA genes. This gene consists sufficient conservation to provide as a PCR target but adequate polymorphisms to make possible typing of species and detection (Roelfsema *et al*., 2011). The ITS region evolves more quickly than the functional domains that flank and phylogenetic studies of various organisms have shown that are helpful to infer about the relationship between the directly related population of organisms (Hillis, 1987; Hillis *et al*., 1991).

Interestingly, CL is an emerging tropical and subtropical disease in underprivileged regions of AJK. The *Leishmania* parasite identification on species level is most important for clinical diagnosis, treatment, and eco-epidemiology. Previously, no report available concerning the occurrence of *L. major* and *L. infantum* in CL patients of all districts of AJK. In AJK, anthroponotic CL caused by *L. tropica* is believed to be the only species causing CL (Shaheen *et al.,* 2020). Hence, this study was designed to identify *Leishmania spp.* circulating in AJK by using two molecular-based techniques: microscopy and the ITS1 PCR-RFLP. The sequencing was done for phylogenetic analysis and genetic variability.

MATERIALS AND METHODS

3.1. Ethical Statement

A district-based active CL surveillance was conducted by the research team members of Quaid-i-Azam University Islamabad, Pakistan. The study was conducted in 2019 with the same approval as described in chapter 2.

3.2. Study Area and Sample Collection

The sample collection was based on the methodology described in chapter 2 from the local population of AJK. The skin scraps (n=13761) from suspected patients of CL infection and blood drops (n=567) were collected for *Leishmania* detection by following methodology same used in chapter 2 in 2019. The suspected inhibitants participated in 2018 (511/20000) and 2019 (567/13761) were different. The study area is shown in the map of AJK in chapter 2 (Fig.2.1).

3.3. DNA Extraction

DNA extraction DBSs on the cards (567) were cut into small pieces and transferred into 1.5ml eppendroph tube and soaked in 40µl PBS, and incubated aovernight at 37^{o}C. The genomic DNA was isolated from blood using the DNeasy Blood and tissue kit (Qiageen, Valencia, USA) by following manufacturerer's protocol.

3.4. DNA Concentration

The DNA concentration were obtained by measuring at 260nm (A_{260}), and assuming that an A_{260} of 1 is equal to 35ng/µl pure ssDNA. The DNA concentrations were measured by a smartspec plus spectrophotometer (Bio Rad, Hercules, USA).

3.5. Amplification of ITS1 Region

The microscopically positive samples (n=567) were subjected to PCR amplification. The reaction mixture, primers, and thermal cyclic conditions used were as same described in chapter 2. The uninfected blood was used as a negative whereas MW139334-340, MW114510- MW114522, MW165326, and MW183336 *Leishmania* spp. confirmed samples were used as a positive control. The amplified products were analyzed by electrophoreses following the same steps used in chapter 2.

3.5. Restriction Fragment Length Polymorphism of Amplified ITS1

For the RFLP analysis, amplified products were digested with BsuR1 enzyme (HaeIII) by following the manufacturer's instructions described in chapter 2.

3.6. Sequencing and Accession Numbers of Nucleotide Sequences

The samples were purified using methodology used in chapter 2. The partial sequences of the ITS1 gene obtained in the current study were deposited in the Gene Bank database under accession numbers MW114510.1-MW114521.1, MW139334.1-MW139340.1, MW165326.1, and MW183336.1.

3.7. Polymorphic Analysis of ITS1 Region

Clustral-W multiple sequence alignment program was used to viewed and analyzed the nucleotide sequences. The sequences were compared using the BLAST algorithm to the homologous sequences in the gene bank database (Camacho *et al*., 2009). The sequences were edited and assembled with the BioEdit (7.2.5.0 version) to identify nucleotide variations by BioEdit (7.2.5.0 version) software (Hall, 1999). A molecular phylogenetic tree was constructed through *L. tropica, L. major,* and *L. infantum* sequences obtained from different areas of AJK as well as available published sequences in the Gene Bank by the Neighbor-Joining (NJ) method using MEGA6 (Tamura *et al*., 2013). The NJ tree reliability was evaluated by the bootstrap method with one thousand replications. The nucleotide diversity was calculated by Tajima test using BioEdit (7.2.5.0 version) software (Tajima, 1989; Nei and Kumar, 200).

3.8. Statistical Analysis

The incidence of CL count was analyzed to the season, gender, age, and species. The initial data investigation confirmed that data count is over-dispersed having 23.625 mean that is lower than variance i.e. 1.9576 and also zero-inflated. For this reason, we used 3 models in R software described in chapter 2. The two diagnostic methods PCR and microscopy Cohen Kappa was calculated in SPSS V.21 (Cunningham *et al*., 2012). Annual parasitic rate, slide positivity rate, and annual bold examination rate was calculated by using same formulas in chapter 2. BioEdit software was for the analysis of the sequence variation. The MEGA 6 software was used to contract the phylogenetic tree.

RESULTS

Out of 13,761 CL suspected inhabitants based on visual inspection of the skin lesions and ulcerative skin, 567 samples were microscopically positive while 13194 were negative. In the current findings, the highest positive cases of CL were observed in district Mirpur 4.87% (118/2431) and Muzaffaarad was the 2nd highly affected district having 5.74% (106/1847) of CL cases. The district Kotli ranked at 3rd number showing 4.41% (88/1995) CL positive cases followed by Bimber 4.24% (56/1321), Bagh 4.65% (54/1161), Sudhnoti 3.42% (48/1402), Hattian 3.82% (46/1205), Poonch 2.14% (23/1076), and Haveli 1.81% (19/1050). The district Neelum showed the lowest positive cases of CL infection, i.e. 3.29% (9/273). The occurrence of CL infection in the local inhabitants of AJK was 4.12% in the total population studies (n=567/13761) (Table 3.1).

Table 3. 1: District-wise Distribution of CL infection in Azad Jammu and Kashmir, Pakistan

District name	**Population studies**	**Suspected cases**	**Microscopic positive slides**	**Negative slides**	**SPR (%)**	**API per 1000**	**BER (%)**
Mirpur	564,045	2431	118	2313	4.85	0.21	0.43
Muzaffarabad	665,744	1847	106	1741	5.74	0.16	0.27
Kotli	957,157	1995	88	1907	4.41	0.09	0.21
Bimber	520,059	1321	56	1265	4.24	0.11	0.25
Hattian	285,026	1205	46	1159	3.82	0.16	0.42
Poonch	618,905	1076	23	1053	2.14	0.04	0.17
Sudhnoti	3667,932	1402	48	1354	3.42	0.01	0.04
Havali	188,086	1050	19	1031	1.81	0.10	0.55
Bagh	459,840	1161	54	1107	4.65	0.12	0.25
Neelum	236,462	273	9	264	3.29	0.04	0.12
Total		13761	567	13194	3.84	0.01	0.27

The total slide positivity rate (SPR) in the present investigation (2019) was 3.84% in all districts of AJK. The district Muzaffarabad showed the highest SPR, i.e. 5.74%, and the lowest observation in Haveli (1.81). The SPR of CL was Mirpur, Bagh, Kotli, Hattian, Sudhnoti, Neelum, and Poonch having 4.85, 4.65, 4.41, 4.24, 3.82, 3.42, 3.29, and 2.14 respectively. The annual parasitic rate (API) was 0.01 per 1000 population in all cases under study. The highest (0.21) API was shown in district Mirpur, while the lowest (0.01) observed in district Sudhnoti. The districts Muzaffarabad and Hattian showed similar API, i.e. 0.16. On the other hand, Bagh, Bimber, Haveli, and Kotli showed 0.12, 0.11, 0.10, and 0.09, respectively. The district Neelum and Poonch 0.04 also showed the same API. The total BER has observed in the current study was 0.27. The district Haveli showed highest (0.55) BER while lowest (0.94) in district Sudhnoti. The observed BER in districts Mirpur, Hattian, Muzaffrabad, Kotli, Poonch, and Neelum were 0.43, 0.42, 0.27, 0.21, 0.17, and 0.12 respectively. The district Bimber and Bagh showed the same BER, i.e. 0.25 (Table.3.1).

3.8. Season-Wise Distribution of CL

The observations of the present investigation stated that CL distribution is temperature-dependent. The highest cases of CL were observed during more temperature months, i.e. summer 47.79% (271/567) (June-September) followed by spring 20.46% (116/567) (March to May), Autumn 28.92% (164/567) (October to November) and Winter 2.81% (16/567) (December-February). The expected log was recorded for CL cases with the highest for summer, i.e. 0.49249 while in winter showed the lowest expected log, i.e. -2.35064. The predicted log for autumn was -1.20716 showed lowest then winter, i.e. -2.35064 (Table 3.2).

Table 3.2: Negative binomial regression analysis presenting the effect of age, gender, season, and *Leishmania* species

Coefficient	Categories	Estimate	Std. Error	Z value	Pr(>Iz I)
Intercept		3.5578	0.3033	11.729	< 2e-16 ***
Age in years	1-20	1.68332	0.12256	-13.73	< 2e-16 ***
	21-40	-1.03770	0.10058	-10.317	< 2e-16 ***
	41-60	-1.87448	0.18074	-10.371	< 2e-16 ***
Gender	Male	0.57252	0.08769	6.529	6.61e-11 ***
	Female	-2.98528	-0.21561	-13.84	4.51e-5
Seasons	Spring	-0.36963	0.13247	-2.790	0.00527 **
	Summer	0.49249	0.10538	4.673	2.96e-06 ***
	Winter	-2.35064	0.26726	-8.795	< 2e-16 ***
	Autumn	-1.20716	0.03604	-33.49	< 2e-16 ***
Species	*L. tropica*	0.13500	0.28444	0.475	0.63507
	L. major	0.09048	0.29351	0.308	0.75787
	L. infantum	-3.46732	0.00979	-11.421	0.06212

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

3.9. Gender and Age-wise Distribution of CL Infection

The proportion of the affected population under the current study revealed that the highest infection rate was observed in males (63.84%) than females (36.16%). The male population showed the highest (0.57252) expected log (incidence) value than females (-2.98528) that were used as a constant for other variables. The most affected age group of CL patients were 1-20 years ie. 66.67% (378/567) while 23.63% (134/567) was observed in 21-40 years old followed by 9.70% (55/567) in 41-60 years old. The males were observed more frequently affected than females in all age groups. The males were more affected by 50.92% (232/567) than females 25.75% (146/567) at the age group of 1-20.

The infection rate was 17.28% (98/567) at the 21-40 years in males and 6.35% (36/567) in females, while 5.64% (32/567) in the age 41-60 years in males and 4.06% (23/567) in females. On base upon log scale, the CL incidence in the 1-20 years old age was 1.68332 which was the highest between the all observed age group i.e. 21-40 (-1.03770) and 41-60 (-1.87448) (Table 3.2).

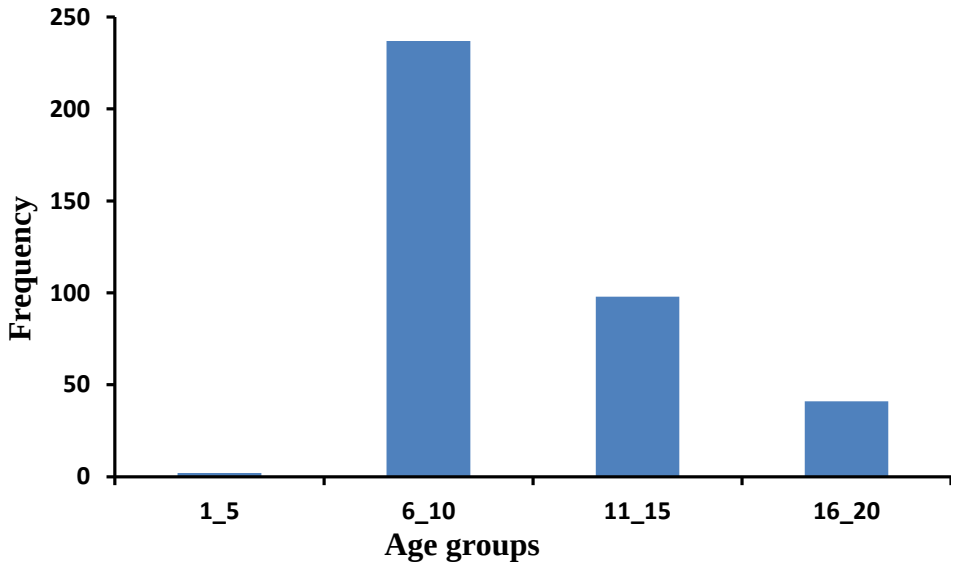


Figure 3.1: Distribution of most affected ages 1-20 in sub-categories

In our previous (Shaheen *et al.,* 2020) and current study, we observed that 1-20 age groups are the most susceptible group for CL infection. Therefore, the most affected age, i.e. 1-20 was sub-divided into other four categories, i.e. 1-5, 6-10, 11-15, and 16-20 year of age group. We have observed 0.53% (2/378) in 1-5, 62.69% (237/378) in 6-10, 25.93% (98/378) in 11-15, and 10.85% (41/378) in 16-20 years of age group (Fig 3.1.).

3.10. ITS1-PCR Amplification of CL Infection

The microscopically positive CL samples (n= 567) obtained from ten districts of AJK, were subjected to ITS-PCR for ITS1 regions amplification. The PCR resulted in the amplification of CL isolates giving about 300-350bp amplification band size under UV transilluminator. The blood samples from uninfected people were used as negative control while SUB8322782.1, MW139334.1-340.1, MW114510.1-MW114522.1, MW165326.1,

and MW183336.1 were used as a positive control. According to the results obtained by PCR diagnosis, 98.94% were found positive, while 1.06% was negative. The district-wise distribution of PCR positive samples was highest in Mirpur 20.81% (118/567) and lowest in district Neelum 1.58% 9/567. The CL positive cases by PCR in district Muzaffarabad 18.69% (106/567), Kotli 15.16% (86/567), Bimber 9.87% (56/567), Bagh 9.52% (54/567), Sudhnoti 7.94% (45/567), Hattian 8.11% (46/567), Poonch 4.06% (23/567) and Haveli 3.17% (18/567). The 1.06% (6/567) samples were unsuccessful for amplification of the ITS1 region. The comparison of two diagnostic tests (microscopy and ITS-PCR) showed 0.821 Kappa agreements with a significant value of 0.000, while 0.0025 was a standard error.

3.11. Analysis of RFLP of Amplified Products

The findings of amplified products digestion with HaeIII enzyme showed the fragments of 210bp and 140bp in 3.71% (21/567) samples such pattern was observed in the positive control MW139334- MW1393340 for *L. major.* The fragments of 200bp and 60bp in 94.89% (538/567) sample the same pattern seen in the positive control MW114510- MW114522 for *L. tropica.* The 0.35% (2/567) fragments of 200bp, 80bp, and 40bp showed *L. infantum* pattern observed in the positive control MW165326, and MW183336. Twenty-one CL patients showed no long term history except to see medical attention. Two unexpected species of *L. infantum* patients had traveled history of Italy and Spain. To date, *L. infantum* has not previously been reported to cause CL in AJK. The *L. tropica* 94.89% (538/567) was observed in all four seasons (winter, spring, summer, and autumn). The 3.71% (21/567) cases of *L. major* was reported in spring, summer, and autumn while the 0.35% (92/567) *L. infantum* in summer. Unsuccessful results were 1.06% (6/567). The most affected age group of *L. tropica* was 1-20 years i.e. 25.75% (364/567) followed by 21-40 22.05% (125/567) and 41-60 years old age 8.64% (49/567). In the case of *L. major* 1.41% (8/567) in 1-20, 1.41% (8/567) in 21-40, and 0.88% in 41-60 years old age. The 0.35% (2/567) cases of *L. infantum* was reported 41-60 years old age group (Fig. 3.2).

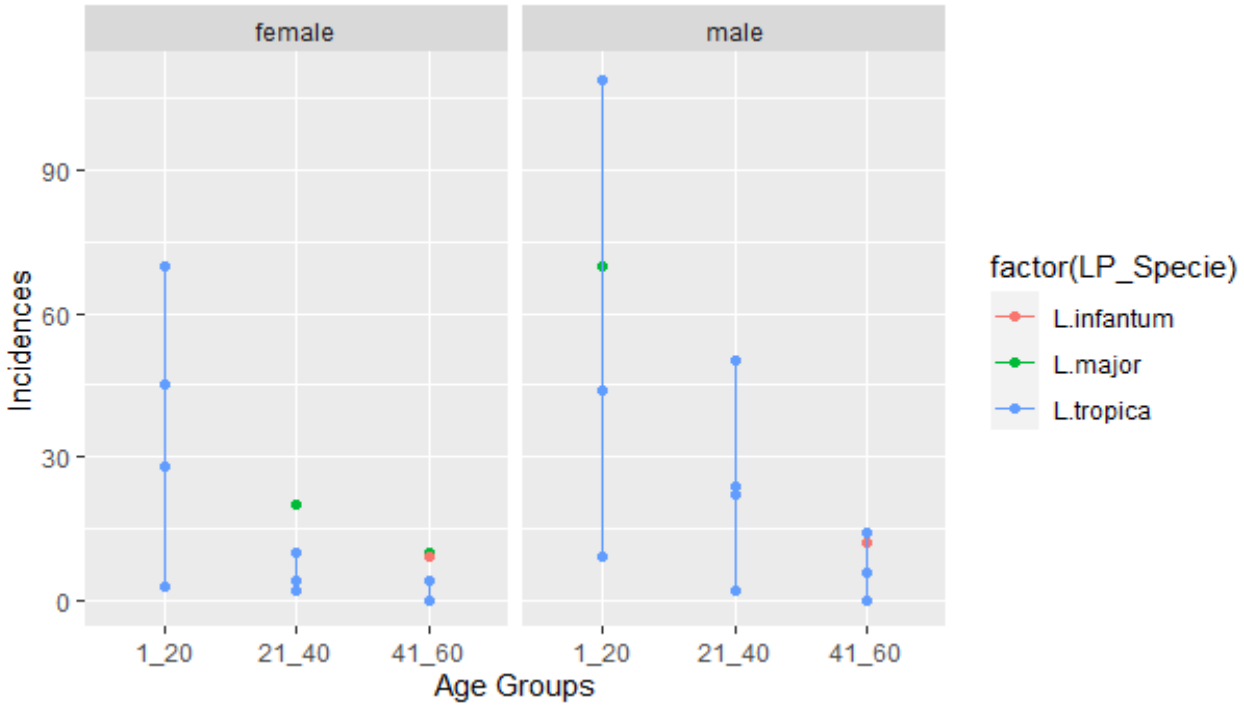


Fig. 3.2: Age-wise distribution of *Leishmania* spp. (*L. tropica, L.major,* and *L. infantum*)

3.12. Phylogenetic Analysis and Polymorphism of ITS1 Region

Two samples from the districts of Muzaffarabad, Poonch, Sudhnoti, Haveli, Hattian, Bimber, Neelum, and Bagh, and three from district Mirpur and Kotli, were subjected to sequence analysis. The ITS1 sequence in the current research from North Eastern Pakistan confirmed that *L. tropica* (according to the BLAST similarity) is the primary cause of ACL, while *L. major* of the ZCL. Two autonomous sequences of *L. infantum* were also observed from districts Mirpur and Kotli. The Neighbor Joining tree was constructed by Mega 6 software. The constructed phylogenetic tree in Fig.3.3. showed the sum of branch length 0.33298561 and the bootstrap test used was 1000 replicates (Felsenstein, 1985). The analysis involved 50 nucleotide sequences and the codon positions included in the analysis were 1^{st} + 2^{nd} + 3^{rd}+ noncoding. The positions had missing data and gaps were removed.

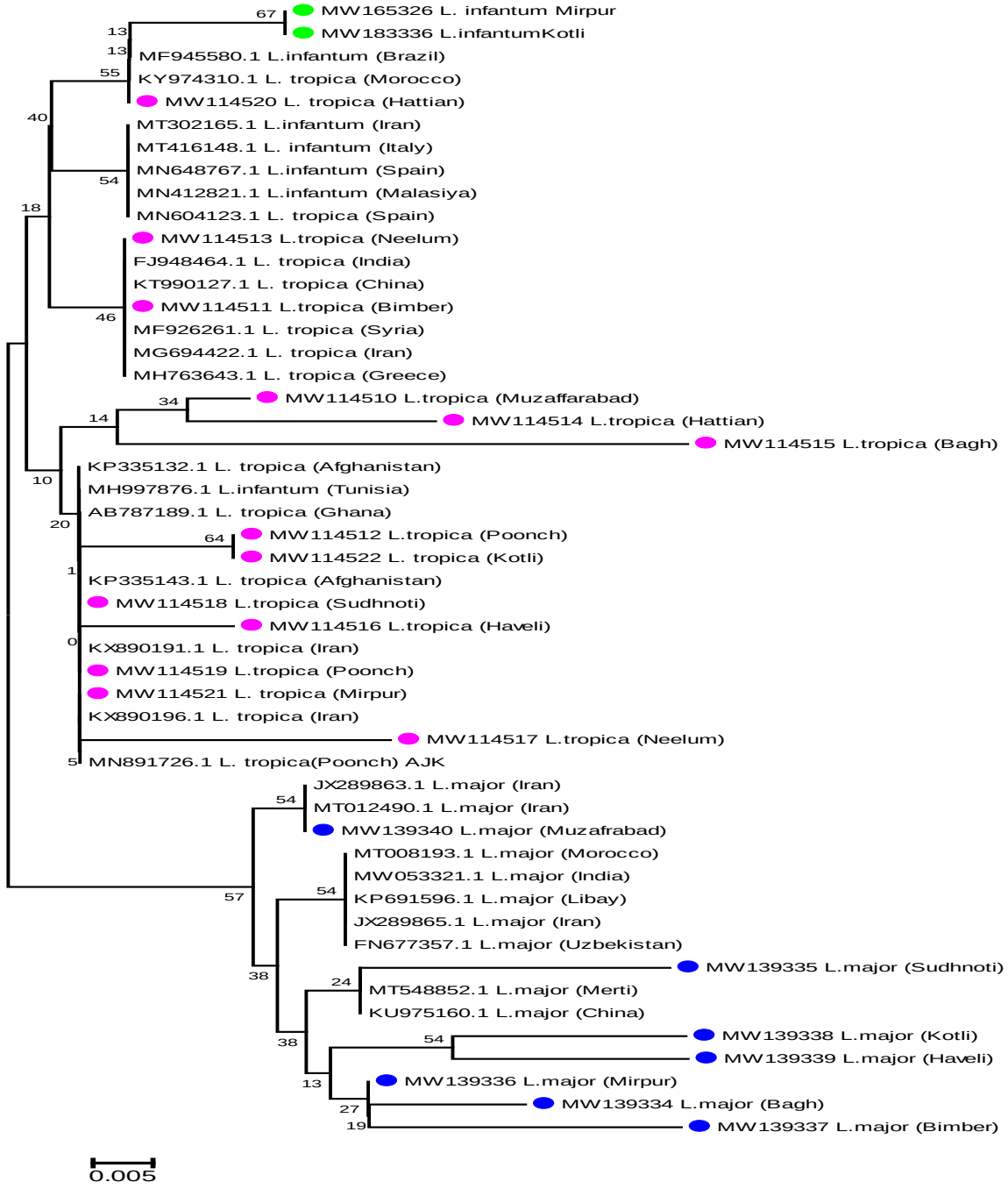


Fig. 3.3: Neighbor-joining phylogenetic tree presents the relationship of *Leishmania* spp. circulating in AJK with other countries based on ITS1 gene sequence using mega software. The published sequences of other countries for the same gene ITS1 of *Leishmania* spp. were taken from NCBI.

Table: 3.3: Comparison of ITS1 sequence similarities and nucleotide variations of *L. tropica, L. major*, and *L. infantum* from AJK with highly similar ITS1 published sequences (NCBI) from different countries.

Species name	Species ID	District name	Country name	Homology %	Genetic variations
L. tropica	MW114510	Muzafrabad	Afghanistan (KP335132.1).	98	Nucleotide # 4 Position # 8 (T), 82 (A), 217 (C), 218 (C).
	MW114511	Bimber	Syria (MF926261.1)	99	Nucleotide # 3 Position # 8 (T), 82 (A), 218 (C).
	MW114512	Poonch	Afghanistan (KP335143.1)	99	Nucleotide # 2 Position # 68 (A), 98 (C)
	MW114513	Neelum	India (FJ948464.1)	98	Nucleotide # 4 Position # 14(T), 89(A), 98(G), 198(C).
	MW114514	Hattian	Afghanistan (KP335132.1).	98	Nucleotide # 9 Position # 76 (A), 77 (T), 84(A), 85(G), 156(A), 157(T), 171(C), 172 (G), 242(T)
	MW114515	Bagh	Afghanistan (KP335132.1).	98	Nucleotide # 5 Position # 65(A), 121(T), 128 (T), 131 (A), 37(A).
	MW114516	Haveli	Iran (KX890191.1)	98	Nucleotide # 4 Position # 16 (C), 73 (C), 94 (C), 242 (A).

	MW114517	Neelum	Iran (KX890196.1)	98%	Nucleotide # 7 Position # 38 (C), 44 (A), 62 (T), 92 (C), 112 (A), 165 (A), 271(C).
	MW114518	Sudhnoti	Afghanistan (KP335143.1)	98	Nucleotide # 6 Position # 71 (A), 72 (G), 168(A), 169(T), 200 (G), 201(G)
	MW114519	Poonch	Iran (KX890196.1)	99%	Nucleotide # 2 Position # 169 (A), 199 (G)
	MW114520	Hattian	Morocco (KY974310.1)	100	-
	MW114521	Mirpur	Iran (KX890196.1)	99%	Nucleotide # 2 Position # 213 (T), 223 (T)
	MW114522	Kotli	Afghanistan (KP335143.1)	98	Nucleotide # 6 Position # 23 (A), 24 (G), 45 (C), 46(C), 52(C), 53(G).
L. major	MW139334	Bagh	China (KU975160.1).	98	Nucleotide # 4 Position # 9(G), 12(A), 16(T), 70(A), 156(G), and 160(T).
	MW139335	Sudhnoti	Merti (MT548852.1)	97	Nucleotide # 4 Position # 18 (A), 92 (G), 150(A), and 105(T).
	MW139336	Mirpur	China (KU975160.1).	98	Nucleotide # 3 Position # 79(T), 1679A), and 191(C).

L. infantum	MW139337	Bimber	China (KU975160.1).	96	Nucleotide # 6 Position # 30(A), 75(T), 123(T), 152(C), 162(A), and 185(C)
	MW139338	Kotli	China (KU975160.1).	97	Nucleotide # 4 Position # 27(G), 138(A), 158(C), and 182(C).
	MW139339	Haveli	China (KU975160.1).	97	Nucleotide # 3 Position # 86 (G), 142(A), 188(C).
	MW139340	Muzaffarabad	Iran (MT012490.1)	98	Nucleotide # 1 Position # 122 (T)
	(MW165326)	Mirpur	Italy (MT416148.1)	97	Nucleotide # 7 Position # 15(A), 36(A), 123(T), 130(C), 1519C), 172(T), 208(A).
	(MW183336)	Kotli	Brazil (MF945580.1)	99	Nucleotide # 1 Position # 137(T).

Despite increasing CL infection cases in AJK, there is no genetic information available to compare the relationship between the causative agents of the disease in different areas of AJK. Current study revealed for the first time the genetic variations of *L. tropica, L. major,* and *L. infantum* circulating in AJK. The ITS1 sequences of *L. tropica, L. major*, and *L. infantum* presents various nucleotide changes from the published sequences in NCBI.

In the current study, the ITS1 gene sequences of *L. tropica* (MW114510-MW114522), *L. major* (MW139334- MW1393340), and *L. infantum* (MW165326, and MW183336) were compared with the published sequences of the countries, which showed a closed relationship in NJ tree. The published sequences were obtained from NCBI. The sequences (MW114520) from the district of Hattian showed the highest similarity (100%) with Morocco (KY974310.1). The sequences from the districts of Neelum (MW114513), Poonch *(*MW114519), Bimber (MW114511), Hattian (MW114514), Bagh (MW114515), Muzaffarabad (MW114510), Neelum (MW114517), Poonch (MW114512), Kotli (MW114522), Sudhnoti (MW114518), Haveli (MW114516), Kotli (MW114522), and Mirpur (MW114521) showed 98-99% homology with published sequences from Afghanistan (KP335132.1), Syria (MF926261.1), India (FJ948464.1), and Iran (KX890191.1). The ITS1 gene sequences of *L. major* Muzafrabad (MW139340.1), Sudhnoti (MW139335), Kotli (MW139338.1), Haveli (MW139339), Mirpur (MW139336.1), Bagh (MW139334.1), and Bimber (MW139337) exhibited 96-98% homology. In the phylogenetic tree, the *L. major* sequences from Bagh (MW139334.1), Mirpur (MW139336.1), Kotli (MW139338), Haveli (MW139339.1), Bimber (MW139337), Sudhnoti (MW139335), and Muzaffarabad (MW139340.1) showed a close relationship with published ITS1 sequences of *L. major* from China (KU975160.1), Merti (MT548852.1), and Iran (MT012490.1). The two unexpected isolates from the districts of Mirpur (MW165326) and Kotli (MW183336) showed a close relationship with published sequences of ITS1 region of *L. major* previously reported from Italy (MT416148.1) and Brazil (MF945580.1) with 97 and 99% identities with respective homologous sequences

3.12.1. Statistical Analysis of Nucleotide Variation in ITS1 Gene

In the current study, the inter-genetic diversity in ITS1 gene sequence of *L. tropica* and *L. major* from different districts ((Mirpur, Muzaffarabad, Poonch, Sudhnoti, Haveli, Hattian, Bimber, Neelum, Bagh, and Kotli) of AJK was determined by using Tajima test. The sequences used in the current study were subjected to Mega 6 software for multiple alignment by CLUSTAL W. The nucleotide diversity was determined by the Tajima test in mega 6 software.

Table 3.4: The nucleotide diversity of the ITS1 sequences between *L. tropica* and *L. major*

Leishmania spp.	***M***	***S***	***Π***	***D***
L. tropica	13	22	0.02	-1.64
L. major	7	14	0.05	-1.10

M = number of sequences, *S* = number of segregating sites, π = diversity of nucleotides, and *D* is the statistic value for the Tajima test.

The analysis for *L. tropica* involved 13 nucleotide sequences. In the final data set, there were a total of 179 positions. For *L. major,* 7 nucleotides were reviewed. There were a total of 82 locations. The analysis removed all positions containing gaps and missing data. Codon positions included were 1st+ 2nd+ 3rd+ noncoding. The nucleotide diversity of *L. infantum* was not calculated via the Tajima test because this study generated only two sequences and this analysis required three or more. There was also no *L. infantum* sequence available in the gene bank from AJK for comparison. The *L. tropica* (0.02) sequences had more nucleotide diversity than *L. major* (0.05). The D value was -1.64 for *L. tropica* and -1.10 for *L. major* sequences (Table.3.4).

DISCUSSION

In Pakistan, the distribution of CL shows broad patterns and it is spread constantly in neighboring areas. Several outbreaks in the Southern and Northern areas of Pakistan are currently being recorded. It is hypothesized that the non-endemic territory that adjoins borders with endemic regions is particularly at risk of CL infection. This study was conducted in North Eastern Pakistan, geographically in contact with Khyber Pakhtunkhwa, Punjab, Gilgit Baltistan, and India Administered Kashmir, which is prone to CL infection (Ayub et al., 2013; Kakarsulemankhel, 2004; Qureshi et al., 2016; Noor and Hussain, 2017; Aara et al., 2013). Our previous investigation (2018) attempts to highlight North Eastern Pakistan as a new CL endemic area (Shaheen et al., 2020). In the current study, we determined the molecular characterization of new foci and genetic diversity of *Leishmania spp*. from North Eastern Pakistan. We found that the spread of CL is positively affected by seasonal distribution as the vector (sand-fly) is more active throughout the more temperate months than in colder months throughout the year (Shaheen et al., 2020). The most cases of CL in Pakistan occur from April to September due to the sand fly activity being elevated in these months. The activity is also higher in regions located at altitudes 1500-1800m above sea level (Marco et al., 2006; Durrani at al., 2011). A study conducted by Zeb et al., in (2021) reported the most cases at high altitudes. The sand flies also annually migrate from sub mountainous terrains to valleys to avoid the environmental conditions (Iftikhar et al., 2003). In their natural habitats, the sand flies were more active in the evening, night, and morning. The sand flies are able to survive in the dry environment by withdrawing to cool, humid resting sites during the day and then becoming active at night when the ambient temperature dropped and the humidity increased (Jaffernay and Nighat, 2001; Kuzeo, 1993).

Taken all together our results show that male patients were predominantly affected by leishmaniasis. This might be due to the fact that males are at the highest risk of acquiring CL due to their occupational exposure such as sleeping outside in summer or traveling (Bashaye *et al*., 2009; Mengesha *et al.,* 2014). Moreover, they may also differ in attitude

toward seeking and providing treatments (Kassi *et al*., 2008). Current findings are inconsistent with a previous study done by El Buni *et al*. (2000).

In the current study, the incidence of disease was observed in all age groups. However, the highest frequencies 66.67% (378/567) and the lowest 9.70% (55/567) were found among 1-20 and 41-60 years-old individuals. Similar results were reported by our previous study conducted in 2018 (Shaheen *et al.,* in 2020). The most affected sub-category amongst 1-20 years old was 6-10 years old in the current investigation. The potential explanation for these distinctions may be associated with incomplete body coverage in children, unawareness of the risks carried by the sand-fly, and the habit to play outside of the home. Other reasons may also be associated with malnutrition and compromised immune system (Karami *et al*., 2013; Zijlstra *et al*., 2016).

However, the age group of 16-50 years old was reported to have the highest prevalence of vectors and reservoir areas such as Isfahan. On the contrary, the five to six-years old age-group was to be reported as the highly infected group (Momeni and Aminjavaheri, 1994; Pourmohammadi *et al*., 2008). Hence, it can be concluded that infection occurrence rates in different regions vary upon the age group and study place. It should be kept in mind that while most people develop immunity against the disease, the incidence rate gradually decreases in older adults (Sadeghi Nejad, 2000). However, the differences were not statistically significant. The female participants in our study was limited, and most of them refused to participate due to tradition and social norms in the North Eastern Pakistan. Many studies showed no gender disparity suggesting equal exposure to infection (Salam *et al*., 2014; Yadon *et al.,* 2013).

In the current investigation, out of 567 microscopic positive cases of CL, 561 were successfully amplified by ITS-PCR. The PCR negative findings might involve various factors, that includes false microscopic identification, poor sample handling resulting in contamination, because microscopy analysis is time-consuming and need more expertise, and less (<5ng/µl) quantity of sample on filter paper (Boggild *et al.,* 2010). Current findings also stated excellent concordance between ITS-PCR and microscopy, i.e. 0.8. A

similar agreement level was also previously reported (Gao *et al*., 2015; Shaheen *et al*., 2020).

In most investigations, the causative agent of CL is either not reported or a specific species is assumed by conventional methods based on previously reported investigations. Yet, various epidemiological studies also described the occurrence of *L. tropica* in all provinces of Pakistan including the North Eastern part of Pakistan (Noyes et al., 1998; Marco *et al.,* 2006; Shaheen *et al.,* 2020). In the current study, conventional and molecular methods (PCR-RFLP and sequencing) show that three different *Leishmania spp*. were found in the study area. The ZCL in Pakistan, caused by *L. major* is mostly reported in Sindh and Southern Baluchistan bordering Iran. Although, it has always been considered anthroponotic in KP (Qureshi *et al.,* 2016). The ZCL focus (*L. major* cases) had dry habitat. Still, broad crop growing, using the canal and irrigation system, favors crowded populations of rodents and vectors. The main elements in the development of new diseases include a rise in the populations of the natural zoonotic CL animal reservoirs and their related vector sand fly. However many animal reservoirs are still unknown in different parts of Pakistan (Kolaczinski et al., 2004; Bhutto et al., 2003). The introduction of new ZCL foci may be closely linked to human activities e.g. people migration, climate change, natural disasters, industrial projects, and agricultural activates (Ben Salah, 2007). The current findings of *L. major* predict the likelihood of encountering new prevalent ZCL foci in North Eastern Pakistan. *Leishmania infantum* is the causative agent of autochthonous VL, CL, and mucocutaneous lesions in Italy, Cyprus, Malta, and France region. CL caused by *L. infantum* is a sporadic infection that has been described in VL endemic patients. Many areas of Pakistan and AJK are known for VL by *L. infantum* (WHO, 1991; Alvar, 2012; Rab *et al*., 1989). To the best of our knowledge, the current investigation is the 1st report of autochthonous *L. infantum* infectivity from CL patients in North Eastern Pakistan. The first two cutaneous *L. infantum* cases reported from the districts of Mirpur and Kotli were imported in this district since the patients have travel histories of Spain and Italy. This region was already suspected to have *L. infantum* transmission (Portu *et al.,* 1986; Pozio et

al., 1985). Unexpectedly, *L. infantum* spp. from CL patients were also obtained by Khan *et al*., (2016) from Pakistan.

Despite the increasing cases of CL in North Eastern Pakistan, there is not enough genetic information to analyze the relationship between the causative agents of Leishmaniasis. In this study, we have used ITS1 gene to evaluate the genetic diversity of *Leishmania* spp. Even though the ITS1 gene serves as a sign for *Leishmania* spp. differentiation, limited studies employed the ITS1 sequence to compare *L. tropica* sequence (Khanra *et al*., 2011). In the current study, the phylogenetic analysis revealed that Pakistani isolates are identical to those found in India, Syria, Afghanistan, Iran, Morocco, Merti, and China. Notably, there are many reports on *Leishmnaia* spp.′ presence from these areas (Fakhar *et al*., 2016; Hayani et al., 2015; Kahime *et al*., 2014; Wang *et al*., 2012; Ghatee *et al*., 2018).

In the current investigation, significant nucleotide variations were found in the ITS region of *L. tropica, L. major,* and *L. infantum* from different areas of North Eastern Pakistan. This genetic variation may associate with the assortment of medical manifestations and geographical distributions. The genetic diversity in *Leishmania spp.* suggested that the spread of CL infection may be attributed to migration, abundant population with impaired immunity, entry of infected population into the disease-free region, and changing travel patterns and geographical disease expansion (Eroglu *et al.,* 2015). The occurrence of genetic variability in *L. tropica*, and *L. major* may be involved in treatment failure and/or emergence of new haplotypes. The occurrence of treatment failure, resistance, and low response are also reported from some endemic areas of the world (Ponte Sucre *et al.,* 2017). Various mechanisms have been anticipated to explain the lack of response to drug treatment (Vanaerschot *et al.,* 2014) such as *Leishmania* RNA (LRV) belongs to family Totiviridae which infect *Leishmania* (Hajjaran *et al.,* 2016; Zangger *et al.,* 2013; Scheffter *et al.,* 1995). The LRV is a double strand RNA, which suggested as a virulence factor and exist in some *Leishmania* isolates in some reports Hajjaran *et al.,* 2016; Zangger *et al*., 2013; Scheffter *et al.,* 1995; Guilbride *et al.,* 1992).

Therefore, more extensive studies on genetic analysis are needed from all regions of North Eastern Pakistan, especially using a larger sample size.

CONCLUSION

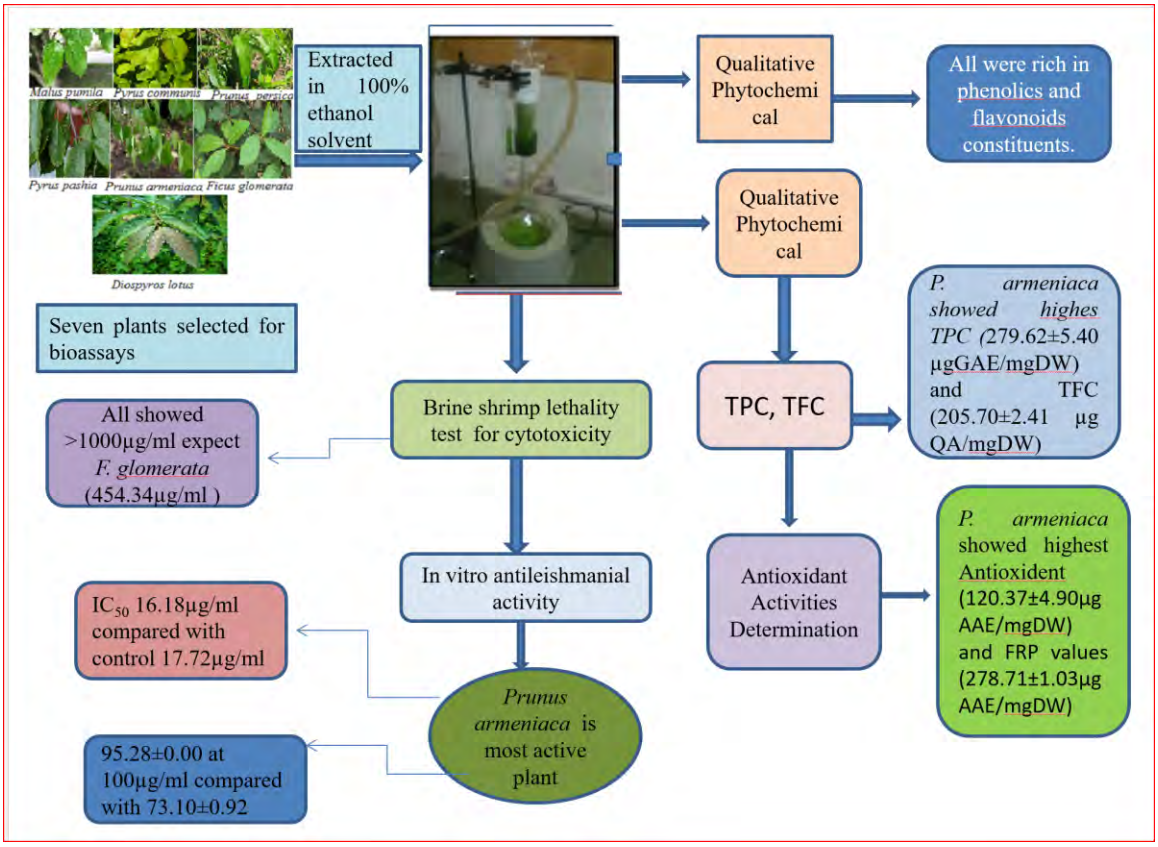
The current investigation concluded that in North Eastern Pakistan CL infection appeared as a severe health issue. The *Leishmania* spp. (*L. tropica* and *L. major*) with remarkable heterogeneity is predominantly circulating in North Eastern Pakistan. The occurrence of nucleotide variability may be causing treatment failure or emergence of new haplotypes. However, extensive investigations are needed from various geographical areas of North Eastern Pakistan for further species identification, using a large scale sample size. Identifying potential host and vectors for *L. infantum* in the study area must be addressed.

Evaluation of Safety, Antileishmanial, and Chemistry of Ethanolic Leaves Extracts of Seven Medicinal Plants: An *In-vitro* Study

ABSTRACT

Cutaneous leishmaniasis (CL) currently affects people among 102 countries and causes significant morbidity and mortality including Pakistan. Current chemotherapeutic interventions are unsatisfactory and have limitations. Thus there is a need to search for new effective and safe medicines for CL. Therefore the current study was based upon the use of medicinal plants to evaluate their antileishmanial potential cytotoxicity and phytochemistry which could be used for drug formulation against CL. The ethanolic leaves extracts of *Pyrus pashia*, *Malus pumila* , *Prunus persica, Pyrus communis*, *Prunus armeniaca*, *Ficus glomerata,* and *Diospyros lotus* were obtained using soxhlet apparatus, diluted to make various concentrations (0.5, 5, 25, 50, and 100µg/ml) and applied in an *in-vitro* bioassay to evaluate antileishmanial activities (*L. tropica*). The cytotoxicity analysis was conducted by using brine shrimp lethality assay. The phytochemical screening was conducted using standard protocols. The antioxidant potential of plant extracts was determined by total antioxidant capacity, ferric reducing power, and DPPH radical scavenging assays. All the experimented plants showed cytotoxicity in safety range >1000µg/ml except *F. glomerata* (LC_{50} 454.34µg/ml). The *P. armeniaca* demonstrated the highest antileishmanial activity (IC_{50} 16.18µg/ml). The phytochemical investigation showed the existence of the highest total phenolic (279.62±5.40µgGAE/mgDW) and flavonoid contents (205.70±2.41µgQA/mgDW) in *P. armeniaca* with the strongest antioxidants (120.37±4.90µgAAE/mgDW) and FRP values (278.71±1.03µgAAE/mg DW). These findings are highly encouraging so, further and extensive investigations of *P. armeniaca* should be carried out; especially bio guided fractionation to identify the active fraction and further chemical characterization of structure.

GRAPHICAL ABSTRACT



INTRODUCTION

The most neglected tropical disease in 102 countries with weak scioecomic condition is cutaneous leishmaniasis (CL) (Neris *et al.,* 2013). In Pakistan, the most prevalent form of CL is *L. tropica* and its effect on public health is immense (Booker *et al.,* 2004; Torres Guerreror *et al*., 2017). The spread of CL increase to non-endemic areas of Pakistan is due to the migration of millions of refugees toward North Western Pakistan (Alvar *et al.,* 2012). Up to now, there is no effective vaccine available for the CL treatment (Bekhit *et al.,* 2018). Chemotherapy is the main approach for the treatment of CL infection and these are not effective and cause serious effects (Camacho *et al.,* 2003).

The pentavalent antimony compounds were used as a first-line dose (20-28mg/kg/day) treatment for CL for one month. It needs intramuscular or intravenously injection administration, and also have many side effects, and resistance. The drug consist in two formulation including sodium stibogluconate (pentostam) and meglumine antimoniate (glucantime) is usually recommended since1950 (Camacho *et al.,* 2003) and it treats amastigotes of both CL and VL (Camacho *et al.,* 2003; Sen and Chatterjee, 2011; Mohapatra, 2014). In Pakistan, pentavalent antimony compound glucantime is commercially available used against CL infection, Iran, Afghanistan, and other parts of the world (Saebi, 2005; Kheirandish *et al*., 2011; Mahmoudvand *et al.,* 2011; Rashid *et al*., 2016). There is still no understanding of the mechanism of action. These drugs stop activity of the fatty aids oxidative and glycolytic pathways to decrease ATP in the amastigote form (Lindoso *et al.,* 2012). Furthermore, the pentavalent form used thiols from the host and parasite cell surface and it changed into trivalent which showed activity and toxicity in macrophages (Lindoso *et al.,* 2012; Alviano *et al*., 2012; Mohapatra, 2014). The antimicrobial resistance is also reported by thiol metabolism and its high intracellular level. The thiol molecule raises oxidative stress, which prevents the production of antioxidents and decreases the trivalent form of pentavalent antimonials. (Jain and Jain, 2013; Mohapatra, 2014).

However, these antimonials have disadvantages and side effects i.e. vomiting, dizziness, fever, arthralgia, anorexia, parenteral administration, prolong time, and high cost

(Fernandez *et al*., 2014; Gamboa leon *et al*., 2014). These drugs have restrictions for individual with hepatic, cardiac, and renal disease, old age, and for the pregnant women because of organs toxicity and pancreases (Neves *et al*., 2011, Sen and Chatterjee, 2011; Lindoso *et al*., 2012). The resistance and treatment failures are also reported for these pentavalent antimonials (Chakravarty and sundar, 2010; Jain and Jain, 2013). Inspite of these side effects and prolonged time people are are using (Salehabadi *et al.,* 2014). When the treatment failure and side effects with these drugs occur, the 2nd line drugs i.e pentamidine and amphotericin B and paromomycin were used (Prajapati *et al*., 2011; Wiwanitkit, 2012).

The amphotericin B is active for both promastigotes and amastigotes at dose of 0.71-1mg/kg for fifteen days (Lindoso *et al.,* 2012; Jain and Jain, 2013). Amphoterocin B exist in 4 forms i.e. amphotericin B deoxycholate, liposomal amphotericin B, amphoterocin colloidal dispersion, and amphotericin lipid complex (Mohapatra, 2014). The ampB target sterols constitue very important sterols for the membrane. It binds to cholesterol and form pores in the cell membrane, leading to the death of parasites. The safety profile of various amphotericin formulations has not been evaluated (Lindoso *et al.,* 2012; Jain and Jain*,* 2013; Brasil, 2010). The resistance occurs due to frequently use and also has severe effects such as vomiting, nausea, anemia, nephrotoxicity, and hapokalema cardiotoxicity (Lindoso *et al*., 2012; Gamboa leon *et al.,* 2014). The paromomycin and imiquimod used against CL and administrated intramuscularly for 21 days (15mg/kg) (Tiuman *et al*., 2012; Jain and Jain, 2013). These drugs inhibiting respiration and protein synthesis (Lindoso *et al*., 2012; Wiwanitkit, 2012). The major side effects include toxicity, hepatotoxicity, and injection site pain (Lindoso *et al*., 2012; Sachdeva *et al*., 2013). The pentamidine cause parasite death by interfers with the DNA synthesis and also affecting on the mitochondrial membrane. This drug used for 4mg/kg for 3 times/week (Jain and Jain*,* 2013). Low efficiency and side effects have restricted its use (Monzote, 2011) and side effects include myalgia, diabetes mellitus, hypotention, and hypoglycemia (Lindoso *et al.,* 2012). Many other therapeutic regimens used, but no agreement exist over which is the best (Gonzales *et al.,* 2009). The drugs, which are effective to some extent are not economically feasible

and are unavailable in the neglected geographical regions. The focus should be on the compounds that are free of side effects on the liver, kidney and renal artery failures, etc (Bahmani *et al.,* 2012; Ghasemipirbalouti *et al.,* 2012).

The lack of vaccine and increasing in resistance to drugs administered for the CL healing, coupled with administration route, toxicity, and expensive have become a huge alarm particularly in the widespread areas of underdeveloped areas. Hence, a new alternative effective drug is urgently needed for the treatment of CL infection because new cases of leishmaniasis are increasing now a day (Brodskyn *et al*., 2003). So, there is a vital need for the screening of natural plants against leishmaniasis. People in countryside areas prefer traditional medicinal usage for curing health services (Chan-Bacab and Pena-Rodriguez 2001). The importance of therapeutic flora due to the presence of various medical agents gaining more interest worldwide (Shabazi, 2017). The natural products having high antileishmanial activity were used for the discovery of the active compounds. About 250,000 medicinal plants have been reported worldwide. However, only 6% have been evaluated for their biological activities. In clinical trials, only about 1% of therapeutic natural products are investigated (Sen and Chatterjee, 2011; Jameel *et al*., 2014). Approximately, 35% of standard drugs developed from semi-synthetic derivatives while about 30% were based on pharmacophore or natural products. However, it's notable, that about 65% of parasitic medications isolated from natural products have been accepted by health authorities from 1981-2006 (Newmam and Cragg, 2012). In Iran, plant-based drugs are inexpensive and are proven to be more effective against different infectious diseases. An important assessment of the clinical data revealed that herbal medication is commonly approved well than synthetic (Delfan *et al.,* 2014).

The antileishmanial potential of some activity has been attributed to the presence of the compound i.e. triterpenoids, quinines, lignans, flavonoids, terpenes, naphthoquinones, alkaloids, steroids, and chalcones (Lage *et al.,* 2013; Sifaoui *et al.,* 2014). The plant extracts and essential oils have been reported to show antilesmanial potential of various *Leishmania* spp. (Rodrigues *et al*., 2013; Monzote *et al.,* 2014). The drug used against leishmaniasis should be chosed based on various factors i.e.

pharmaceutical characteristic of drug, *Leishmania* spp, patient's characteristic, geographical area and risk factors (Lindoso *et al*., 2012; Fernandez *et al.,* 2014). The drug used against *Leishmania* parasite should provide several requirements for affected people including minimum 1 or few dose, cheap, unteratogenic, free from side effect, no resistance, and no need for hospitalization (Alviano *et al*., 2012).

Kashmir is well known as a global center for the diversity of plants (Shinwari and Shinwari, 2010).The wide topographical variations in plant species ranging from alpine subtropical flora and higher altitude flora plains (Afshan *et al*., 2011). About 80% of Pakistan's plant species are confined to Kashmir and the Western Mountains (Ali, 2008). Because of vastness and inaccessibility with climate variation numerous regions of AJK remain unexplored. Trar Khel was given less attention by taxonomists as an integral part of the Western Himalayan Kashmir. Trar Khel is a mountainous region topographically situated in the humid Himalayan climate. So it deserves special consideration for the preservation of the environment and also for the suitable development of natural products (Haq *et al*., 2010). In Azad Jammu and Kashmir, there is no report of the test conducted on medicinal plants for antileishmanial activity (Ali, 2008). Therefore, this study aims to explore the phytochemical properties, antioxidant activities, and *in-vitro* antileishmanial activity of *P. pashia*, *M. pumila*, *P. persica, P. communis*, *P. armeniaca*, *F. glomerata,* and *D. lotus* extracts. If the promising activity against promestigotes of *L. tropica* of these medicinal plants is evaluated by *in-vitro* assay, the more effective natural antileishmanial component can be prepared in further studies due to the availability of raw material in high access.

MATERIALS AND METHODS

4.1. Study Area

Azad Jammu and Kashmir is a Western Himalayan foothill with a surface area of 13,269km^2 lies in northeastern Pakistan. Trar Khel is one of the tensile of district Sudhnoti AJK. It is located between 73°41' 9" East longitudes and 33°42'54" North latitude with an elevation range of 1372m. The area of study is mountainous and hilly and can be divided into the temperate, subtropical, and alpine zone. The climate of Trar Khel is with moderate hot summer, and cold winter is predominantly moist temperate to alpine (Fig.4.1).

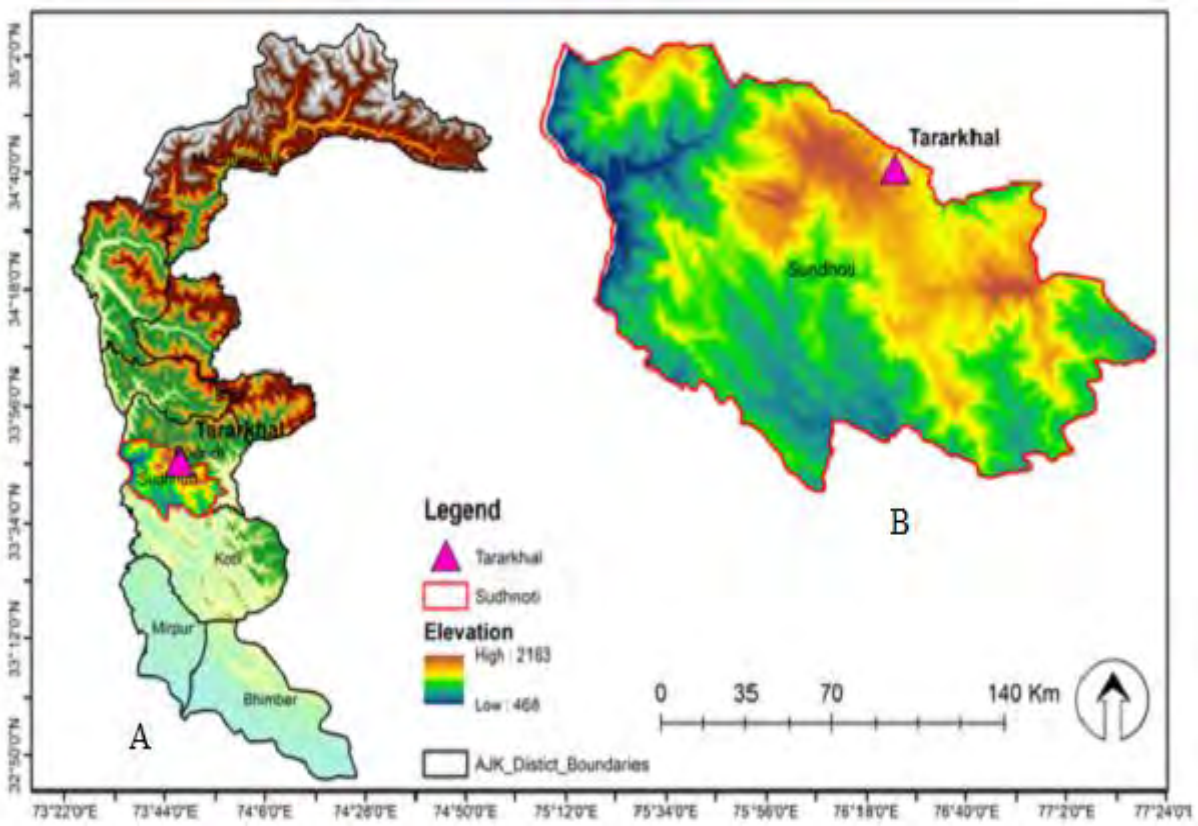


Fig. 4.1: Map shows the study area of plant collection in AJK. A: Showing ten districts of AJK B: showing study area Trarkhel of district Sudhnoti Azad Jammu and Kashmir.

4.2. Plants Selection

Table.4.1: The plants selected for antileishmanial activity and their previously reported biological activaties are listed in

Sr. #	Plant names	Biological activities	References
1	*P. pashia*	Antimicrobial activities, antioxidant, stomachic, anthelmintics, and diuretic, dynsentery, febrifuge, and laxative properties	Maga, 1978; Shui and Leong, 2002; Chettri *et al.,* 2005; Kala, 2002
2	*M. pumila*	Antioxidant, cardiovascular disease	Abbas and Sultana, 2020
3	*P. persica*	Insecticidal, diuretic, vermicidal, sedative, antifungal, antimicrobial	Kritikar and Basu, 1984
4	*P. communis*	Anti-inflammatory, wound healing, antibacterial	Parle and Arzoo, 2016; Velmurugan and Bhargava, 2014
5	*P. armeniaca*	antiparasitc, antiaginal, anticancer, antiatherosc lerating, antioxidant, cardioprotective, renoprotective	Yigit *et al.,* 2009; Yilmaz *et al.,* 2010; Raj *et al*., 2012; Sharma *et al.,* 2014; Minaiyan *et al.,* 2014
6	*F. glomerata*	anti-inflammatory, antioxidant, antiulcer, antiasthmatic, antidiabetic, hepatoprotective antioxidant, antipyretic, antidiarrheal	Jagatap supriya *et al*., 2012

7	*D. lotus*	Antifungal, antihypertensive, vermicide, dysponea, anti-inflammatory	Tezuka *et al.,* 1973; Thomas *et al.,* 2006; Chopra *et al.,* 1956

Fig. 4.2: Plants selected for biological assays

4.3. Plant Collection and Identification

The leaves of *P. pashia*, *M. pumila*, *P. persica, P. communis*, *P. armeniaca*, *F. glomerata,* and *D. lotus* were collected from Trar Khel in 2018. The collected plant species were identified through Pakistan flora (Nasir *et al*., 1972; Nasir and Ali, 1994-2010) and the voucher specimens were deposited in Herbarium of Pakistan, Quaid-i-Azam University Islamabad. The plant families' names, common name, and voucher numbers are presented in Table 4.2. After collection, the leaves of plants were washed with tap water and shadow dried for three weeks at room temperature (27-37ºC).

Table. 4.2: Plants scientific names, family names, common names, and voucher number

Plant name	Family	Common name	Voucher number
P. pashia	Rosaceae	Tangi, wild pear	130871
M. pumila	Rosaceae	Sab, apple	130869
P. persica	Rosaceae	Arwari, peach	130866
P. communis	Rosaceae	Pear	130870
P. armeniaca	Rosaceae	Khubani, apricot	130872
F. glomerata	Moraceae	Toshi, wild fig	130867
D. lotus	Ebenacea	Date plum	130868

4.4. Extracts Preparation for Biological Assays

After drying, the collected leaves were crushed in an electric mill (mesh, IKA MF: pore diameter 0.5mm). The powder (30g) obtained was used for extraction in (100%) ethanol solvent (250ml) by using the Soxhlet apparatus (Shanghai Heqi, China) at 40-60ºC (6 cycles per hour) for eight hours. The resultant extracts were filtered by Whatman No. 1 filter and the solvent was removed using a rotary vacuum evaporator (R-300, Rotavapor, Germany). The resultant extracts were stored at 4ºC for further analysis.

Fig.4.3: Soxhelet apparatus used for plant extract preparation

4.5. Phytochemical Analysis

A standard qualitative phytochemical test was conducted in the current study for the determination of saponins, terpenoids, flavonoids, alkaloids phenols, tannins, and coumarins in selected ethanolic leaves extracts (Table. 4.3).

Table. 4.3: Summary of phytochemical test conducted for selected plant extracts

Sr. #	Tested compound	Test name	Reagents added	Results	References
1	Alkaloids	Mayer test	Plant extract (2ml), con. HCL (2ml), mayer reagent	White precipitates	Harborne, 1984
2	Flavonoids	Ferric chloride	Plant extract (1ml), FeCL3 (drops)	Blackish red preci	Harborne, 1984
		Alkaline reagent	Plant extract (1ml), 2N NaOH (1ml)	Yellow color	Onwukaeme *et al.,* 2007
3	Saponins	Foam or forthing	Distilled water (2ml) in plant extract (2ml), shake (15 min)	Foam	Parekh and Chanda, 2007
4	Terpenoids	Salkowski's test	Plant extract (5ml), chloroform (2ml) Con. H_2SO4 (3ml)	Red brown color at the interface	Edeoga *et al*., 2005
5	Phenols	Ellagic acid test	Plant extract (1ml), 5% glacial few drops acetic acid and $NaNO_2$	Muddy brown color	Harborne, 1884
6	Coumarins	-	Plant extract (2ml), 10% NaOH (3ml)	Yellow color	Sofowora, 1993
7	Tannins	Ferric chloride	Plant extract (1ml),5% FeCL3 (2ml)	Greenish black	Kumar *et al*., 2007
8	Quinones	-	Plant extract (1ml), H2SO4 con. (1ml)	Red color	Harborne, 1984

4.6. Total Phenolic Content

For quantitative phytochemical analysis, the Folin-Ciocalteu (FC) method was used to determine total phenolic content (TPC) in the plant extracts Ranalli *et al.,* 2006). Briefly, about 20ul of each crude extract (DMSOmg/ml) was transferred by micropipette to the wells of the 96 well microtiter plate and then added FC reagent (90ul). After incubation at room temperature for 5min, 90ul of Na_2CO_3 (6% w/v) was added. At 630nm, absorbance was measured by the microplate reader (microplate reader ELX 800, Biotek, USA). The TPC was expressed as µg gallic acid equivalent (GAE)/ mg of dry weight (DW).

4.7. Total Flavonoid Content

Total flavonoid content (TPC) was determine using the method of aluminum chloride colorimetric method previously reported by Bouyahya *et al.* 2016, 2017. About 20µl of each sample (4mg/ml dimethylsulfoxide), 1M potassium acetate (10ul), 10µl of distilled water (160ul), and 10% (w/v) aluminum chloride were added in the 96 well plates. The absorbance was assessed after 30 minutes at 415nm of incubation. The quercetin 2.5-40ug/ml concentration was used and resultant TFC was shown to be equal to ug quercetin equivalent (QE) per mg DW. The test was repeated three times.

4.8. Antioxidant Activities Determination

4.8.1. Total Antioxidant Capacity (TAC)

The \ TAC capacities of extracts were determined by phosphomolybdenum assay spectrophotometrically viaprocess reported by Jafri *et al.* (2014). This assay is used daily to estimate the TAC of plant extracts, founded on acidic molybdenum (VI) reduction to molybdenum (V) by the complex natural extract (antioxidant compound) and green phosphomolybdenum (V) absorption at 695nm (Prassad *et al*., 2009). Briefly, 0.1ml with the final concentration of 100µg/ml of crude extract in methanol was combined with 1ml of reagent solution (28mM sodium phosphate, 4mM ammonium molybdate, and 0.6M sulfuric acid) and 90 minutes of incubation at 95ºC were carried. After incubation, the

sample was cooled at room temperature. Mixture absorbance was observed on the UV-spectrophotometer at 695nm against a blank reagent (methanol 0.1ml without plant extract).

4.8.2. Ferric Reducing Power Test

The ferric reducing power (FRP) assay of crude extract was conducted as reported by Zhao *et al.* (2008). In brief, the stock solution extract (500µl) was mixed with Phosphate buffer (500µl) (0.2 M; pH 6.6) and 1% potassium ferricyanide (500µl), and incubation at 50ºC for 20 minutes followed by the addition of 10% trichloroacetic acid (500µl). The centrifugation of the tube was carried for 10min at 10,000rpm. After centrifugation, the upper layer was transferred to a new tube and mixed with the same dH_2O volume and 0.1% ferric chloride (100ml). In the presence of crude extract or normal, the FRP operation is based on reducing Fe (III) to Fe (II). At 700nm, the development of Perl's Prussian blue color indicates Fe (II). The absorbance strength is parallel to power reduction. The results were expressed as equal to ascorbic acid (AAE µg per mg test dry weight of sample).

4.8. 3. DPPH Assay

Evaluation of DPPH (2, 2 -diphenyl-1- picrylhydrazyl) free radical scavenging potential was done via the technique already described by Brand Williams *et al.* (1995). New radical DPPH solution in methanol was prepared before the measurements of absorbance. About 3ml DPPH solution was mixed with plant extract (100µl) at final concentrations of 0.5, 5, 25, 50, and 100µg/ml against blank reagent. The mixture was shaken at room temperature and kept in the dark for 1h. The ability of the resultant extract to donate electron or hydrogen atom was measured, from a purple color to light yellow colored, on a UV spectrophotometer (Bio-Rad, USA) at 517nm. Ascorbic acid was used as a positive control and the experiment was conducted in triplicate.

The DPPH radical percent inhibition was measured by the following formula:

Effect of % DPPH scavenging = $[A_\circ - A1/A_\circ] \times 100$

A1 and $A_{\circ}$ = Blank reagent and sample absorbance recorded respectively. The antiradical activity was expressed in µg/ml. less IC_{50} value showed higher antioxidant activity.

4.9. Toxicity Assay

The brine shrimp lethality test was used to predict the toxicity assessment of selected plants. Briefly, the brine shrimp were hatched using brine shrimp eggs at an ambient temperature of 23±1ºC in a conically shaped vessel (1L), filled with artificial seawater (38g sea salt/L, pH 8.5 using 1N NaoH) with a constant supply of oxygen for 2 days. After hatching, the nauplii were collected and used for bioassay. Sample stock solution was prepared by dissolving the necessary quantitiy of extracts with a particular quantity of 0.5% DMSO (dimethyl sulfoxide) and sea water. Reletive sample volumes were then transferred to the test tube from stock solution to achive final concentration of 10, 100, and 1000 ppm (µg/mL) (Hamidi *et al*., 2014). The nauplii (10 per vial) were placed in the vial (containing 5ml of brine solution) through glass capillary with samples and maintained at room temperature. After 24 hr, the survived larvae in the sample environment were counted and tricaine methanesulfonate was used as control. All tests were conducted in triplicates.

4.10. Preparation of Stock Solution and Dilutions for Antileishmanial Activity

The stock solution for the antileishmanial test was prepared by dissolving 1mg/ml of dimethyl sulfoxide in the sterile glass bottles. The stock solution was divided serially from 0.5, 5, 25, 50, and 100µg/ml using dimethyl sulfoxide. The 0.45mg/ml syringes were used to filter all samples (Eltayeb and Ibrahim, 2012).

4.11. Parasite Culture

The *L. tropica* (Accession # MN891719) was previously isolated from a patient in AJK. The promastigotes form of *L. tropica* were cultured in M199 medium with HEPES buffer, 10% fetal calf serum (FSC), penicillin, and streptomycin (Shah *et al.,* 2014).

4.12. Evaluation of Anti Promastigotes Activity

The antileishmanial activity assays were done by the methods already described Ogeto *et al.* (2013) with some modifications. The log phase of promastigotes at $1x10^6$/100µl was used for the current assay. About 90µl of 199 media, 10µl each plant dilution and 50µl promastigotes log phase culture was dispensed to various wells of microtiter plates. Along with amphotericin b as the drug standard and dimethyl sulfoxide as negative control were used. Afterword the micropipette plate was incubated at 26ºC for 72h. The experiment was repeated three times. After incubations, 10µl of all dilution were pipette on the neubaur chamber and counted under the electron microscope.

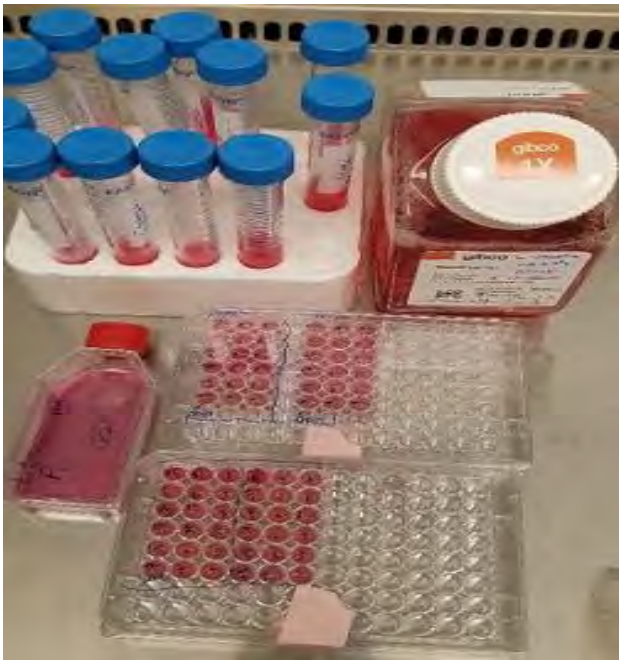


Fig. 4.4: *In-vitro* antileishmanial assays in 96 well plate

4.13. Statistical Analysis

All experiments were conducted in triplicates and the data analysis was shown as mean ± standard deviation (SD). The coefficient associations between the methods of antioxidant activate and total phenolics were confirmed by Microsoft Excel 2010. The lethal concentrations were measured by Probit Analysis at a confidence interval of 95%. When the value of $p<0.05$ then it is consider as significant.

RESULTS

4.14. Phytochemical Screening

The alkaloids were absent in *D. lotus* and *P. persica*. The absence of alkaloids in *P. persica* is supported by previous studies in Labia and Jammu Kashmir (Edrah *et al*., 2015; Hussain *et al*., 2015). Tannins were only absent in *F. glomerata.* Terpenoids were present in all extracts expect *P. persica* and *F. glomerata*. The plant extracts of *P. armeniaca, F. glomerata* and *D. lotus* showed the absence of saponins while present in the remaining all extracts. *M. pumila, P. communis*, *P. pashia, D. lotus,* and *P. armeniaca* showed the presence of steroids except for *P. persica* and *F. glomerata*. The quinone was absent in *D. lotus* and *F. glomerata*. The coumarins were present in *P. armeniaca* among others (Table 4.4).

Table 4.4: The phytochemical screening of leaves extracts of seven selected plants.

Test	***M. pumila***	***P. communis***	***P. persica***	***P. pashia***	***P. armeniaca***	***F. glomerata***	***D. lotus***
Alkaloids	+	+	-	+	+	+	-
Phenolics	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+
Tannin	+	+	+	+	+	-	+
Terpenoids	+	+	-	+	+	-	+
Saponin	-	+	+	+	-	-	-
Quinone	+	+	+	+	+	-	-
Coumarins	-	-	-	-	+	-	-

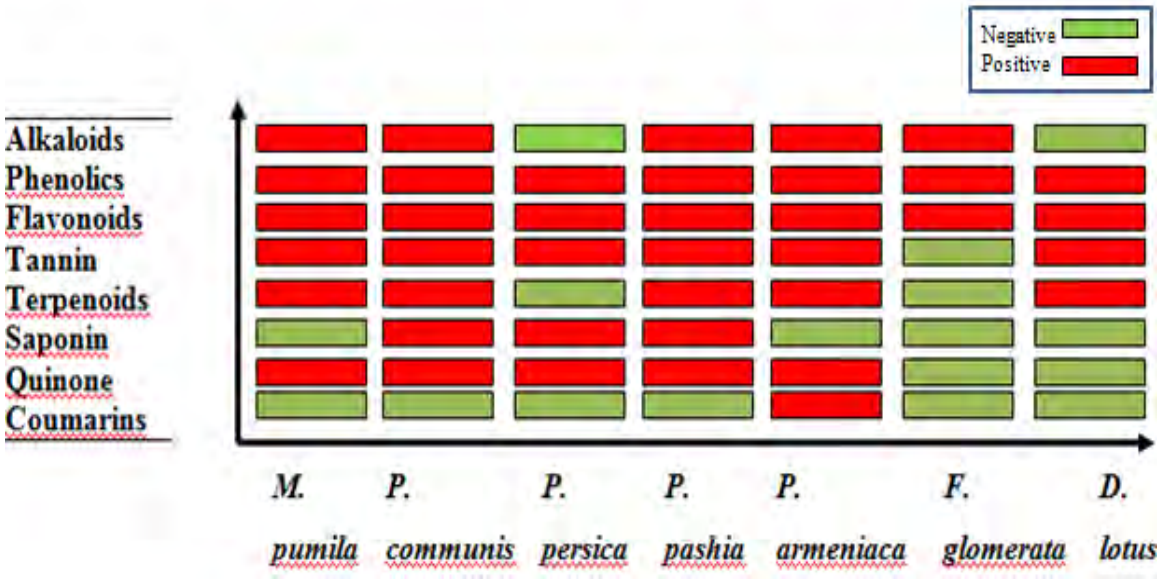


Fig. 4.5: The phytochemical screening of leaves extracts of seven selected plants.

4.15. Total Phenolic and Flavanoid Content

In Table 4.5, the ethanolic extract from the selected plants revealed a significant difference in the TPC rate. The highest TPC level was observed in *P. armeniaca (*279.62±5.40µgGAE/mgDW), followed by *P. pashia (*241.71±4.27µgGAE/mgDW) and *P. communis* (180.52 ±4.22µgGAE/mgDW). *M. pumila* and *P. persica* exhibited the TPC in a comparable quantity 54.91±3.90 and 53.32±9.30 µg GAE/mg DW respectively. The *F. glomerata (*12.82±6.80 µg GAE/mg DW) and *D. lotus (*10.91±6.70 µgGAE/mgDW) was found to possess comparatively minor TPC. Moreover, the ethanolic extract of selected plants showed differences in TFC. The TFC level of *P. armeniaca* remained the highest and that of *M. pumila* remained the lowest. *P. persica* and *P. pashia* showed a similar quantity (54.90±3.90 and 51.62±9.81µgQA/mg DW respectively). The *P. communis, D. lotus,* and *F. glomerata* exhibited 136.42±2.10, 98.62±1.50, and 106.90±2.90µgQA/mg

DW respectively. The current investigations showed that the tested plants contained a distinctive but significant amount of flavonoid and phenolic contents (Table 4.5).

Table 4.5: Total phenolic (TPC) and total flavonoid (TFC) content of selected plants in crude ethanolic extract.

Plant name	TPC (µg GAE/mg DW)	TFC (µg QA/mg DW)
M. pumila	54.91±3.90	12.70±2.42
P. communis	180.52 ±4.22	136.42±2.10
P. persica	53.32±9.30	54.90±3.90
P. pashia	241.71±4.27	51.62±9.81
P. armeniaca	279.62±5.40	205.70±2.41
F. glomerata	12.82±6.80	106.90±2.90
D. lotus	10.91±6.70	98.62±1.50

In the table, each value is presented as a mean ±SD (n=3), GAE= gallic acid equivalent; QE= quercetin equivalent; DW dry weight

4.16. Antioxidant Activities Determination

In Table 4.7, the TAE of seven selected plants (ethanolic crude extracts) were measured in the presence of antioxidant compounds based on the principle of Mo (V1) reduction to Mo (V). The reduction of Mo (V1) into Mo (V) leads to the formation of a green-colored phosphomolybdenum (V) complex observed at 695nm spectrophotometrically. The TAC of ethanolic extracts was calculated and expressed as ascorbic acid equivalent i.e AAEµg/mgDW. The TAC of ethanolic extracts of *P. armeniaca* (120.37±4.90µgAAE/mgDW) and *P. pashia* (100.42±5.20µgAAE/mgDW) were higher than *P. persica* (94.21±4.30µgAAE/mgDW) and *P. communis* (91.80±3.90µgAAE/mgDW) respectively. The *M. pumila* (83.60±3.00µgAAE/mg DW), and *F. glomerata* (80.60±2.71µgAAE/mgDW) showed high TAC than *D. lotus*

*(*76.71±3.21µgAAE/mgDW). These results revealed that ethanolic extracts from all plants contained compounds with high antioxidant potential and association with high levels of flavonoids and phenolic.

A parallel correlation between TAC and FRP was shown in the current investigation. The plant extract from *P. armeniaca* (120.37±4.90µgAAE/mgDW) showed the highest FRP values (278.71±1.03µgAAE/mgDW). The *P. pashia, P. persica, P. communis, F. glomerata, M. pumila,* and *D. lotus* showed 166.58±8.80, 108.21±4.62, 102.61±3.27, 67.62±7.01, 62.20±7.25, and 60.30±6.91µgAAE/mgDW respectively. The current study showed that the higher the maximum phenolic content of the plant extract, the greatest it will have the reducing power ability (Table 4.6).

The free radical scavenging (%) of ethanolic extracts were observed at various test concentrations in the following order: *P. armeniaca> P. pashia> P. persica> P. communis> M. Pumila> D. lotus> F. glomerata.*

Table 4.6: Total antioxidant capacity (TAC) and ferric reducing power (FRP) in selected plant extracts.

Plant name	TAC (µgAAE/mgDW)	FRP (µg AAE/mg DW)
M. pumila	83.60±3.0 0	62.20±7.25
P. communis	91.80±3.90	102.61±3.27
P. persica	94.21±4.30	108.21±4.62
P. pashia	100.42±5.20	166.58±8.80
P. armeniaca	120.37±4.90	278.71±1.03
F. glomerata	80.60±2.71	67.62±7.01
D. lotus	76.71±3.21	60.30±6.91

In the table, each value is presented as a ± SD (n=3) mean;

AAE=ascorbic acid; DW=dry weight.

The DPPH free radical scavenging activity (%) of ethanolic plant extract was greater at the highest extracts concentration. Interestingly, *P. armeniaca* extract showed the highest (53.94±1.24%) DPPH scavenging activity than other tested plants, which were as compared to other rich in phenolics. However, *P. pashia* showed maximum antioxidant potential (94.21±4.30%) through scavenging at the highest concentration of 400µg/ml, which showed that *P. pashia* may contain some compounds that have a higher dose effect. The IC_{50} for all plants was also observed in following the order: *P. armeniaca> P. pashia> P. persica> P. communis> M. pumila> D. Lotus> F. glomerata* (Table 4.7).

Table 4.7: DPPH free radical scavenging % activity from selected plants of ethanolic crude extracts.

Plant name	**Concentrations (µg/ml) % inhibition by plants and the Vitamin C**					**IC50**
	0.5	5	25	50	100	µg/ml
M. pumila	41.21±1.35	44.72±1.54	51.24±1.32	56.47±1.22	70.62±2.71	66.90
P. communis	43.90±3.06	54.12±1.12	60.94±0.71	71.90±1.25	86.90±1.90	44.03
P. persica	44.62±1.31	51.07±0.91	55.45±1.50	60.30±2.13	74.47±1.12	59.51
P. pashia	47.92±1.20	50.51±0.87	59.40±0.92	82.48±1.46	94.32±1.24	35.70
P. armeniaca	53.94±1.24	55.54±1.51	61.36±1.14	74.47±1.84	86.33±1.34	19.54
F. glomerata	37.86±1.08	41.21±1.32	48.70±1.30	53.75±1.60	60.82±1.83	87.52
D. lotus	38.28±1.07	42.31±0.61	47.93±1.38	51.50±1.45	60.40±1.17	73.42
Vitamin C	71.30±1.32	67.87±1.32	77.67±1.04	99.45±1.22	100±1.07	5.51

In the table, each value is presented as a ± SD (n=3) mean

4.17. Cytotoxicity Assays

A total of 7 crude extracts of *M. pumila, P. communis, P. persica, P. pashia, P. armeniaca,* and *D. lotus* showed no toxicity with LC_{50} values >1000µg/ml. The *M. pumila, P. communis,* and *P. persica* showed LC_{50} values of 1283.67, 1411.30, and 1659.90 µg/ml with the significance value of p= 0.003, 0.001, and 0.001 respectively. The extracts of *P.*

pashia, P. armeniaca, and *D. lotus* showed LC_{50} values of 1230.66, 1912.31, and 1671.56µg/ml having values of *p*=0.999, 0.001, and 0.000 respectively. The *F. glomerata* extract showed moderate toxicity having LC_{50} values of 454.34µg/ml having the significance value of *p*= 0.147.

4.18. Antileishmanial Activity

In Table 4.8, the preliminary evaluation of crude ethanolic extracts of seven plants revealed that among the total crude extracts tested *P. communis* and *P. pashia* having moderate antileishmanial activities with IC_{50} 56.68 and 60.95µg/ml values respectively. These findings may lead to this raw material for other parasitic diseases. *M. pumila*, *P. persica, D. lotus,* and *F. glomerata* have shown no antileishmanial activity having IC_{50} >100µg/ml. One prominent extract, *P. armeniaca* showed the highest antileishmanial activity with IC_{50} 16.18µg/ml which is less than the standard drug named as amphotericin B used as positive control i.e 17.72µg/ml. The standard error for positive control and tested plant extracts was calculated with a 95% confidence interval having a significance value of 0.000. The percent mortality recorded was 95.28±0.000 at 100µg/ml of *P. armeniaca* while control showed 73.10±0.92. Moderate inhibition of parasitic growth was shown by *P. communia* and *P. pashia* at 100µg/ml were 59.76±0.60 and 63.74±0.70. Less than 50% inhibition of parasite growth was shown by *P. persica, F. glomerata,* and *D. lotus* at 100µg/ml.

Table 4.8: *In-vitro* antileishmanial activity of ethanolic plant extract (0.5-100µg/ml) on the promestigotes of *L.tropica*. The activity was estimated as growth inhibition percent.

Plants Name	**Inhibition growth (%)**					**IC_{50} (µg/ml)**	**Significant value**
Concentration (µg/ml)	0.5	5	25	50	100		0.000
M. pumila	4.33±0.51	7.53±0.07	10.56±0.95	20.69±0.75	26.69±0.85	>100	0.000
P. communis	21.16±0.67	35.86±0.52	45.81±0.87	53.73±0.71	59.76±0.60	56.68	0.000
P. pashia	10.59±0.21	26.61±0.15	40.02±0.52	51.31±0.51	63.74±0.70	60.95	0.000
P. persica	0	6.99±0.07	12.01±0.97	22.16±0.65	31.43±0.54	>100	0.000
P. armeniaca	28.46±0.21	46.95±0.05	55.04±0.44	83.78±0.78	95.28±0.00	16.18	0.000
F. glomerata	9.07±0.05	13.25±0.23	16.56±0.91	31.48±0.57	43.15±0.83	>100	0.000
D. lotus	2.12±0.32	2.98±0.32	4.71±0.72	7.08±0.81	13.46±0.76	>100	0.000
Control	34.41±0.21	46.33±0.07	57.89±0.05	70.23±0.78	73.10±0.92	17.72	0.000

DISCUSSION

The natural products are rich sources of selective and new agents for curing imperative tropical diseases caused by protozoan and many other parasites (Shabazi, 2017). The use of herbal medicines becomes a common practice in all developing countries, where basic health services are not accessible worldwide including AJK. The medicinal flora of AJK has been described as diversified and rich but limited studies investigated the potential use in the curing of parasitic diseases (Shinwari and Shinwari, 2010). The phytochemical analysis is the first step in bioactive compounds identification. It has been stated that the total flavonoid and phenolic contents are directly correlated with antioxidant activity. Such compounds are referred to as strong antioxidant chain breakers (Ksouri *et al.,* 2008). In this context, we investigated the raw ethanolic crude extracts of seven selected plant leaves for phytochemical analysis along with antioxidant and *in-vitro* antileishmanial activities. In the current study, the phytochemical screening showed all leaf extracts were rich in phenolics and flavonoids constituents. Similar observations were reported by Saeed *et al*., (2012) for *Fagonia olivieri* and *Torilis leptophylla* extracts.

The therapeutic and biological advantages are due to the presence of flavonoids and phenolics constituents in all tested leaf extracts. Highest TPC *(*279.62±5.40) and TFC were shown by *P. armeniaca* among all tested extracts. The existence of such compounds explains the use of these plants in folk medicine (Amarowicz *et al*., 2004). The antioxidant role of the phenolic compound is well established that attributed their action to scavenge free radicals, give chelated metal ion, electron, or proton (Amarowicz *et al*., 2004). The antioxidant activity of the hydroxyl group number and location to the carboxyl group and as high as the hydrolation increases (Karishniah *et a.,* 2011). Furthermore, the flavonoid mechanism of action was correlated with free radical scavenging or metal ion chelation (Kessler *et al.,* 2003). This power to scavenge free radicals gives them antioxidant ability (Sahreen *et al*., 2010). In the current investigations, a parallel correlation was observed between TAC and FRP. This parallel correlation was also reported by Kumar and Jain (2015) for *Lanneacoro mandelica*. TAC *(*120.37±4.90µgAAE/mgDW) and FRP (278.71±1.03µgAAE/mg DW) of *P. armeniaca* was highest among other plants extracts.

This capacity is because antioxidants are essentially protons or electrons that reduce ferric ion (Fe^{3+}) by electron donation to ferrous ion (Fe^{2+}) (Shon *et al*., 2004). The present study claimed that the higher the TPC in plant extracts, the more the reduction capacity it will have (Jafri *et al*., 2014). The phenolic compounds minimize the risk of health problems because they are immune to reactive oxygen (ROS) damage. Prior investigations stated that the strongest antioxidant compounds were also antileishmanic (Jain *et al.,* 2013). The DPPH (517nm absorption) is a stable radical capable of antioxidant scavenging (Lu and Foo, 2001). It is widely used to assess the ability of compounds as free radical scavengers and hydrogen donors, as well as the antioxidant activity of important medicinal plant extracts (de Porto *et al.,* 2000). In the reaction, DPPH absorbs radical hydrogen or electron from antioxidants and changes its color, which can be calculated by spectrophotometer at 517nm (Canadanovic Brunet *et al*., 2014).

Plant pharmacological assessment provides an attractive and good source for safe and novel medical plant growth. It is necessary to check the cytotoxicity of selected plants to improve safety. For the assessment of toxicity determination, brine shrimp toxicity assay is considered as rapid, reliable, and low cost (Olila *et al.,* 2001). The plant extracts with LC_{50} values between 100 and 500µg/ml are considered as moderately toxic and those with <100 are strongly toxic. Values of LC_{50} >1000 are considered nontoxic (Nguta *et al.,* in 2012). The *F. glomerata* extract showed moderate toxicity having LC_{50} values of 454.34µg/ml following the statement of Nguta *et al.* (2015). The crude extract of *F. glomerata* showed moderate activity and is need to not be left as irrelevant because Bussmann *et al.* (2011) and Nguta *et al.,* (2012) noticed that toxicity showed variations significantly because of different collection location, the tissue of plants, time of harvest and solvent extraction. Due to this natural variability, the leaves identified LC_{50} between 100 and 500µg/ml cytotoxicity have served us for more research on biologically active extracts against fleas (*Xenopsylla cheopis*), mosquitoes (*Ades aegypti*), ticks (*Ixodes scapularis*), microbes affecting forest and health of living thing Johnston *et al.,* 2001; Dietrich *et al.,* 2006).

The current findings may lead to this raw material for other parasitic diseases. Biological activates of *P. pashia* were also reported by Guven *et al.* (2006). *M. pumila* and *P. persica, D. lotus* and *F. glomerata* have shown no antileishmanial activity having IC_{50} >100µg/ml. The *M. pumila* and *P. persica* have anti-inflammatory (Kashyap *et al.,* 2015), *D. lotus* used as antiseptic, antitumor, and antidiabetic (Uddin *et al.,* 2011, 2014), and *F. glomerata* reported for bacterial infection (Menezes *et al.,* 2013). One prominent extract, *P. armeniaca* showed the highest antileishmanial activity with IC_{50} 16.18µg/ml which is less than the control i.e 17.72µg/ml. A wide range of pharmacological effects of *P. armeniaca* has been reported such as antioxidant (Guclu *et al*., 2006), antimicrobial activities, (Sochor *et al*., 2010) and anti-asthmatic (Erdogan and Kartal, 2011). Minaiyan *et al.* in (2014) reported that *P. armeniaca* has been used in many parasitic diseases.

However, pharmacological data of *P. armeniaca* regarding the antileishmanial activity has not been documented in the literature, some phytochemicals constitute such as phenols, flavonoids, alkaloids, saponins, terpenoids, coumarins, tannins, and quinone was present in this plant in the current study. The coumarian have been already purified from *Calophyllum brasiliense* (Calophyllaceae) reported antileishamanial activity against *L. amazonesis* (Brenzan *et al.,* 2008). The alkaloids from the genus *Prosopis* showed significant *in-vitro* parasitic activity against *L. donovania* with compared to the control drug (Samoylenko *et al.,* 2009). Some other investigations reported the presence of phenolic (Bodiwala *et al*., 2007) and flavonoids (Nour *et al.,* 2010) from *Ageratum conyzoides* (*Asteraceae*) possess antileishmanial activities against promastigotes of *L. donovania*. Torres Santos *et al.* (2004) has reported the terpenoids from *Pourouma guianensis* (*Moraceae*) as an antileishmanial agent against *L. amazonesis*. The antileishmanial activity against *L. donovania* and *L.major* was demonstrated by quinone from *Zhumeria majdae* (*Labiateae*) (Moein *et al.,* 2008) and *Perovskia abrotanoides* (*Lamiaceae*) (Sairafianpour *et al.,* 2001). Paolini *et al.* (2004) verified the anti-parasitic activity of tannins.

CONCLUSION

It is concluded from the current investigation that, plant extracts could be an effective alternative for synthetic drugs against *L. tropica*. The plant *P. arminiaca* contains chemical compounds that may lead to the development of an affordable and effective antileishmanial drug against cutaneous leishmaniasis (*L. tropica*). In most developing countries, these results provide a different way to used natural plant-based remedies that might be less toxic, safer, and cheaper than available recommended medicines. AJK is an area rich in possibilities, and world flora represents an enormous source of material for testing. Therefore, extensive studies are needed, particularly bio-guided fractionation for the identification of active fraction and more chemical characterization.

In-vitro anti-leishmanial Activity of *Prunus armeniaca* Fractions on *Leishmania tropica* and Molecular Docking Studies

ABSTRACT

Prunus armeniaca (L.) is a member of the Rosaceae, subfamily Prunoideae, shows anticancer, antitubercular, antimutagenic, antimicrobial, antioxidant, and cardioprotective activities. Here we fractionated the leaves extract of this highly medicinally important plant for antileishmanial activity. In the current study, the leaves extract was fractionated and characterized using column and thin layer chromatography by n-hexane, ethyl acetate, and methanol solvents. Twelve fractions were isolated and subjected for evaluation of their cytotoxicity on peritoneal macrophages. The *in-vitro* antileishmanial activity was conducted against promastigotes and amastigotes of *L. tropica.* Among all fractions used, the fraction (F7) exhibited the strongest antileishmanial activity. The bioactive fraction was further characterized by spectroscopy (FTIR, UV-Vis), and GC-MS analysis. The *in-silico* docking was carried out to find the active site of PTR1. All derived fractions exhibited toxicity on peritoneal macrophages in the safety range IC_{50}>100µg/ml. The fraction (F7) showed significantly the highest antipromastigotes activity with IC_{50}11.48±0.82µg/ml and antiamastigotes activity with IC_{50} 21.03±0.98µg/ml compared with control i.e. 11.60±0.70 and 22.03±1.02µg/ml respectively. The UV-Vis spectroscopic analysis revealed the presence of six absorption peaks and the FTIR spectrum revealed the presence of alkane, aldehyde, carboxylic acid, thiols, alkynes, and carbonyls compounds The GC-MS chromatogram exhibited the presence of nine compounds: (a) benzeneethanol, alpha, beta dimethyl, (b) carbazic acid, 3-(1 propylbutylidene)-, ethyl ester, (c)1, 2-benzenedicarboxylic acid, diisooctyl ester, (d) benzeneethanamine a-methyl, (e) 2aminononadecane, (f)2-heptanamine-5-methyl, (g) cyclobutanol, (h) cyclopropyl carbine, and (i) nitric acid, nonyl ester. Among all compounds, the 1, 2-benzenedicarboxylic acid, diisooctyl ester bound well to the PTR1 receptor. Fraction (F7) showed acceptable results with no cytotoxicity. However, *in-vivo* studies are required in the future.

INTRODUCTION

As previously described, lack of an appropriate vaccine for the treatment of CL and drug resistance, coupled with administration mode, toxicity, and high cost has been regarded as an enormous intrest particularly in prevalent areas of underdeveloped countries. Therefore there isno doubt that the exploration for new antileishmanial agents is one of the most important challenges in the recent drug discovery field and is a universal alarm (Guimaraes *et al*., 2010; Iqbal *et al*., 2012; Wink, 2012; Ogeto *et al*., 2013; Mahmouvand *et al.,* 2014). Various studies are conducted by researchers looking for new alternative in nature (Wink, 2012; Sifaoui *et al*., 2014). Approximately, 250,000 medicinal plant species are found worldwide but biological activities of only 6% have been done. In addition only 0.75% medicinal herbal compounds have been investigated in medical trials (Sen and chatterjee, 2011; Jameel *et al*., 2014).

In small communities, herbal therapies are ideal due to the unavailability of standard therapies. In this reference, natural plants are a good source of bioactive compounds for human beings (Lavinas *et al.,* 2019). Kashani *et al*., in (2012) highlight the pharmacological properties of secondary metabolites. Various researchers also reported the activity of less known and new terpenoids, phenolic compounds, and alkaloids which gives a direct role in modern pharmaceuticals (de Andrade *et al.,* 2012; Sarala *et al*., 2011). Although the quantity of secondary metabolites are found very little and, their characterization, purification, and extraction are still a huge challenge in the drug discovery process (Chan *et al.,* 2011; Winjngard *et al*., 2012; Tang *et al.,* 2012). For the initiation of the drug discovery process, extraction is the 1st step. Various common methods have been planned for obtaining extracts that presents the polarity range (Wall *et al*., 1996) and also enriched the secondary metabolites i.e. saponins (Cordell, 1981), and alkaloids (Hoststtmann *et al.,* 1991). The bioassays approach is essential to authenticate the conventional use and to find the most active crude or partially crude extracts. The *in-vitro* biological assays are very faster and little quantity of compound is required, even if they are irrelevant to clinical conditions (Sarker *et al.,* 2005). *In-vitro* assays are micro plate-based and are sensitive, specific, and are extensively used. The bioassay complexity must

be defined by quality available personnel and laboratory facilities (Valgas *et al.,* 2007). The crude extract or pure compound having IC_{50} value is below 100µg/ml and below 25µM is getting more interest (Cos *et al*., 2006). The compound presence with active potential can be assessed by using chemical assays based on spectrophotometric measurements (Ellman *et al.,* 1961; Beretta and Facino, 2010; Pohanka *et al.,* 2011). Once the activity potential has been done, the crude extract needs to be purifying to bioactive compound isolation. Various separation methods are generally used (Sticher, 2008).

The column chromatography technique is used for quantification and isolation of active phytocompounds from the crude extract of plants (Church *et al*., 2005; Abdulhamid *et al.,* 2017). The thin-layer chromatography (TLC) is used for compound identification and their purity determination (Abdulhamid *et al.,* 2017). The UV-VIS spectroscopy is used for phytocomponents detection (Gunasekaran, 2003) while FTIR (Fourier Transform Infrared Spectrophotometer) is used to identify the existence of functional groups/chemical bonds in phytochemicals (Grube *et al.,* 2008). However, the use of UV-Visible spectrophotometer in the complex analysis is limited by the inherent difficulty of assigning the absorption peaks to any particular component in the system. Therefore, UV-VIS findings need to be supplemented with some other analytical techniques such as GC/MS etc. to allow proper extract characterization and identification of constituents (Karpagasundari and Kulothungan, 2013). The bioactive constituents are scrutinized by using the finest method of GC-MS (gas chromatography-mass spectroscopy) and the mass spectrum of each component can also be recorded by this technique (Krishnakumari and Nagaraj, 2012).

Currently, all available drugs against parasites have been identified by *in-silico* approach and this strategy provides a source of drug candidates (Moine *et al*., 2015). In the case of neglected diseases where a specific drug development approach is not affordable and no market return for huge investment, an elegant variation of in silico strategy is the repurposing of drug candidates (Andrews *et al.,* 2014). The best example for repurposing is the aminoglycoside paromomycin was approved in India against leishmaniasis (Sundar *et al*., 2007). In the post-genomic, a 2nd method has come into the

focus, known as target-based drug design, in which the reorganization of potential drug targets occur by in silico method including genome and protein data-base (Egner *et al*., 2005).

The molecular in silico docking is a computational technique conducted on structure-based rational drug design for the identification of correct conformation of ligands and protein-ligand interaction, which generally involve 1 ligand and 1 receptor (Yuriev *et al.,* 2009; Mura and McAnany, 2014; Tantar *et al.,* 2008). The most common software and programs include Autodock Vina (Trott and Olson, 2010), Flex X (Rarey *et al*., 1996), GOLD (Jones *et al*., 1997), and Autodock (Morris *et al*., 2009). Up to now, these and various other similar methods generally focus on the docking between 2 molecules by non-covalent interaction including the hydrogen bonding, electro statistic interaction, and van der waals interaction or by scoring functions for the identification of these interactions (non-covalent) (Rarey *et al*., 1996). Though, all drugs are not made non-covalent interaction to the active site; there is a lot of drugs classification known as covalent drugs (Singh *et al*., 2011). Non-covalent interaction of ligand to receptors is more conventional now a day. Various docking methods have been used in focused on the successful prediction of the binding mode of non-covalent inhibitors (Yuriev and Ramsland, 2013; Jacob *et al.,* 2012; Fukunishi *et al.,* 2010). The ligand-receptor interactions can be predicted by using a major computational method known as molecular docking (Kellenberger *et al.,* 2004) and is an important tool for the discovery of antileishmanial compounds.

In *Leishmania* parasite the pteridine reductase 1 (PTR 1) gene is an excellent drug target because of unusual pterin salvage from the hostwhile the host lack PTR1 activity and synthesizes pterin derivatives de novo from GTP (Nichol *et al*., 1985). Various biochemical studies showed that PTR1 enzyme is a NADPH dependent pterin reductase. It demonstrates its enzymatics activity as a tetramer (Bello *et al.,* 1994; Wang *et al.,* 1997; Kumar *et al.,* 2004). The enzyme reduces biopterin to H2 and H4 biopterin. This enzyme also able to reduce folate to 7, 8, dihydrofolate and tetrahydrofolate. The dihydroopyrimidiones and 1 phenyl 4 glycosyl dihydropyridines arrest PTR1 of

Leiashmania and also showed significant antileishmanial activities (Kumar *et al.,* 2007; Kumar *et al*., 2008). Various studies showed that the docking results matched with the results observed experimentally (Ramirez and Caballero, 2016).

The *P. armeniaca* bark has been used as an astringent to irritation while apricots are used for infertility, spasm, hemorrhages, and eye infection. The healing of vaginal infection is reported by using apricot kernel paste while kernel oil is used as the pharmaceutical agent and also in cosmetics (Asma, 2002). The fresh juice of apricot leaves was used in Chinese traditional medicine with fruitful results in skin ailments such as eczema, itching, and scabies (Yeung, 1985; Amasiatsi *et al*., 1990). The *P. armeniaca* leaves, kernels, and, fruits were also the best antimicrobial agents (Karapetyan *et al.,* 2011; Yigit *et al*., 2009; Rashid *et al.,* 2007). The *P. armeniaca* kernels, leaf, and fruits extracts showed antioxidant activities (Scebba *et al*., 2001; Rashid *et al.,* 2005; Durmaz and Alpaslan, 2007). A strong inhibitory potential against tyrosinase was reported in *P.armeniaca*. The tyrosinase is the key enzyme on trypsin and melanin biosynthesis (Gahloth and Sharma, 2010). The dry fruits of *P. armeniaca* showed cardioprotective activity in urethane anesthetized rates (Parlakpinar *et al*., 2009). The anti-inflammatory, antinociceptive, antitubercular, and anticancer activity was also reported in *P. armeniaca* (Change *et al*., 2005; Hwang *et al*., 2008; Arya, 2011; Akcicek *et al.,* 2005). To best our knowledge, this highly medicinal important plant has not been investigated against *L. tropica* in Azad Jammu and Kashmir, Pakistan. However, in current research we are reporting for the first time, the *in-vitro* antileishmanial activity of *P. armeniaca* fractions against *L. tropica* and also assessed their cytotoxicity. The active fraction of *P. armeniaca* was then characterized for phytocomponent identification along with the molecular docking studies for determining the active binding site of the PTR1 gene.

MATERIALS AND METHODS

5.1. Plant Collection and Identification

The leaves of *P. armeniaca* (Fig. 5.1) were collected from Trar Khel in 2019. After collection, the leaves of plants were washed with tap water and shadow dried for three weeks at room temperature (27-37ºC).

Fig.5.1. *P. armeniaca* plant used in the current study

5.2. *P. armeniaca* Crude Extract Preparation

The ethanolic crude extract was prepared by following the methodology described in chapter 4. The resultant extract was stored at 4ºC for further column chromatography.

Fig.5.2: *P. armeniaca* extract prepration in Soxhlet appratus

4.3. Fractionation through Column Chromatography

For isolation of various fractions from *P. armeniaca* extract, a column (28 x 3 inches) with silica gel (0.063-0.200mm) was prepared in dry powder form and left overnight. The column was run with n-hexane (300ml) for settling down. The ethanol extract of *P. armeniaca* was poured to a silica gel open chromatographic column and step gradient technique was followed to run the column in three various solvents i.e. n-hexane, ethyl acetate, and methanol in ascending order of polarity. Different ratios of n-hexane and ethyl acetate (pure n-hexane, 20:80, 40:60, 80:20, and purr acetate) then ethyl acetate and methanol, (pure ethyl acetate, 20;80, 40;60; 80;20, and pure methanol) were used for the isolation of various fractions (5.3). Based on the thin-layer chromatography technique, the same types of fractions were mixed. The solvent was evaporated and crude fractions were used for further analysis.

Fig 5.3: Fraction isolation by column chromatogragy

5.4. Model Animal

The male BALB/c mice (six weeks old) were taken from the National Institute of Health Islamabad, Pakistan. The mice were placed in a small steel cage in the animal house of Quaid-i-Azam University. The animals were nourished on a standard pellet diet and water of *libitum.* The room temperature was maintained from 26-29ºC with a light-dark cycle (12 hrs). They were acclimatized for two weeks before the experiment. The animals were kept in a hygienic environment and handled properly (Fatima *et al*., 2017).

5.5 Isolation of Murine Macrophages

In the current study, the murine macrophages were collected from healthy eight weeks old male BALB/c mice by injecting cold (3-5ml) RPMI-1640 medium (Sigma) (Roswell Park Memorial Institute) in the peritoneal cavities. The aspirated macrophages were washed two times with 0.5% PBS and suspended in RPMI 1640 medium with

penicillin (100 U/ml), and (100µg/ml) streptomycin (Sigma Aldrich, St. Louis, MO, USA) (Carrio *et al.,* 2000).

5.6. Control Drug

We have used standard drug amphotericin B (Havan city, Cuba, IMEFA) at a concentration of 1mg/ml as positive control. The drug was diluted in sterile water for various (0.5, 5, 25, 50, and 100µg/ml) concentrations.

5.7. Cytotoxicity Assay on Peritoneal Macrophages

The peritoneal macrophages (BALB/c mice) were used to determine the CC_{50} value of the fraction. The macrophages were collected in RPMI 1640 medium (Sigma, St. Louis, Mo, USA) with 200µg/ml streptomycin, 200UI penicillin and cultured at 10^5 Cells/ml in 96 well Lab Tek (Costar USA) and then incubated at (37ºC in 5% CO_2) for 2 hrs. The non-adherent cells were removed by washing with a phosphate buffer solution. Then 2µl fractions of various dilutions (previously prepared in medium) were added in medium (198µl) with (10%) HFBS and antibiotics (200µg/ml streptomycin, 200 UI penicillin). The macrophages were treated with the fractions extract from 50, 100, 150, and 200µg/ml for 48 hrs. The culture with (0.5%) DMSO was added as a negative control. The toxicity was determined by the colorimetric assay with 3-[4-4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (Sigma, USA) solution (MTT). The MTT solution (15µl at 5mg/ml in PBS) was added to each well and incubated for 4hrs at the same conditions. Then the medium was removed and formazan crystals were dissolving in DMSO (100µl). The EMS reader MF (version 2.4-0) was used to determined absorbance at 560nm test wavelength (reference wavelength= 630nm) (Sladowski *et al.,* 1993). The IC_{50} value was determined using the equation for the sigmoidal E_{max} model from dose-response curves fit data in graph pad prism. The test was conducted in triplicates.

5.8. Preparation of Stock Solution and Dilutions for Antileishmasnial Activity

The stock solution for the antileishmanial test was prepared by dissolving 1mg/ml of 0.5% dimethyl sulfoxide (DMSO) in the sterile glass bottles. The stock solution was

divided serially from 0.5, 5, 25, 50, and 100µg/ml using 0.5% DMSO. The 0.45mg/ml syringes were used to filter all samples (Eltayeb and Ibrahim, 2011).

5.9. Parasite Culture

The *L. tropica* (MN886941) was previously isolated from a patient in AJK. The promastigotes form of *L. tropica* were cultured in M199 medium with HEPES buffer [4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid], (10%) FSC, penicillin (100U/ml), and (100µg/ml) streptomycin (St. Louis, MO, Sigma Aldrich, USA) (Qureshi *et al*., 2014).

5.10. Evaluation of Anti promastigotes Activity

The antileishmanial activity test was conducted by following the process already explained Ogeto *et al.* (2013) with little changes. The log phase of promastigotes at $1x10^6$/100µl was used for the current assay. About 90µl of M199 media, 10µl each plant dilution (0.5, 5, 25, 50, and 100µg/ml) and 50µl promastigotes log phase culture was dispensed to various wells of microtiter plates. Along with amphotericin B as the drug standard and culture media as negative control were used. Afterword the plate was incubated at 26°C for 72h. The experiment was repeated three times. After incubations, 10µl of all dilution were pipette on the neubauer chamber and counted under the binocular microscope (Optica, 500 series). The IC_{50} value for antileishmanial activity was determined by SPSS software (version 19).

5.11. Antiamastigote Activity

For the evaluation of the antileishamanial activity of *P. armeniaca* fractions of *L. tropica* amastigotes (mammalian infected form), the murine macrophages were used in accordance with the method already described by Mahmoudvand *et al*., (2014). $1cm^2$ sterile cover slips were positioned in the six chambers slides wells (Nalge Nunc Lab Tek international NY, USA), and then 200µl murine macrophages (10^5 cells/ml) were placed in each well. After two hours (incubation at 37ºC, 5% CO_2 and 95% relative humidity), the promastigotes of *L. tropica* strain were added in each well, and incubation was carried out in a CO_2 incubator at same conditions for 24hrs. The RPMI 1640 medium (sigma) was

used to remove the free parasites.The infected macrophages were treated with different concentrations (0.5, 5, 25, 50, and 100µg/ml) of *P. armeniaca* fractions extract for 72 hours at 37ºC. Lastly, dried slides were fixed with methanol, stained by Giemsa stain, and examined under the light microscope. The antiamastigotes effect was determined by counting the number of amastigotes in each macrophage by examining 100 macrophages on each cover slip (Shokri *et al*., 2012). Infected macrophages without treatment were used as positive control while non-infected with no drug was as negative control respectively. All experiments were conducted in triplicates. The IC_{50} values were calculated by Probit analysis. The percentage of inhibition of *L. tropica* intra macrophage was determined by counting 200 cells in triplicate. The selective index (SI) was calculated by CC_{50} for macrophages divided by IC_{50} for amastigotes. The SI was used to compare the cytotoxicity of fractions for murine macrophages and the potential against intracellular amastigotes of *L. tropica.* When S>1 is considered more selective for activity in opposition to a parasite while SI<1 is considered more selective for potential against cells (Shioji *et al*., 2005). The ANOVA (analysis of variances) test was used to determine the possible significant different effect among the fractions and control drug using SPSS software. We also used student's *t-test* to compare the IC_{50} values of fractions and control drug. The $p<0.05$ was considered as significant.

5.12. Characterization of the Most Active Fraction

5.12.1. UV-Vis Spectroscopy and Fourier Transform Infrared Spectroscopy

The most active fraction was examined using UV-Vis spectroscopy analysis for its spectral profile. The fraction extract was centrifuged at 3000rpm for ten minutes and sieved by filter paper Whatman No. 1. The spectra were obtained by scanning the diluted (1:10) fraction in the wavelength at 200-800nm using the spectrophotometer (UV-1601, Shimadzu) and characteristic values of a peak were noted. The FTIR analysis of the most active fraction was performed for the detection of functional group and the characteristic peaks values were observed and recorded in the infrared region from 400-4000cm-1. The sample was prepared by mixing 2mg leaves extract in 100mg of KBr (FTIR grade). About 3mm diameter salt disc were loaded in the FTIR spectroscope (Shimadzu FTIR

spectrophotometer) and scanned to confirmed infrared spectra at variouswavelengths (400-4000cm-1) (Hemavathy *et al.,* 2019).

5.12.2. Gas Chromatogram Mass Spectroscopy

The GC-MS analysis of active fraction was conducted by chromatographs attached with a mass spectrophotometer (GC-2010, Shimadzu) employing the following conditions: the GC system was installed with autosampler AOC20s, injector AOC-20I, and mass selective detector QP2010. The DB-5Ms fused silica capillary column (0.25mm ×30m ×0.25µm). About 1µL sample was injected at 250^{o}C temperature. The program of GC was initiated at 100^{O}C for 0.5 minutes, then increase to 280^{o}C for 3 minutes. The Helium was supply as carrier gas (1.0ml/min). The mass spectral scan range was 50-350m/z and the ionization voltage was 70eV. The spectral peaks and the chromatogram were visualized. The specific compounds presenting the sample were recognized by comparing their retention time and mass spectra of the relevant peaks with known and unknown compounds stored in the National Institute of Standards and Technology mass spectral database (NIST-MS).

5.13. Molecular Docking

PTR1 is a vital enzyme responsible for *Leishmania* pteridine salvage. The PTR1 take part to the mechanism of antifolate resistance. PTR1 is accountable for conventional therapies breakdown for example methotrexate against these protozoans. The studies on PTR1 by gene knockout in *Leishmania*, and knockdown in *Trypanosoma brucei* showed that the enzyme is essential for parasite survival (Hemavathy *et al.,* 2019; Sienkiewicz *et al.,* 2010)). Hence, in the current study, we focused to explore PTR1 (from *L. tropica*) as a possible target for our nine identified compounds from fraction (F7) by using molecular docking studies. The three-dimensional structure of PTR1 was retrieved through protein data bank (PDB) and it was predicted by the X-ray diffraction method. This selected protein under prevailed for docking purpose and PyRx software was used to study the binding affinity of test compounds with protein PTR1 in the form of E value. A three-dimensional grid with coordinates (x, y, and z) was set as maximum to provide maximum area for

compounds binding and further default parameters availed for PyRx docking. The PyRx is a virtual screening software for computer-aided drug designing and is used to monitor lead compounds against possible drug targets. The two-dimensional interaction of docked complexes was analyzed and visualized (Discovery studio visualizer) and used for systematic presentation of several hydrogen bonds, hydrophobic interactions, and residues (Khalid *et al*., 2018; Kumalo *et al.,* 2015)

RESULTS

5.14. Fraction Collected by Column Chromatography

Considering the efficacy of *P. armeniaca* in the treatment of skin ailments, infertility, spasm, hemorrhages, eye infection, and vaginal infection, *P. armeniaca* was fractionated by column chromatography in the present study. The column chromatography gave 142 fractions (20ml each) which were pooled following their TLC profiles. The same fractions were mixed resulting in 12 fractions (F1-F12) fractions. The fraction (F1) did not show any band due to hexane.

5.15. Cytotoxicity of Derived Fractions

In the derived fractions of the current study, CC_{50} of all fractions ranged from 103.01±1.93 to 251.62±1.87µg/ml (Table 5.1). The fraction (F2) exhibited the lowest (103.01±1.93) while fraction (F8) presents the highest (251.62±1.87) CC_{50} values. The CC_{50} values for fractions (F3-6) were 125.09±0.87, 113.54±2.12, 206.23±3.02, and 152.76±2.54µg/ml respectively. The fractions (F7 and F9-12) presented 214.44±1.74, 247.00±2.43, 169.04±0.93, 109.09±2.02, and 121.45±1.34µg/ml respectively. Interestingly all fractions showed CC_{50} >100µg/ml which indicates the safety range of all fractions for antileishmanial assays. When the fraction were compare with each other they showed significant value (0.000).

5.16. *In-vitro* antipromastigote Activity of Fractions

In the present investigations, the antileishmanial activity of fractions (F2-12) isolated from the crude extracts of *P. armeniaca* showed that the highest potential was found in fraction (F7) i.e. 11.48±0.82µg/ml compared with amphotericin B i.e. 11.60±0.70µg/ml against promastigotes of *L. tropica.* Fraction (F5) (IC_{50} 45.55± 0.71µg/ml), (F6) (25.21± 0.77µg/ml), (F8) (39.39± 0.87µg/ml), (F9) (51.55±1.02 µg/ml) present moderate activity with IC_{50} values varies between 45-51µg/ml. The fractions (F2), (F3), (F4), (F10), and (F11) showed the lowest antipromastigote activity against *L. tropica* with IC_{50} values between 63-95µg/ml i.e. IC_{50} 95.18±1.31, 93.50± 1.39, 63.04± 0.85, 73.56± 1.15, and 80.80±1.33µg/ml. The fraction (F12) was considered as inactive against promastigotes of *L. tropica* with IC_{50}>100µg/ml (Table. 5.1). The fractions (F1-F12) showed significant value 0.000 for IC_{50}. The fractions (F2) and (F3) showed 50.75±0.54,

and 48.21±0.05 % inhibition at the higher concentration of extract i.e 100µg/ml followed by fractions (F4-F6) which present 62.01±0.21, 70.63±0.23, and 74.51±0.65 %inhibition respectively. The fraction (F7) reported the highest % inhibition at 100µg/ml i.e 85.61±1.00 while the control showed 80.11±0.89. The fractions (F8-F11) showed 67.95±0.87, 62.09±0.92, 53.85±0.76, and 51.75±0.98 % inhibition. The fraction (F12) reported 39.42±0.54 at the highest concentration of 100µg/ml.

5.17. Antiamastigote Activity of Derived Fractions

Similarly to the promastigote stage of *L.tropica*, the highest antiamastigote activity was observed in fraction (F7) (IC_{50} 21.03±0.98µg/ml). The highest SI of fraction (F7) i.e. 10 showed the existence of the highest amounts of compounds with reasonable effectiveness against *L. tropica* The fractions (F5) (53.51±0.77µg/ml), (F6) (34.84±0.89µg/ml), (F8) (47.49±0.86µg/ml), and (F9) (58.24±1.04µg/ml) showed IC_{50} value between 34-58µg/ml having moderate antiamastigote activity and the SI were 3, 4, 5, and 4 respectively (SI>1). The fraction (F4) (78.30±0.01µg/ml), (F10) (83.43±1.72µg/ml), and (F11) (89.74±2.15µg/ml) present IC_{50} value between 78-89µg/ml having little antiamastigote potential and SI were 1, 2, and 1 (SI>1). The fractions (F2) (198.97±5.13µg/ml), (F3) (157.25±3.98µg/ml), and (F12) (141.98±4.74µg/ml) were failed to show activity against amastigotes having IC_{50} >100 and having SI values were 0.52, 0.79, and 0.85 respectively (SI<1) (Table 5.1). The significant value of p=0.000. Similarly to the promastigote stage of *L. tropica,* a high growth inhibited effect was demonstrated for amastigotes after 72h incubation. Furthermore, results indicated that the antiamastigote effect of the fraction (F7) was based on a dose-dependent manner, so the fraction (F7) was able to inhibit the growth rate of 72.75±1.00 amastigote form at 100µg/ml concentration compared with control 80.13±0.89. The fractions (F2-F6) showed 50.75±0.54, 48.21±0.05, 54.59±0.21, 70.63±0.23, and 74.51±0.65 at highest (100µg/ml) concentration. The fractions (F8-F12) exhibited 69.06±0.87, 59.12±0.92, 54.89±0.76, 49.82±0.98, and 40.31±0.54 inhibition respectively at 100µg/ml concentration. The most effect fraction (F7) caused 80.12±1.00 inhibition of antipromastigote growth at 100µg/ml concentration compared with control (85.62±1.00). Based on these results we decided that further experiments would be only with the fraction (F7).

Table 5.1: *In-vitro* antileishmanial activity and cytotoxicity of *P. armeniaca* fractions extracts (F2-F12).

Fractions	**[a]IC_{50}±SD (µg/ml)**		**[b]CC_{50}±SD macrophages µg/ml**	**SI**
	Promastigotes	Amastigotes		
F 2	95.18±1.31	198.97±5.13	103.01±1.93	0.52
F 3	93.50± 1.39	157.25±3.98	125.09±0.87	0.79
F 4	63.04± 0.85	78.30 ± 0.01	113.54±2.12	1
F 5	45.55± 0.71	53.51±0.77	206.23±3.02	3
F 6	25.21± 0.77	34.84±0.89	152.76±2.54	4
F 7	11.48± 0.82	21.03± 0.98	214.44±1.74	10
F 8	39.39± 0.87	47.49±0.86	251.62±1.87	5
F 9	51.55±1.02	58.24± 1.04	247.00±2.43	4
F 10	73.56± 1.15	83.43± 1.72	169.04±0.93	2
F 11	80.80±1.33	89.74±2.15	109.09±2.02	1
F 12	110.07±1.26	141.98±4.74	121.45±1.34	0.85
Control	11.60±0.70	22.00±1.02	252.43±2.98	11

[a]IC_{50}: concentration of fractions extracts that cause 50% of mortality of amastigotes of *L. tropica*; [b]CC_{50}: concentration of fractions extract that cause 50% of mortality of amastigotes of *L. tropica*; SD: Standard deviation; SI: Selectivity index. SI= IC50 macrophage/ IC_{50} *L. tropica* amastigote. The amastigote of fractions (F2-12) compare with control showed value of p= 0.000 (t= -16.94), 0.000 (t= -11.995), 0.001 (t= -9.616), 0.000 (t= -35.065), 0.000 (t= -13.414), 0.373 (t= 1.001), 0.000 (t= 15.906), 0.020 (t= -3.747), 0.029 (t= -3.326), 0.061 (t= -2.582) and 0.000 (t= -46.616). The promastigotes of fractions (F2-F12) compared with control showed p values of 0.156 (t= -1.742), 0.16 (t= -4.032), 0.003 (t= -6.339), 0.148 (t= 1.788), 0.019 (t= -3.795), 0.12 (t= 1.969), 0.001 (t= -8.088), 0.000 (t= -11.847), 0.000 (t= -22.726), 0.000 (t= -17.398) and 0.000 (t= -11.625).

5.18. Characterization of the Fraction (F7)

5.18.1. UV-Vis Spectroscopic Analysis

The UV-Vis spectroscopic analysis of fraction (F7) showed a spectrum at 300-800nm because of proper baseline and peaks sharpness. The spectrum showed peaks at 665.00, 607.00, 536.00, 505.50, 359.00, and 321.00nm with the absorption of 0.27, 0.08, 0.09, 0.10, 0.89, and 1.36 respectively (Table.5.2. and Fig. 5.4.).

Table: 5.2. UV-Peaks values and absorption

Sr. no	Peaks (nm)	Absorption
1	665.00	0.27
2	607.00	0.08
3	536.00	0.09
4	505.50	0.10
5	359.00	0.89
6	321.00	1.36

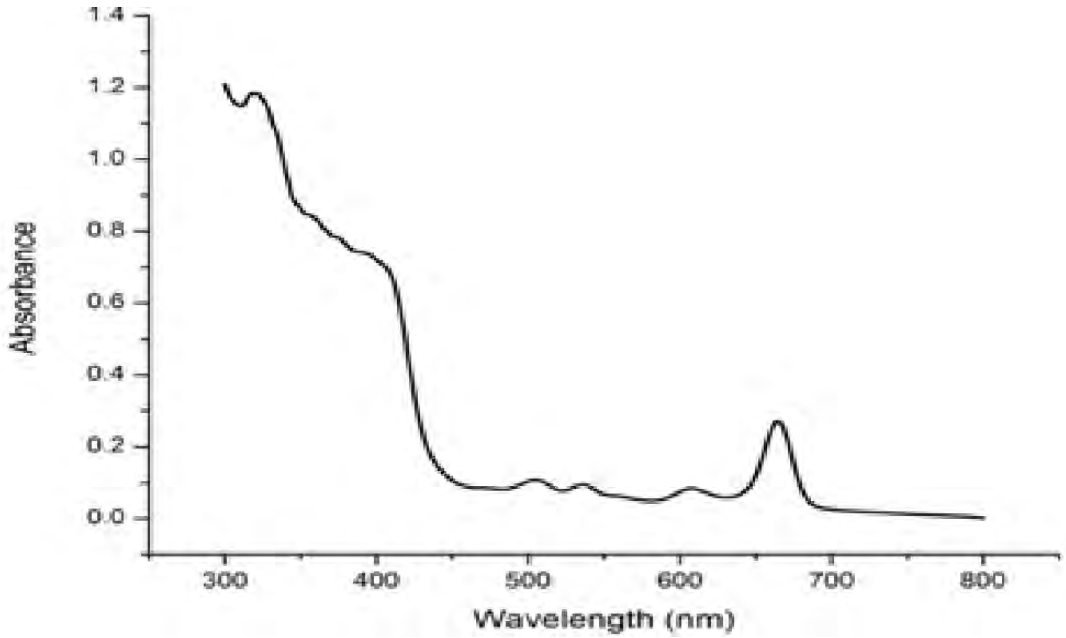


Fig. 5.4: UV–Vis spectrum of active fraction (F7) extract of *P. armeniaca*

5.18.2. FTIR Analysis of Most Active Fraction (F7)

In the current study, the characteristic peaks were exhibited at 2947, 2920, 2892, 2870, and 2848cm^{-1} assigned to the C-H stretching (alkane). The peaks obtained at 2807 and 2758cm^{-1} showed the presence of C-H stretching (aldehyde). The peaks observed at 2680, 2650, and 2610cm^{-1} indicated the presence of O-H stretching (carboxylic acid). The peak at 2587cm^{-1} was due to the S-H stretching (thiols). The peaks at 2521, 2480, 2449, and 2411cm^{-1} showed O-H stretching (carboxylic acid). The peaks at 2233 and 2158cm^{-1} showed the presence of carbon carbon triple bonds (alkynes). In addition, the peaks at 2094, 2052, and 2009cm^{-1} indicated carbonyl compounds (carbonyls and nitrile). The peaks at 2387, 2360, and 2330cm^{-1} were unknown (Table.5.3. Fig.5.5). The FTIR spectrum confirm the functional constituent's presence in the given extract and parts. In the current analysis, various functional groups (alkane, aldehyde, carboxylic acid, thiols, alkynes, and carbonyls compound) were observed in fraction (F7). Identified groups in fraction (F7) might be the reason for antileishmanial potential against *L. tropica*.

Table 5.3: FTIR interpretation of the fraction (F7)

Wavelength $cm^{-1}_{(test\ sample)}$	Frequencies range cm^{-1}(reference)	Functional group assignments	Phytocompound
2947	3000-2840	C-H stretching	Alkane
2920	3000-2840	C-H stretching	Alkane
2892	3000-2840	C-H stretching	Alkane
2870.29	3000-2840	C-H stretching	Alkane
2848	3000-2840	C-H stretching	Alkane
2807	2830-2695	C-H stretching	Aldehyde
2758	2830-2695	C-H stretching	Aldehyde
2680	3300-2500	O-H stretching	Carboxylic acid
2650	3300-2500	O-H stretching	Carboxylic acid

2610	3300-2500	O-H stretching	Carboxylic acid
2587	2600-2550	S-H stretch	Thiols
2521	3500-2400	O-H stretching,	Carboxylic acid
2480	3500-2400	O-H stretch,	Carboxylic acid
2449	3500-2400	O-H stretch,	Carboxylic acid
2411	3500-2400	O-H stretch,	Carboxylic acid
2387	-	Unknown	-
2360	2360-2100	Si-H	Silicon
2330	2360-2100	Si-H	Silicon
2233	2260-2100	Carbon carbon triple bond	Alkynes
2158	2260-2100	Carbon carbon triple bond	Alkynes
2094	2100-1800	Carbonyl compound frequency	Carbonyls
2052	2100-1800	Carbonyl compound frequency	Carbonyls
2009	2100-1800	Carbonyl compound frequency	Nitrile compounds

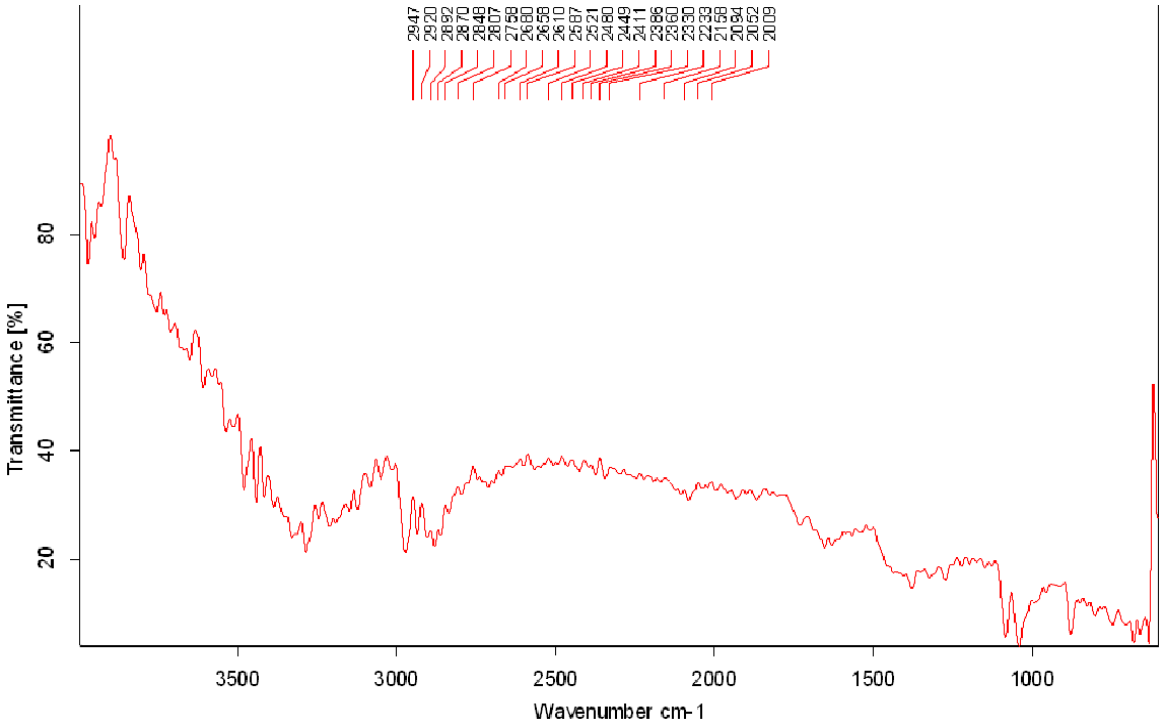
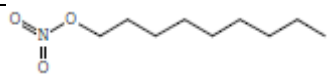


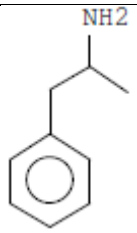
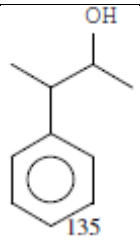
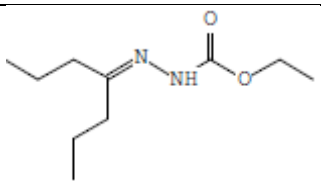
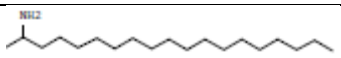
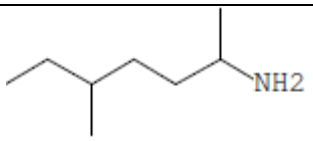
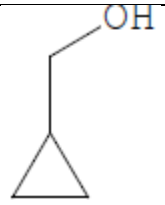
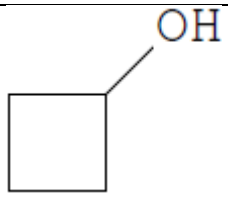
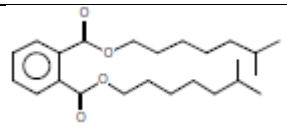
Fig. 5.5. FTIR intensity range spectrum of the fraction (F7)

5.18.3. GC-MS Analysis of Active Fraction (F7)

The anitileishmanial bioassays revealed that the fraction (F7) posses the highest antileishmanial activity against promastigotes and amastigotes of *L. tropica.* The bioactive fraction (F7) upon GC-MS analysis presents a chromatogram showing nine peaks (Fig. 5.6) which exhibited the existence of nine bioactive compounds. The retention time, area percentage, molecular weight, compound name, and structure of the recognized component are listed in Table 5.4.

Table 5.4: GC-MS analysis showed constituents present in bioactive fraction (F7) of *P. armeniaca*

Peak	Rt time	Area%	MW	Compound name	Structure
1	4.992	2.47	189	Nitric acid, nonyl ester	

2	5.200	10.16	135	Benzeneethanamine a-methyl	
3	5.375	39.38	150	Benzeneethanol,alpha,beta dimethyl	
4	5.997	18.45	200	Carbazic acid, 3-(1-propylbutylidene),ethyl ester	
5	18.691	6.03	283	2-aminononadecane	
6	19.275	4.93	129	2- heptanamine-5-methyl	
7	20.510	2.80	72	Cyclopropyl carbinol	
8	25.486	3.31	72	Cyclobutanol	
9	28.777	12.46	390	1, 2-benzenedicarboxylic acid, diisooctyl ester	

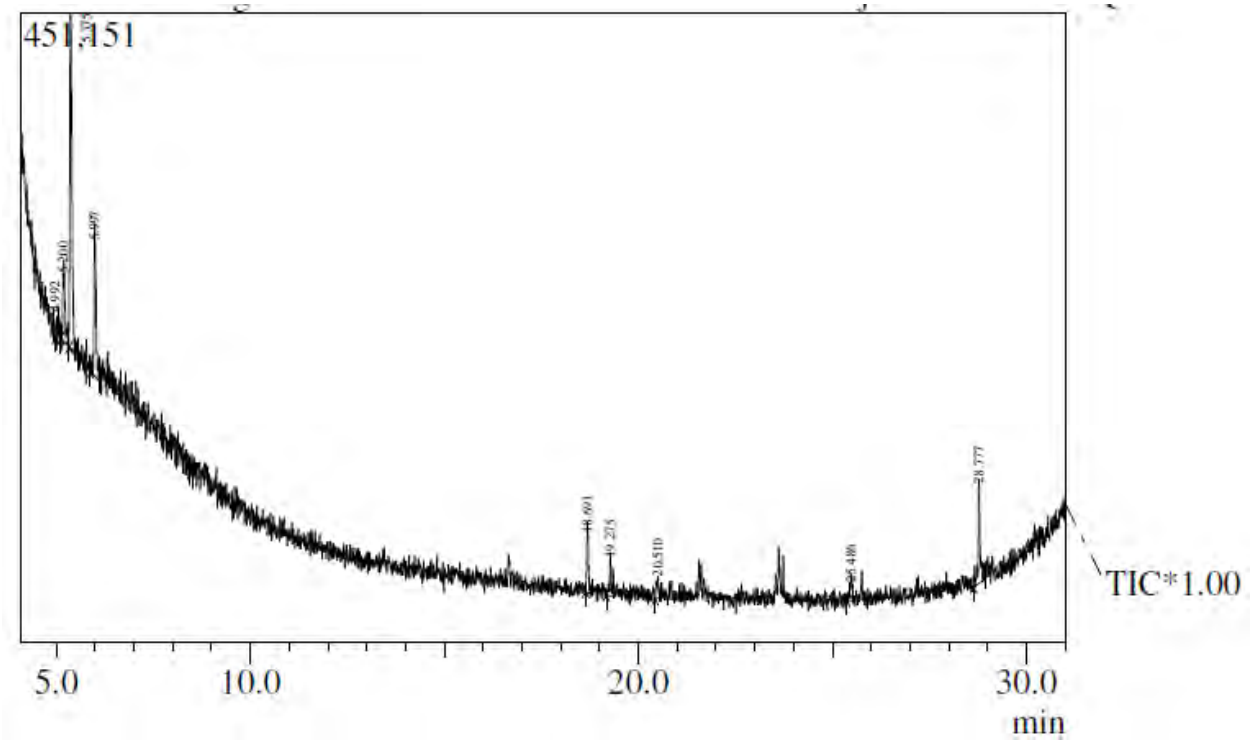


Fig. 5.6: GC-MS chromatogram of the bioactive fraction (F7) of *P. armeniaca*

5.19. Molecular Docking

The three-dimensional crystal structure of PTR1 as selected for the docking. The nine compounds from an isolated fraction (F7) (Fig. 5.7) were assessed based on binding affinity with PTR1, to rationalize the antileishmanial potential. The best docked was calculated by values of binding affinity, hydrogen-bonds, and residues of hydrogen bonding. The E-values of compound 1, 2-benzenedicarboxylic acid, diisooctyl ester, benzene ethanol alpha-beta dimethyl, benzeneethanamine alpha methyl, carbazicacid and 2 aminonona decane were -6.7, -5.8, -5.4, -5.6 and -5 kcl/mol respectively. The compounds nitric acid nonyl ester and 2 heptanamine 5 methyl showed -4.7 and -4.25kcl/mol. The lowest E value was shown by cyclobutanol and cyclopropyl carbinol i.e. -3.55kcl/mol. Out of all compounds, 1, 2-benzenedicarboxylic acid, diisooctyl ester showed significant results with the highest E value (Table.5.5).

Table 5.5: E -value and post docking analysis of the best pose of 1, 2-benzenedicarboxylic acid, diisooctyl ester compound with PTRI protein.

Compound name	E value	H bond	Bonding residues
1**, 2-benzenedicarboxylic acid, diisooctyl ester**	-6.7	4	TYR191, ALA197, LYS198, ASP181

The 2D interaction diagram showing hydrogen bonds of compound 1, 2-benzenedicarboxylic acid, diisooctyl ester with PTR1 are presented in Fig. 5.7. The vital contact residues for the docked legends were TYR191, ALA197, LYS198, and ASP181. The docking study of the 1, 2-benzenedicarboxylic acid, diisooctyl ester compound with the protein indicates its interaction with the protein through different chemical forces, i.e., bonds. Four hydrogen bonds are seemed to be involved; one is between the acidic hydrogen of the compound and electronegative part of the protein residual, two hydrogen bonds between the electronegative oxygen and positive part of the residual protein, and a fourth hydrogen bond between the electronegative nitrogen of the compound and residual protein. Other then hydrogen bonding many other important electrochemical forces are also involved, i.e., Van der Waals, carbon-hydrogen interaction, covalent bond etc. as clear from Fig. 5.7. The irreversible inhibition is achieved by using a strategy known as covalent inhibition. The irreversible inhibitors act together with their specific targets in a time-dependent manner and the reaction proceeds to end before proceeding into equilibrium. There are some significant advantages possessed by covalent inhibitors for example the covalent warheads can target exceptionally, the particular protein having non-covalent residue and thus aid in the development of strongly selected inhibitors, shallow binding cleavage in particular protein targets can be affected by covalent inhibitors, which form novel inhibitors with the highest potency than non-covalent inhibitors. The covalent interactions can be simulated by using various computational approaches; this is still a demanding area to explore. The covalent interaction (between inhibitors and biological

targets) can be described by covalent molecular docking which can be implemented in the computer-aided drug design workflows (Kumalo *et al*., 2015). In current study, a strong interaction is possible between the 1, 2-benzenedicarboxylic acid, diisooctyl ester compound, and the protein. This showed that compound (1, 2-benzenedicarboxylic acid, diisooctyl ester) bound well to the PTR1 receptor.

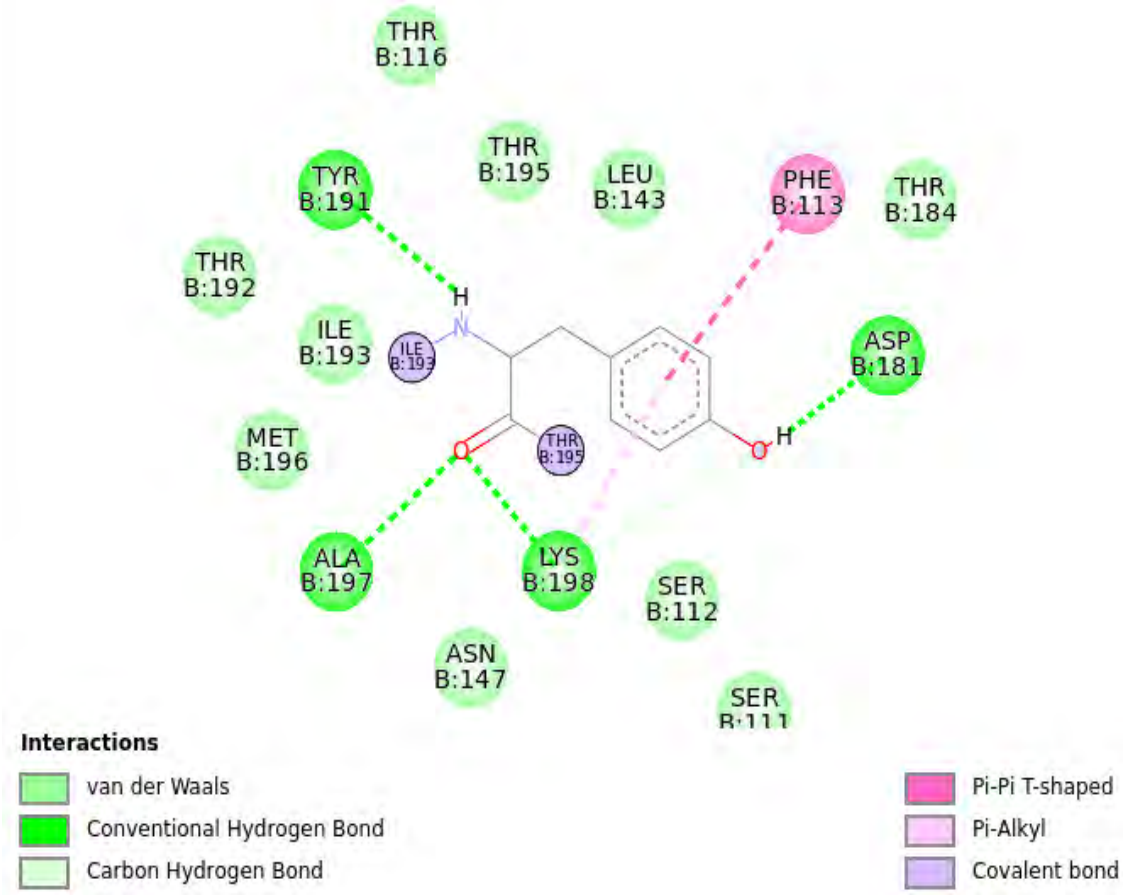


Fig. 5.7: Interaction of compound 1, 2-benzenedicarboxylic acid, diisooctyl ester with its target.

DISCUSSION

In the present investigations, the antileishmanial activity of fractions (F2-12) isolated from the crude extracts of *P. armeniaca* showed that the highest potential was found in fraction (F7) i.e. 11.48±0.82µg/ml compared with amphotericin B i.e. 11.60±0.70µg/ml against promastigotes of *L. tropica.* Similar results were also reported by previous studies where *Artemisia annua, Mimosa tenuiflorw,* and *Arnebia euchroma* present antileishmanial potential against promastigotes of *L. major* as compared to control (Shamsuddini *et al*., 2006; Sozangar *et al.,* 2012; Heidari *et al*., 2012). In the case of *L. tropica*, the test is generally conducted on promastigotes form but the promastigotes form in vertebrate host is not an infectious form of the *Leishmania* parasite. The current preliminary assessment must therefore be complemented by an assessment of intracellular amastigotes in macrophages.Simultaneously, using non-parasitized macrophages, an assessment of the potential cytotoxicity of fractional extracts must be carried out. The activity of derived fractions against intracellular amastigotes was constrasted with the toxicity exhibited against macrophages, which determined weather the fraction in-vitro potentional was due to their general toxicity or whether they had a selective potential against *L. tropica* (Chan-Bacab *et al.,* 2001; Croft *et al*., 2006).

Various advanced techniques have been reported for compounds identification from plant extracts, which exhibited antileishmanial potential. UV-VIS and FTIR spectroscopy area reliable and sensitive tools for detecting bimolecular composition (Kumar and Devi Prasad, 2011). The UV-Vis spectroscopic analysis of fraction (F7) showed a spectrum at 300-800nm because of proper baseline and peaks sharpness. The spectrum showed peaks at 665.00, 607.00, 536.00, 505.50, 359.00, and 321.00nm (Fig. 5.4.). In our investigation peak at 662.50nm wavelength was observed which corresponds to the presence of alkaloids also reported by the previous study (Leelavathi *et al*., 2018). Jain *et al.,* in (2016) reported 665nm peak wavelength in *Mentha spicata* plant extract supports our finding. The wavelength of 607.00nm was observed in the current study which was supported by the nearest peaks of 600nm in *Ferula oopoda* (Iqbal *et al*., 2019). This wavelength showed the presence of terpenoids (Santos-Buelga *et a*l., 1995) which are

reported for strong antioxidants in *P. armeniaca* (Kurus *et al*., 2014). The absorption peak at 505.00 and 536.00nm ensure the presence of the phenolic compounds (Santos-Buelga *et al.,* 1995). The presence of phenolics with antimicrobial and antioxidant potentials in *P. armeniaca* was also reported by some authors (Voi *et al*., 1995; Guclu *et al*., 2006; Sochor *et al*., 2010). The 359.0nm peak was observed in the current study and the same peak was also reported by Kalaichelvi and Dhivya in (2017). These bands showed the presence of polyphenolic compounds also supported by Santos Buelga *et al*., in (1995). The peak wavelength at 321.00nm showed valuable information on the nature of flavonoids. This is following the previous report on *Acorus calamus* (Mamta and and Jyoti, 2012). The antimicrobial activities of flavonoids were reported in *P. armeniaca* by Rashid *et al.,* (2007).

The compound benzeneethanamine a-methyl (10.16%) present in the current study already reported by Jabir *et al*., (2010) in *Solanum torvum*. The compound 2-aminononadecane (6.03%), showed antimicrobial activity (Srinivasan *et al*., 2014). Previously, cyclobutanol (3.31%) was reported in *Jugans regia* and its antimicrobial activity was also reported (Ara *et al.,* 2012). Nitric acid, nonyl ester (2.47%) compound was previously reported in *Bridelia stipularis,* and no biological activity of this compound was reported (Yusufzai *et al*., 2019). The carbazic acid, 3-(1-propylbutylidene), ethyl ester (18.45%), was also already reported in *Andrographis paniculata* (Sithar *et al*., 2017). The compounds cyclopropyl carbinal (2.80%) and 2 heptanamine, 5 methyl (4.93%) were reported by Ara *et al.*, (2013), and their biological activities are not reported. Vimalavady and Kadavul in (2013) reported 1, 2-benzenedicarboxylic acid, diisooctyl ester (12.46%) compound in *Hugonia mystax* (*Linaceae*) having no biological activity reported. The compound benzeneethanol, alpha, beta dimethyl (39.38%) is not reported previously in plants and this plant showed the highest quantity present in our study (Fig. 5.6.).

The complete genome sequence availability opens new windows for the drug target identification (Ivens *et al.,* 2005). Various enzymes e.g. PTR1 are widely explored as drug targets.The important role for unconjugated pteridines indicate that PTR1 gene deletion is lethal for the promastigotes stage of *Leishmania* (Kaur *et al*., 1988; Thony *et al.,* 2000;

Bello *et al*., 1994). A metabolic by pass of DHFR-TS inhibition was provided by the PTR1 expression and it cause complete or partial reversal of anti-pteridine inhibition in the *Leishmania* promastigotes (Kaur *et al.,* 1988; Thony *et al.,* 2000; Nare *et al*., 1997). The 3D structure of PTR1 parasite provides a strong base for new inhibitor designing which are selective for only for parasite (Gourley *et al*., 1999; Knighton *et al*., 1994). The covalent interaction (between inhibitors and biological targets) can be described by covalent molecular docking which can be implemented in the computer-aided drug design workflows (Kumalo *et al*., 2015). This study indicates that a strong interaction based on electrochemical interaction forces is possible between the 1, 2-benzenedicarboxylic acid, diisooctyl ester compound, and the protein. This showed that compound 1, 2-benzenedicarboxylic acid, diisooctyl ester bound well to the PTR1 receptor. Out of nine compounds only one compound i.e. 1, 2-benzenedicarboxylic acid, diisooctyl ester showed significant results.

Conclusion

Our finding confirmed that extract of the fraction (F7) had potent antileshmanial activity against promastigote and amastigote of *L. tropica* whereas; all fractions exhibited no cytotoxicity effect on murine macrophages. Among all compounds recognized in the fraction (F7), the 1, 2-benzenedicarboxylic acid, diisooctyl ester bound well to the PTR1 receptor by *in-silico* studies that could be an ideal candidate for the production of a novel drug against CL caused by *L. tropica.* Further *in-vivo* investigation is required to evaluate the *P. armeniaca* fractions effect and particularly 1, 2-benzenedicarboxylic acid, diisooctyl ester compound on *L. tropica,* and other *Leishmania* spp.

REFRENCES

Aara, N., Khandelwal, K., Bumb R.A., Mehta, D.R., Ghiya, C.B., Jakhar, R., Dodd, C., Salotra, P. & Satoskar A.R. (2013). Clinco-Epidemiologic Study of cutaneous leishmaniasis in Bikaner, Rajasthan, India Naushin. *Am. J. Trop. Med. Hyg,* 89(1): 111-115.

Abbas, S. R. & Sultana, N. (2020). Medicinal Significance of Malus Pumila [A Review] (2): 41-46.

Abdulhamid, A., Fakai, I.M. & Ogwihi, O.J. (2017). Phytochemical Screening, Chromatographic Studies and Antibacterial Activity of *Carica Papaya* Leaves Extracts. *Int. J. Medi Plant Natur Prod*, 3(1): 11-15.

Abdulhamid, A., Onyenonachi, I. C. & Ismail, A. M. (2017). Phytochemical Screening, Chromatographic Studies and Antibacterial Activity of *Eclipta alba* L. Leaves Extracts. *Indo Global J. Pharmaceut. Sci.,* 7: *143-147*.

Achakzai, J. K., Panezai, M. A., Akhtar, B., Kakar, S., Akbar, A., Kakar, A. M. & Achakzai, T. (2020). In Vitro Anti-Inflammatory, Anticancer (MCF-7, 3T3, and HeLa Cell Lines), and Brine Shrimp Lethality Assay and FTIR Analysis of the Extract and Fractions of the Whole Plant of Heliotropium europaeum. *Media. inflamma*, *2020*.

Afghan, A.K., Kassi, M., Kasi, P.M., Ayub, A., Kakar, N. & Marri, S.M. (2011). Clinical manifestations and distribution of cutaneous leishmaniasis in Pakistan. *J. Trop. Med. Vol,* ID 359145: 1-8.

Afshan, N. S., Iqbal, S. H., Khalid, A. N. & Niazi, A. R. (2011). Some additions to the uredinales of Azad Jammu and Kashmir (AJ & K), Pakistan. *Pak. J. Bot, 43*(2): 1373-1379.

Ahmed, A. S., Elgorashi, E. E., Moodley, N., McGaw, L. J., Naidoo, V. & Eloff, J. N. (2012). The antimicrobial, antioxidative, anti-inflammatory activity and

cytotoxicity of different fractions of four South African Bauhinia species used traditionally to treat diarrhoea. *J. Ethnopharm*, 143(3): 826-839.

Akcicek, E., Otles, S. & Esiyok, D. (2005) Cancer and its prevention by some Horticultural and field crops in Turkey. *Asian Pac J Cancer Prev*, 6: 224-230.

Akhoundi, M., Hajjaran, H., Baghaei, A. & Mohebali, M. (2013). Geographical distribution of Leishmania species of human cutaneous leishmaniasis in Fars Province, southern Iran. *Iranian J. parasitology*, 8(1): 85.

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

Alavinia, S., Arzamani, K., Reihani, M. & Jafari, J. (2009). Some epidemiological aspects of cutaneous leishmaniasis in Northern Khorasan Province, Iran. *Iran. J. Arthropod. Borne Dis*, 3(2): 50.

Alborzi, A., Rasouli, M. & Shamsizadeh, A. (2006). *Leishmania tropica* infected patient with visceral leishmaniasis in southern Iran. American *J. Tropical Medicine Hygiene,* 74: 306-307.

Aleixo, J.A., Nascimento, E.T., Monteiro, G.R., Fernandes, M.Z., Ramos, A.M., Wilson, M.E., Pearson, R.D. & Jeronimo, S.M. (2006). Atypical American visceral leishmaniasis caused by disseminated *Leishmania amazonensis* infection presenting with hepatitis and adenopathy. *Transactions Royal Society Tropical Medicine Hygiene*, 100: 79-82.

Al-Gindan, Y., Kubba, R., El-Hassan, A., Omar, A.H., Kutty, M.K. & Saeed, M. B. (1989). Dissemination in cutaneous leishmaniasis. 3. Lymph node involvement. *Int. J. Dermatol*, 28: 248 -54.

Ali, A., Rehman, T. U., Qureshi, N. A. & Rahman, H. U. (2016). New endemic focus of cutaneous leishmaniasis in Pakistan and future epidemics threats. *Asian Pacific J. Tropi Dise*, *6*(2): 155-159.

Ali, B. H., Blunden, G., Tanira, M. O. & Nemmar, A. (2008). Some phytochemical, pharmacological and toxicological properties of ginger (*Zingiber officinale Roscoe)*: a review of recent research. *Food chemical Toxicology, 46*(2): 409-420.

Ali, M.M., Mikhail, N.N. & Haq, M.S. (1978). A class of bivariate distributions including the bivariate logistic. *J. multivariate analysis*, *8*(3): 405-412.

Ali, S.I. (2008). Significance of flora with special reference to Pakistan. *Pak. J. Bot,* 40(30): 967-71.

Al-Jaser, M.H. (2005). Treatment trends of cutaneous leishmaniasis in Saudi Arabia. *Saudi Med J,* 26(8): 1220-24.

Al-Jawabreh, A., Barghuthy, F., Schnur, L. F., Jacobson, R. L., Schoenian, G. & Abdeen, Z. (2003) Risk assessment of cutaneous leishmaniasis in the endemic area of Jericho in Palestine. *East. Med. Hlth. J*, 9(4): 805-15.

Al-Jawabreh, A., Schnur, L. F., Nasereddin, A., Schwenkenbecher, J. M., Abdeen, Z., Barghuthy, F. & Schoian, G. (2004). The recent emergence of Leishmania tropica in Jericho (Ariha) and its environs, a classical focus of *L. major. Tropical Medicine & International Health*, 9(7): 812-816.

Alsamarai, A.M. & Alobaidi, H.S. (2009). Cutaneous Leishmaniasis in Iraq. *J Infect Dev Countr*. 3: 123-129.

Alten, B., Caglar, S. S., Kaynas, S. & Simsek, F. M. (2003). Evaluation of protective efficacy of K-OTAB impregnated bednets for cutaneous leishmaniasis control in Southeast Anatolia-Turkey. *J. Vect. Ecolo. J. Soci. Vect. Ecol*, 28(1): 53-64.

Alvar, J., Croft, S. & Olliaro, P. (2006). Chemotherapy in the treatment and control of leishmaniasis. *Advances in parasitology*, *61*: 223-274.

Alvar, J., Velez, 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.

Alvar, J., Velez, I.D. & Bern, C. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*, 7: 356-371.

Alviano, D. S., Barreto, A. L. S., Dias, F. D. A., Rodrigues, I. D. A., Rosa, M. D. S. D. S. R., Alviano, C. S. & Soares, R. M. D. A. (2012). Conventional therapy and promising plant-derived compounds against trypanosomatid parasites. *Frontiers in microbiology*, 3(1): 1-10.

Amarowicz, R., Pegg, R., Rahimi-Moghaddam, P., Barl, B. & Weil, J. (2004). Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem, 84(4): 551-562.

Amasiatsi, A., Gabrielyan, E.S., Revazova, L.V., Aleksandrovskaya, O.A. & Commentary: Vardanyan, S.H. (1990). Moscow. "Nauka". (Science Publishers). 294.

Anand, T. & Gokulakrishnan, K. (2012). Phytochemical analysis of *Hybanthus enneaspermus* using UV, FTIR and GC-MS. *J. Pharmacy*, *2*(3): 520-524.

Andrews, K. T., Fisher, G. & Skinner-Adams, T. S. (2014). Drug repurposing and human parasitic protozoan diseases. *Inter. J. Parasito. Drug. Resis*, *4*(2): 95-111.

Ara, M.A., Bakir, W.N., Hozzein, T. & Kudo, (2013). Population, morphological and chemotaxonomical characterization of diverse rare actinomycetes in the mangrove and medicinal plant rhizosphere. *Afr. J. Microbiol. Res*, 7:1480-1488.

Araujo, A.C., Gonçalves, N.N.V.M., Dantas-Torres, F., Ferreira, F. & Horta, M.C. (2016). Visceral Leishmaniasis in Petrolina, State of Pernambuco, Brazil, 2007–2013. *Rev. Inst. Trop, São Paulo* (in press).

Arevalo, I., Ward, B., Miller, R., Meng, T. C., Najar, E., Alvarez, E. & Alejandro, L. C. (2001). Successful treatment of drug-resistant cutaneous leishmaniasis in humans by

use of imiquimod, an immunomodulator. *Clinical infectious diseases*, *33*(11): 1847-1851.

Arfan, B. & Rahman, S. (2006). Correlation of clinical, histopathological, and microbiological finding in 60 cases of Cutaneous Leishmaniasis. Ind. Dermato. Venereol Leprol, (72): 28-32.

Arya, V. (2011). A review on anti-tubercular plants. *Int J Pharm Tech Res,* 3: 872-880.

Ashokkumar, R. & Ramaswamy, M. (2014). Phytochemical screening by FTIR spectroscopic analysis of leaf extracts of selected Indian Medicinal plants. *Int.J.Curr. Microbiol. App.Sci,* 3(1): 395-406.

Asma, B.M. (2002). Geçmisten gunumuze Malatyada kayisi yetistiriciligi. *Izollu Dergisi,* 5: 18-20.

Ayaz, M.M., Nazir, M.M., Ullah, N., Zaman, A., Akbar, A., Zeeshan, M. & Lindsay, D.S. (2018). Cutaneous leishmaniasis in the metropolitan City of Multan, Pakistan, a neglected tropical disease. *J. medi. entomo*, *55*(4): 1040-1042.

Ayub, S., Gramiccia, M., Khalid, M., Mujtaba, G. & Bhutta, R.A. (2003). Cutaneous leishmaniasis in Multan: species identification. *J. Pak. Med. Assoc,* 53(10): 445-447.

Ayub, S., Khalid, M., Mujtaba, G. & Bhutta, R. A. (2001). Profile of patients of cutaneous leishmaniasis from Multan. *J. Pak. Medi. Asso, 51*(8): 279-281.

Azizi, N., Torkiyan, L. & Saidi, M. R. (2006). Highly efficient one-pot three-component Mannich reaction in water catalyzed by heteropoly acids. *Organic Letters*, *8*(10): 2079-2082.

Baghaei, M. (2005). Intraspecific variation in *Leishmania major* isolated from different forms of zoonotic Cutaneous Leishmaniasis. *IJMS,* 30: 51-54.

Bahmani, M. & Eftekhari, Z. (2013). An ethnoveterinary study of medicinal plants in treatment of diseases and syndromes of herd dog in southern regions of Ilam province, Iran. *Comparative clinical pathology*, 22(3): 403-407.

Bailey, B. N. & Mahaffee, W. F. (2017). Rapid, high-resolution measurement of leaf area and leaf orientation using terrestrial LiDAR scanning data. *Measurement Sci. Technolo*, *28*(6): 064006.

Bailey, M. S. & Lockwood, D. N. (2007). Cutaneous leishmaniasis. *Clinics in dermatology*, *25*(2): 203-211.

Balavignesh, V., Srinivasan, E., Ramesh Babu, N. G. & Saravanan, N. (2013). Molecular docking study ON NS5B polymerase of hepatitis c virus by screening of volatile compounds from Acacia concinna and ADMET prediction. *Int J Pharm Life Sci*, 4: 2548-58.

Bañuls, A.L., Hide, M. & Prugnolle, F. (2007). *Leishmania* and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. *Adv. Parasitol,* 64: 1-458.

Bashaye, S., Nombela, N., Argaw, D., Mulugeta, A., Herrero, M., Nieto, J., Chicharro, C., Canavate, C., Aparicio, P., Velez, I.D., Alvar, J. & Bern, C. (2009). Risk factors for visceral leishmaniasis in a new epidemic site in Amhara Region, Ethiopia. *Am J Trop Med Hyg,* 8: 34-39.

Baytop, T. (1999). Turkiye'de Bitkiler ile Tedavi. Istanbul, *Nobel Tp Kitapevleri,* 268-374.

Bekele, B., Adane, L., Tariku, Y. & Hailu, A. (2013). Evaluation of antileishmanial activities of triglycerides isolated from roots of Moringa stenopetala. *Medicinal Chemistry Research*, *22*(10): 4592-4599.

Bekhit, A. A., El-Agroudy, E., Helmy, A., Ibrahim, T. M., Shavandi, A. & Bekhit, A.E.D.A. (2018). *Leishmania* treatment and prevention: Natural and synthesized drugs. *European journal of medicinal chemistry*, *160*: 229-244.

Bello, A. R., Nare, B., Freedman, D., Hardy, L. & Beverley, S. M. (1994). PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. *Proceedings National Acad Sci*, *91*(24): 11442-11446.

Ben Salah, A. (2007). Zoonotic cutaneous leishmaniasis in central Tunisia: spatio-temporal dynamics. *Int J Epidemiol*, 36: 991-1000.

Bensoussan, E., Nasereddin, A., Jonas, F., Schnur, L.F. & Jaffe, C.L. (2006). Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. *J Clin Microbiol*, 44(4): 1435-1439.

Beretta, G., & Facino, R. M. (2010). Recent advances in the assessment of the antioxidant capacity of pharmaceutical drugs: from in vitro to in vivo evidence. *Analytical and bioanalytical chemistry*, *398*(1): 67-75.

Berhow, M. A., Wagner, E. D., Vaughn, S. F. & Plewa, M. J. (2000). Characterization and antimutagenic activity of soybean saponins. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 448(1): 11-22.

Berman, H., Henrick, K. & Nakamura, H. (2003). Announcing the worldwide protein data bank. *Nat. Structural & Mol Bio*, *10*(12): 980-980.

Bern, C., Joshi, A.B., Jha, S.N., Das, M.L., Hightower, A., Thakur, G.D. & Bista, M.B. (2000). Factors associated with visceral leishmaniasis in Nepal: bed-net use is strongly protective. *Am. J. Trop. Med. Hyg.* 63(3-4): 184-188.

Bern, C., Maguire, J.H., Alvar, J. (2008). Complexities of assessing the disease burden attributable to leishmaniasis. *PLoS Negl Trop Dis*, 2(10): 313.

Beverley, S.M., Ismach, R.B. & Pratt, D.M. (1987). Evolution of the genus Leishmania as revealed by comparisons of nuclear DNA restriction fragment patterns. Proceedings National Academy Sciences United States America, 84: 484-488.

Bhutto, A.M., Soomro, R.A., Nonaka, S. & Hashiguchi, Y. (2003). Detection of new endemic areas of cutaneous leishmaniasis in Pakistan: a 6-year study. *Int J Dermatol*, 42(7): 543-8.

Bifeld, E. & Clos, J. (2015).The genetics of *Leishmania* virulence. Med.Microbiol. Immunol. 204: 619-634.

Blum, J.A., Hatz. & C.F. (2009). Treatment of cutaneous leishmaniasis in travelers. *J. Travel Med.* 16(2):123-131.

Bodiwala, H. S., Singh, G., Singh, R., Dey, C. S., Sharma, S. S., Bhutani, K. K. & Singh, I. P. (2007). Antileishmanial amides and lignans from *Piper cubeba* and *Piper retrofractum. J. Natural Medi, 61(4): 418-421.*

Boggild, A.K., Valencia, B.M., Espinosa, D., Veland, N., Ramos, A.P., Arevalo, J. & Llanos-Cuentas, A. (2010). Low DE Detection and species identification of *Leishmania* DNA from filter paper lesion impressions for patients with American cutaneous leishmaniasis. *Clin Infect Dis,* 50(1): 1-6.

Bonecchi, R., Bianchi, G., Bordignon, P. P., D'Ambrosio, D., Lang, R., Borsatti, A. & Sinigaglia, F. (1998). Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. experimental medicine*, 187(1): 129-134.

Bouyahya, A., Abrini, J., Talbaoui, A., Et-Touys, A., Chatoui, K., Harhar, H., Bakri, Y. & Dakka, N. (2017). Phytochemical screening, antiradical and antibacterial activities of *Cistus crispus* from Morocco. *J. Mater Environ. Sci,* 8: 1560-1566.

Bouyahya, A., Bensaid, M., Bakri, Y. & Dakka, N. (2016). Phytochemistry and ethnopharmacology of *Ficus carica. Int. J. Biochem. Res. Rev*, *14*: 1-12.

Bouyahya, A., Et-Touys, A., Bakri, Y., Talbaui, A., Fellah, H., Abrini, J. & Dakka, N. (2017). Chemical composition of *Mentha pulegium* and *Rosmarinus officinalis*

essential oils and their antileishmanial, antibacterial and antioxidant activities. *Microbial pathogenesis*, *111*: 41-49.

Brand-Williams, W., Cuvelier, M.E. & Berset, C.L.W.T. (1995). Use of a free radical method to evaluate antioxidant activity. *Food Sci. Technol*. 28(1): 25-30.

Brenzan, M. A., Nakamura, C. V., Dias Filho, B. P., Ueda-Nakamura, T., Young, M. C. M. & Cortez, D. A. G. (2007). Antileishmanial activity of crude extract and coumarin from Calophyllum brasiliense leaves against *Leishmania amazonensis. Parasitolo research*, 101(3): 715-722.

Britten, R.J. (1986). Rates of DNA sequence evolution differ between taxonomic groups. *Science*, 231: 1393-1398.

Brodskyn, C., De Oliveira, C. I., Barral, A. & Barral-Netto, M. (2003). Vaccines in leishmaniasis: advances in the last five years. *Expert revie vaccin*, *2*(5): 705-717.

Brooker, S., Mohammed, N., Adil, K., Agha, S., Reithinger, R., Rowland, M., Ali, I. & Kolaczinski, J.(2004). Leishmaniasis in refugee and local Pakistani populations. *Emerg. Infect. Dis, 10*(9): 1681-1684.

Bucheton, B., Abel, L., Kheir, M.M., Mirgani, A., El-Safi, S.H., Chevillard, C. & Dessein, A. (2003). Genetic control of visceral leishmaniasis in a Sudanese population: candidate gene testing indicates a linkage to the NRAMP1 region. *Gene Immunit,* 4: 104-109.

Bulle, B., Millon, L., Bart, J.M., Gállego, M., Gambarelli, F., Portús, M., Schnur, L., Jaffe, C.L., Fernandez-Barredo, S., Alunda, J.M. & Piarroux, R. (2002). Practical approach for typing strains of *Leishmania infantum* by microsatellite analysis. *J. Clin. Microbiol,* 40: 339

Bussmann, R. W., Malca, G., Glenn, A., Sharon, D., Nilsen, B., Parris, B. & Townesmith, A. (2011). Toxicity of medicinal plants used in traditional medicine in Northern Peru. *J. ethnophar*, 137(1): 121-140.

Calla-Magariños, J. (2012). Bioactive leishmanicidal alkaloid molecules from *Galipea longiflora Krause* with immunomodulatory activity (Doctoral dissertation, The Wenner-Gren Institute, Stockholm University).

Camacho, C., Coulouris, G., Avagyan, V. Ma N., Papadopoulos, J., Bealer, K. & Madden, T.L. (2009). BLAST+: architecture and applications. *BMC Bioinf,* 10: 421.

Camacho, M. D. R., Phillipson, J. D., Croft, S. L., Solis, P. N., Marshall, S. J. & Ghazanfar, S. A. (2003). Screening of plant extracts for antiprotozoal and cytotoxic activities. *J. ethnopharmacology*, 89(2-3): 185-191.

Canadanovic-Brunet, J., Cetkovic, G., Saponjac, V. T., Stajcic, S., Vulic, J., Djilas, S. & Popović, B. (2014). Evaluation of phenolic content, antioxidant activity and sensory characteristics of Serbian honey-based product. *Industrial Crop. Prod*, *62*: 1-7.

Cantacessi, C., Dantas-Torres, F., Nolan, M. J. & Otranto, D. (2015). The past, present, and future of *Leishmania* genomics and transcriptomics. *Trends parasitology*, *31*(3): 100-108.

Carrio, J., De Colmenares, M., Riera, C., Gállego, M., Arboix, M. & Portus, M. (2000). *Leishmania infantum*: stage-specific activity of pentavalent antimony related with the assay conditions. *Experim parasito*, 95(3): 209-214.

Catsburg, A., van der Zwet, W.C., Morre, S.A., Ouburg, S., Vandenbroucke-Grauls, C.M. & Savelkoul, P.H. (2007). Analysis of multiple single nucleotide polymorphisms (SNP) on DNA traces from plasma and dried blood samples. *J. Immunol Methods,* 321 (2): 135-41.

Chakravarty, J. & Sundar, S. (2010). Drug resistance in leishmaniasis. *Journal of global infectious diseases*, *2*(2), 167.

Chan, C. H., Yusoff, R., Ngoh, G. C. & Kung, F. W. L. (2011). Microwave-assisted extractions of active ingredients from plants. *J. Chromatography A*, *1218*(37): 6213-6225.

Chan-Bacab, M. J. & Peña-Rodríguez, L. M. (2001). Plant natural products with leishmanicidal activity. *Natur prod repo*, 18(6): 674-688.

Chang, H.K., Yang, H.Y, Lee, T.H., Shin, M.C., Lee, M.H. & Shin MS (2005). Armeniacae semen extract suppresses lipopolysaccharide-induced expressions of cycloosygenase-2 and inducible nitric oxide synthase in mouse BV2 microglial cells. *Biol Pharm Bull*, 28: 449-455.

Chettri, N., Sharma, E. & Lama, S.D. (2005). Non-timber forest produces utilization, distribution and status in a trekking corridor of Sikkim, India. *Lyonia,* 8:89-101.

Cheuk, A. & Houde, M. (2016). Genome wide identification of C1-2i zinc finger proteins and their response to abiotic stress in hexaploid wheat. *Mol. Genet Genomi*, *291*(2): 873-890.

Chew, Y.L., Goh, J.K. & Lim, Y.Y. (2009). Assessment of *in vitro* antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia. *Food Chem,* 119: 373-378.

Chouhan, H. S. & Singh, S. K. (2011). Phytochemical analysis, antioxidant and anti-inflammatory activities of *Phyllanthus simplex*. *J. Ethnoph*, 137(3): 1337-1344.

Chowdhury, R., Chowdhury, V., Faria, S., Islam, S., Maheswary, N.P., Akhter, S., Islam, M. S., Dash, A. P., Kroeger, A. & Banu, Q. (2018). Indoor residual spraying for kala-azar vector control in Bangladesh: A continuing challenge. PLoS neglected tropical diseases, 12(10): e0006846.

Chowdhury, R., Dotson, E., Blackstock, A.J., McClintock, S., Maheswary, N.P., Faria, S., Islam, S., Akter, T., Kroeger, A., Akhter, S. & Bern C. (2011). Comparison of insecticide-treated nets and indoor residual spraying to control the vector of visceral

leishmaniasis in Mymensingh District, Bangladesh. *Am J Trop Med Hyg*, 84(5): 662-7.

Chowdhury, R., Mondal, D., Chowdhury, V., Faria, S., Alvar, J. &Nabi S.G. (2014). How Far Are We from Visceral Leishmaniasis Elimination in Bangladesh? An Assessment of Epidemiological Surveillance Data. *PLoS Negl Trop Dis,* 8(8): e3020.

Church, W.H. (2005). Column Chromatography Analysis of Brain Tissue: An Advanced Laboratory Exercise for Neuroscience Majors. *J. Undergraduate Neuroscience Edu*, 3(2): 36-41.

Coleman, M., Foster, G.M., Deb, R., Singh, R.P., Ismail, H.M., Shivam, P., Ghosh, A.K., Dunkley, S., Kumar, V., Coleman, M. & Hemingway, J. (2015). DDT-based indoor residual spraying suboptimal for visceral leishmaniasis elimination in India. *Proceed National Acad. Sci,* 112(28): 8573-8578.

Copeland N.K. & Aronson N.E. (2015). Leishmaniasis: Treatment updates and clinical practice guidelines review. *Curr Opin Infect Dis,* 28(5): 426-437.

Cordell, G. A. (1981). *Introduction to alkaloids: A biogenetic approach*. John Wiley & Sons. Cos, P., Vlietinck, A. J., Berghe, D. V. & Maes, L. (2006). Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. *J. ethnopharmacology*, 106(3): 290-302.

Couppie, P., Clyti, E., Sainte-Marie, D., Dedet, J.-P., Carme, B. & Pradinaud, R. (2004). Disseminated cutaneous leishmaniasis due to *Leishmania guyanensis*: case of a patient with 425 lesions. *American J. Tropical Medicine Hygiene,* 71: 558-560.

Croft, S. L., Sundar, S. & Fairlamb, A. H. (2006). Drug resistance in leishmaniasis. *Clinical microbio reviews*, *19*(1): 111-126.

Cunningham, J., Hasker, E., Das, P., El, S., Goto, H. & Mondal, D. (2012). A globalcomparative evaluation of commercial immunochromatographic rapid

diagnostictests for visceral leishmaniasis. *Clin. Infect. Dis,* 55 (10): 1312-1319.

Cupolillo, E., Brahim, L.R., Toaldo, C.B., de Oliveira-Neto, M.P., de Brito, M.E., Falqueto, A., de Farias Naiff, M. & Grimaldi, G., (2003). Genetic polymorphism and molecular epidemiology of *Leishmania (Viannia) braziliensis* from different hosts and geographic areas in Brazil. *J. Clinical Microbiology,* 41: 3126-3132.

Cupolillo, E., Junior, G., Momen, H. & Beverley, S.M. (1995). Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of *Leishmania. Mol Biochem Parasitol.* 73(1-2): 145-155.

Cupolillo, E., Momen, H. & Grimaldi Jr, G. (1998). Genetic diversity in natural populations of New World Leishmania. *Memorias do Instituto Oswaldo Cruz*, *93*(5): 663-668.

Da Porto, C., Calligaris, S., Celotti, E. & Nicoli, M.C. (2000). Antiradical properties of commercial cognacs assessed by the DPPH test. *J. Agric. Food Chem*, 48(9): 4241-4245.

da Silva L.A., de Sousa C.S., da Graca G.C., Porrozzi R. & Cupolillo E. (2010). Sequence analysis and PCR-RFLP profiling of the *hsp70* gene as a valuable tool for identifying *Leishmania* species associated with human leishmaniasis in Brazil. *Infect. Genet. Evol.* 10(1): 77-83.

Das, M., Banjara, M., Chowdhury, R., Kumar, V., Rijal, S., Joshi, A., Akhter, S., Das, P. & Kroeger, A. (2008). Visceral leishmaniasis on the Indian sub-continent: a multi-centre study of the costs of three interventions for the control of the sand fly vector, *Phlebotomus argentipes*. *Ann Trop Med Parasitol,* 102 (8): 729-741.

David, C. V. & Craft, N. (2009). Cutaneous and mucocutaneous leishmaniasis. *Dermatologic ther*, *22*(6): 491-502.

Davies, C.R., Llanos-Cuentas, E.A., Sharp, S.J., Canales, J., Leon, E., Alvarez, E., Roncal, N. & Dye C. (1997). Cutaneous leishmaniasis in the Peruvian Andes: factors

associated with variability in clinical symptoms, response to treatment, and parasite isolation rate. *Clin Infect Dis*, 25(2): 302-10.

Dawit, G., Girma, Z. & Simenew K. (2013). A review on biology, epidemiology and public health significance of leishmaniasis. *J Bacteriol Parasitol,* 4: 166.

de Albuquerque Melo, G. M., Silva, M. C. R., Guimaraes, T. P., Pinheiro, K. M., da Matta, C. B. B., de Queiroz, A. C. & Viegas Jr, C. (2014). Leishmanicidal activity of the crude extract, fractions and major piperidine alkaloids from the flowers of Senna spectabilis. *Phytomedicine*, *21*(3): 277-281.

de Andrade, J. P., Pigni, N. B., Torras-Claveria, L., Berkov, S., Codina, C., Viladomat, F. & Bastida, J. (2012). Bioactive alkaloid extracts from Narcissus broussonetii: Mass spectral studies. *J. pharmaceut. Biomedi. Anal, 70*: 13-25.

de Araujo, F. J., da Silva, L. D. O., Mesquita, T. G., Pinheiro, S. K. & Vital, W. (2016). Polymorphisms in the Tollip Gene Influence Susceptibility to Cutaneous Leishmaniasis Caused by Leishmania guyanensis in the Amazonas State of Brazil. *PLoS Negl Trop Dis*. 9(6): e0003875.

De Medeiros, M. D. G. F., Da Silva, A. C., Citó, A. M. D. G. L., Borges, A. R., De Lima, S. G., Lopes, J. A. D. & Figueiredo, R. C. B. Q. (2011). In vitro antileishmanial activity and cytotoxicity of essential oil from Lippia sidoides Cham. *Parasitology International*, 60(3): 237-241.

de Oliveira, A. B., de Brito Neto, J. F., Cardoso, G. D. & do Vale, L. S. (2017). Growth and yield of castor bean (Ricinus communis L.) cv.'BRS Energia'under different spacings. *Tropic. Subtropi. Agroecosys*, 20(2).

de Vries, H.J., Reedijk, S.H. & Schallig, H.D. (2015). Cutaneous leishmaniasis: recent developments in diagnosis and management. *Amer clinical der, 16*(2): 99-109.

Dedet, J.P. (1999). *Leishmaniasis*. Paris: Ellipses, French.

Delfan, B., Bahmani, M., Hassanzadazar, H., Saki, K. & Rafieian-Kopaei, M. (2014). Identification of medicinal plants affecting on headaches and migraines in Lorestan Province, West of Iran. *Asi. Pacific J. tropi medi*, *7*: 376-379.

Desjeux, P. (2004). Leishmaniasis: current situation and new perspectives. Comp. *Immunol. Microbiol. Infect. Dis,* 27: 305-318.

Dietrich, G., Dolan, M. C., Peralta-Cruz, J., Schmidt, J., Piesman, J., Eisen, R. J., & Karchesy, J. J. (2006). Repellent activity of fractioned compounds from Chamaecyparis nootkatensis essential oil against nymphal Ixodes scapularis (Acari: Ixodidae). *J. medical entomol*, 43(5): 957-961.

Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P. & Vidal, N. (2006). Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food chemistry*, *97*(4): 654-660.

Doudi, M., Hejazi, SH., Razavi, M.R., Narimani, M., Khanjani, S. & Eslami, G. (2010). Comparative molecular epidemiology of *Leishmania major* and *Leishmania tropica* by PCR-RFLP technique in hyper endemic cities of Isfahan and Bam, Iran. Med Sci Monit. 16(11): 530-5.

Du, R., Hotez, P. J., Al-Salem, W. S. & Acosta-Serrano, A. (2016). Old World Cutaneous Leishmaniasis and Refugee Crises in the Middle East and North Africa. *PLoS neglected tropical diseases*, 10(5): 1-11.

Durmaz, G. & Alpaslan, M. (2007). Antioxidant properties of roasted apricot (*Prunus armeniaca* L.) kernel. *Food Chem*, 100:1177-1181.

Durrani, A.Z., Durrani, H.Z., Kamal, N. & Mehmood, N. (2011). Prevalence of cutaneous leishmaniasis in humans and dogs in Pakistan. *Pak. J. Zool,* 43: 263-271.

Edeoga, H.O., Okwu, D.E. & Mbaebie, B.O. (2005).Phytochemical constituents of some Nigerian medicinal plants. *Afri. J. Biotechnol,* 4: 685-688.

Edrah S., Alafid, F. & Kumar, A. (2015). Preliminary Phytochemical Screening and Antibacterial Activity of Pistacia atlantica and Prunus persica Plants of Libyan Origin. *Inter. J. Sci. Research*, 4(2): 1552-1555.

Egner, U., Krätzschmar, J., Kreft, B., Pohlenz, H. D. & Schneider, M. (2005). The target discovery process. *Chembiochem, 6*(3): 468-479.

El Tai, N. O., El Fari, M., Mauricio, I., Miles, M. A., Oskam, L., El Safi, S. H. & Schönian, G. (2001). Leishmania donovani: intraspecific polymorphisms of Sudanese isolates revealed by PCR-based analyses and DNA sequencing. *Experimental parasitology*, *97*(1): 35-44.

El-Buni, A., Jabeal, I. & Ben-Darif, A. (2000) Cutaneous leishmaniasis in the Libyan Arab Jamahiriya a study of the Yafran area. *East Mediterr Health J,* 6: 884-887.

Ellman, G. L., Courtney, K. D., Andres Jr, V. & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical pharmacology*, *7*(2): 88-95.

Eltayeb, A. & Ibrahim, K. (2011). Potential Antileishmanial Effect of Three Medicinal Plants. *Indian J Pharm Sci,* 74(2): 171-174.

Emami, S. A., Rabe, S. Z. T., Ahi, A. & Mahmoudi, M. (2012). Inhibitory activity of eleven Artemisia species from Iran against Leishmania major parasites. *Iranian J. basic medi. Sci*, *15*(2): 807.

Enami, M.M., Yazdi, M. & Nilofroushzadeh, M. (2009). Emergence of cutaneous leishmaniasis due to *Leishmania major* in a new focus of Central Iran. *Trans. R. Soc. trop. Med. Hyg.* 103(12): 1257-1262.

Erdogan, B., Bauer, T. N., Peiró, J. M. & Truxillo, D. M. (2011). Overqualified employees: Making the best of a potentially bad situation for individuals and organizations. *Industrial and organizational psychology*, 4(2): 215-232.

Erdogan-Orhan, I. & Kartal, M. (2011). Insights into research on phytochemistry and biological activities of *Prunus armeniaca L.* (apricot). *Food Research International*, 44(5): 1238-1243.

Eroglu F, Koltas I.S. & Alabaz D. (2015). Clinical manifestations and genetic variation of *Leishmania infantum* and *Leishmania tropica* in Southern Turkey. *Exp Parasitol.* 154: 67-74.

Esch, K.J. & Petersen, C.A. (2013). Transmission and epidemiology of zoonotic protozoal diseases of companion animals. Clin. Microbiol. Rev. 26: 58-85.

Fajardo, E., Metcalf, C.A., Chaillet, P., Aleixo, L., Pannus, P., Panunzi, I., Trivino, L., Ellman, T., Likaka, A. & Mwenda R. (2014). Prospective evaluation of diagnostic accuracy of dried blood spots from finger prick samples for determination of HIV-1 load with the NucliSENS Easy-Q HIV-1 Version 2.0 assay in Malawi. *J. Clin. Microbiol,* 52: 1343-1351.

Fakhar, M., PazokiGhohe, H., Rasooli, S.A., Karamian, M., Mohib, A.S, ZiaeiHezarjaribi, H., Pagheh, A.S. & Ghatee MA. (2016). Genetic diversity of *Leishmania tropica* strains isolated from clinical forms of cutaneous leishmaniasis in rural districts of Herat province, Western Afghanistan, based on ITS1-rDNA. *Infect Genet Evol,* 41: 120-127.

Fang, G., Zhou, J., Pan, A. & Liang, S. (2018). Recent advances in aqueous zinc-ion batteries. *ACS Energy Letters*, *3*(10): 2480-2501.

Fatima, H., Qureshi, N. A., Shaheen, N., Rashid, U. & Qamar, M. F. (2017). In vivo antiplasmodial potential of aqueous extracts of Trachyspermum ammi and Punica granatum seeds in male BALB/c mice. *Acta polo phar*, 74(6): 1781-1795.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* 39: 783-791.

Fernandes, O., Murthy, V.K., Kurath, U., Degrave, W.M. & Campbell, D.A. (1994). Mini-exon gene variation in human pathogenic Leishmania species. Mol Biochem Parasitol. 66(2), 261-271.

Fernández, O. L., Diaz-Toro, Y., Ovalle, C., Valderrama, L., Muvdi, S., Rodríguez, I. & Saravia, N. G. (2014). Miltefosine and antimonial drug susceptibility of Leishmania Viannia species and populations in regions of high transmission in Colombia. *PLoS Negl Trop Dis*, 8(5): 2871.

Firdous, R., Yasinzai, M. & Ranja, K. (2009). Efficacy of glucantime in the treatment of Old World cutaneous leishmaniasis. *International J. dermatology*, 48(7): 758-762.

Fouladvand, S., Schernich, S., Libuda, J., Grönbeck, H., Pingel, T., Olsson, E. & Carlsson, P. A. (2013). Methane oxidation over Pd supported on ceria–alumina under rich/lean cycling conditions. *Topics in Catalysis*, 56(1-8): 410-415.

Fukunishi, Y. & Structural (2010). Rensemble in computational drug screening. *Expert Opin. Drug Metab. Toxicol,* 6: 835-849.

Gadisa, E., Tsegaw, T., Abera, A., Elnaiem, D. E., den Boer, M., Aseffa, A. & Jorge, A. (2015). Eco-epidemiology of visceral leishmaniasis in Ethiopia. Parasit & vect, *8*(1): 1-10.

Gahloth, D. & Sharma, A.K. (2010). Identification and partial characterization of trypsin inhibitory activity in seed of some fruit plants. *J Plant Biochem Biotech*, 19: 17-19.

Galgamuwa, L.S., Sumanasena, B., Yatawara, L., Wickramasinghe, S. & Iddawela, D. (2017). Clinico-epidemiological patterns of Cutaneous Leishmaniasis patients attending the Anuradhapura teaching hospital, Sri Lanka. *Korean J Parasitol,* 55(1): 1-7.

Gamboa-Leon, R., Vera-Ku, M., Peraza-Sanchez, S. R., Ku-Chulim, C., Horta-Baas, A. & Rosado-Vallado, M. (2014). Antileishmanial activity of a mixture of Tridax procumbens and Allium sativum in mice. *Parasite*, *21*.

Gao, C.h., Yang, Y.T., Shi, F., Wang, J.Y., Steverding, D. & Wang, X. (2015). Development of an Immunochromatographic Test for Diagnosis of Visceral

Leishmaniasis Based on Detection of a Circulating Antigen. *PLoS Negl Trop Dis,* 9(6): 1-12.

Gharavi, M. J., Nobakht, M., Khademvatan, S., Fani, F., Bakhshayesh, M. & Roozbehani, M. (2011). The effect of aqueous garlic extract on interleukin-12 and 10 levels in Leishmania major (MRHO/IR/75/ER) infected macrophages. *Iran. J. public hea*, 40(4): 105-111.

Ghasemi, P. A., Momeni, M. & Bahmani, M. (2013). Ethnobotanical study of medicinal plants used by Kurd tribe in Dehloran and Abdanan districts, Ilam province, Iran. *African J. Traditional, Complement. Alternative Medici*, 10(2): 368-385.

Ghasemzadeh, A. & Jaafar, H.Z.E. (2011). Anticancer and antioxidant activities of Malaysian young ginger *(zingiber officinale Roscoe)* varieties grown under different CO2 concentration. *J. Med. Plant Res,* 5(14): 3247-3255.

Ghatee, M., Sharifi, I., Mirhendi, H., Kanannejad, Z. &Hatam, G. (2013). Investigation of double-band electrophoretic pattern of ITS-rDNA region in Iranian isolates of *Leishmania tropica*. *Iran. J. Parasitol,* 8(2): 264-272.

Ghosh, M. K. & Ghosh, U. K. (2011). Utilization of pine needles as bed material in solid state fermentation for production of lactic acid by lactobacillus strains. *BioResources*, 6(2): 1556-1575.

Ghosh, S., Debnath, S., Hazra, S., Hartung, A., Thomale, K., Schultheis, M. & Hazra, B. (2011). Valeriana wallichii root extracts and fractions with activity against *Leishmania* spp. *Parasitol resear*, 108(4): 861-871.

González, U., Pinart, M., Rengifo-Pardo, M., Macaya, A., Alvar, J. & Tweed, J. A. (2009). Interventions for American cutaneous and mucocutaneous leishmaniasis. *Cochrane database sys rev*, 15: 4-34.

Gourley, D. G., Luba, J., Hardy, L. W., Beverley, S. M. & Hunter, W. N. (1999). Crystallization of recombinant Leishmania major pteridine reductase 1

(PTR1). *Acta Crystallographica Section D: Biological Crystallography*, *55*(9): 1608-1610.

Grogl, M., Thomason, T. N. & Franke, E. D. (1992). Drug resistance in leishmaniasis: its implication in systemic chemotherapy of cutaneous and mucocutaneous disease. *The American journal of tropical medicine and hygiene*, 47(1): 117-126.

Grube, M., Muter, O., Strikauska, S., Gavare, M. & Limane, B. (2008). Application of FT-IR spectroscopy for control of the medium composition during the biodegradation of nitro aromatic compounds. *J. Industrial Microbiolo. Biotechnol*, 35(11): 1545-1549.

Guclu, K., Altun, M., Özyürek, M., Karademir, S. E. & Apak, R. (2006). Antioxidant capacity of fresh, sun-and sulphited-dried Malatya apricot *(Prunus armeniaca*) assayed by CUPRAC, ABTS/TEAC and folin methods. *International J. food sci. Technol*, *41*: 76-85.

Guerbouj, S., Guizani, I., Speybroeck, N., Le Ray, D. & Dujardin, J. C. (2001). Genomic polymorphism of Leishmania infantum: a relationship with clinical pleomorphism?. *Infection, Genetics and Evolution*, 1(1): 49-59.

Guerin, P. J., Olliaro, P., Sundar, S., Boelaert, M., Croft, S. L., Desjeux, P. & Bryceson, A. D. (2002). Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *The Lancet infectious diseases*, *2*(8): 494-501.

Guimarães, L. R., Rodrigues, A. P. D., Marinho, P. S., Muller, A. H., Guilhon, G. M., Santos, L. S. & Silva, E. O. (2010). Activity of the julocrotine, a glutarimide alkaloid from Croton pullei var. glabrior, on *Leishmania (L.) amazonensis*. *Parasitology research*, 107(5): 1075-1081.

Gunasekaran, S. (2003). UV-VIS spectroscopic analysis of blood serum. *Asian J. Microbiol. Biote. Environ. Sci*, 5(4): 581-582.

Güven, K., Yücel, E. & Cetintaş, F. (2006). Antimicrobial activities of fruits of Crataegus. and Pyrus. Species. *Pharmaceu. biolo*, 44(2): 79-83.

Haddad-Kashani, H., Seyed-Hosseini, E., Nikzad, H. & Aarabi, M. H. (2012). Pharmacological properties of medicinal herbs by focus on secondary metabolites. *Life Sci. J*, *9*(1): 509-520.

Hadighi, R., Mohebali, M., Boucher, P., Hajjaran, H., Khamesipour, A. & Ouellette M. (2006). Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drugresistant *Leishmania tropica* parasites. *PLoS Med,* 3(5): 659-667.

Hailu, T., Yimer, M., Mulu, W. & Abera, B. (2016). Challenges in visceral leishmaniasis control and elimination in the developing countries: a review. *J. Vector Borne Dis,* 53(3): 193.

Hakkour, M., Hmamouch, A., El Alem, M.M., Rhalem, A., Amarir, F., Touzani, M. (2016). New epidemiological aspects of visceral and cutaneous leishmaniasis in Taza, Morocco. *Parasit. Vectors,* 9: 612.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp Ser,* 41: 95-8.

Hamarsheh, O., Azmi, K., Amro, A., Schultheis, M., Abdeen, Z., Firdessa, R. & Moll, H. (2017). Antileishmanial potential of crude plant extracts derived from medicinal plants in palestine. *Ann Clin Cytol Pathol, 3(4): 1-7.*

Hamidi, M.R., Jovanova, B. & Panovska, T.K. (2014). Toxicological evaluation of the plant products using Brine Shrimp (Artemia salina L.) model, *Maced. pharm. Bull,* 60(1): 9-18.

Haq, F.U., Ahmad, H., Alam, M., Ahmad, I. & Rahatullah (2010). Species Diversity of Vascular Plants of Nandiar Valley Western Himalaya, Pakistan. *Pak. J. Bot,* 42: 213-29.

Harborne, J.B. (1984). Methods of plant analysis. In *Phytochemical methods,* Springer, Dordrecht, 1-36.

Hawkins, P. & Skillman, G. (2006). Ligand-based design workflow. https ://image s.apple .com/ science /pdf/ ligand based design workflow.

Hayani, K., Dandashli, A. &Weisshaar, E. (2015).Cutaneous leishmaniasis in Syria: clinical features, current status and the effects of war. *Acta DermVenereol,* 95(1): 62-6.

Heidari, F. I., Ghaffarifar, F., Dalimi, A., Mortazavi, N. & Dehkordi, S.G.N. (2012). In vitro study of the effect of artimisinin on promastigotes and amastigotes of *Leishmania major*. *Modares J Med Sci Pathobiol*, *15*: 33-43.

Hejazi, S. H., Shirani-Bidabadi, L., Zolfaghari-Baghbaderani, A., Saberi, S. E. D. I. G. H. E. H., Nilforoushzadeh, M. A., Moradi, S. H. & Ataei, A. T. O. U. S. A. (2009). Comparison effectiveness of extracts of Thyme, Yarrow, Henna and Garlic on cutaneous leishmaniasis caused by *L. major* in animal model (Balb/c). *J. Medic. Plants*, 8(30): 129-160.

Hemavathy, A., Shanthi, P., Sowndharya, C., Thiripura, S. S. & Priyadharshni, K. (2019). Extraction and isolation of bioactive compounds from a therapeutic medicinal plant-Wrightia tinctoria (Roxb.) R. *Br Int J Pharmacogn Phytochem. Res*, 11(3): 199-204.

Herwaldt, B. L. (1999). Leishmaniasis. *Lancet*, 354: 1191-1199

Hijjawi, N., Kanani, K.A., Rasheed, M., Atoum, M., Abdel-Dayem, M. & Irhimeh, M.R. (2016). Molecular diagnosis and identification of *Leishmania* species in Jordan from saved dry samples. *BioMed resear international*, 1-7.

Hillis, D. M. (1987). Molecular versus morphological approaches to systematics. *Annual Review Ecology Systematics*, 18: 23-42.

Hillis, D.M. & M.T. Dixon. **(**1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* 66**:** 411-453.

Ho, M., Siongok, T.K., Lyerly, W.H. & Smith, D.H. (1982). Prevalence and disease spectrum in a new focus of visceral leishmaniasis in Kenya. Transacti Royal Society Tropical Medi Hyg, 76: 741-746.

Hollegaard, M.V., Grauholm, J., Nielsen, R., Grove, J., Mandrup, S. & Hougaard, D.M. (2013). Archived neonatal dried blood spot samples can be used for accurate whole genome and exome-targeted next-generation sequencing. *Mol Genet Metab,* 110(1-2): 65-72.

Hossain, M. A., Shah, M. D., Gnanaraj, C. & Iqbal, M. (2011). In vitro total phenolics, flavonoids contents and antioxidant activity of essential oil, various organic extracts from the leaves of tropical medicinal plant Tetrastigma from Sabah. *Asian Pacific J. tropi. medi*, 4(9): 717-721.

Hosseinzadeh, M., Omidifar, N. & Lohrasb, M.H. (2012). Use of fine needle aspiration cytology in the diagnosis of cutaneous leishmaniasis: a comparison with the conventional scraping method. *Trop Doct.* 42(2): 112-113.

Hostettmann, K., Hostettmann, M., Marston, A., Saponins, in: Charlwood, D.V. & Banthorpe B.V. (Eds.). (1991). Methods in Plant Biochemistry, Terpenoids, vol. 7, Aca-demic Press, San Diego, CA, 435-471.

Hundson, M. M., Mertens, A. C., Yasui, Y., Hobbie, W., Chen, H., Gurney, J. G. & Childhood Cancer Survivor Study Investigators. (2003). Health status of adult long-term survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *Jama*, 290(12): 1583-1592.

Hughes, J. P., Rees, S., Kalindjian, S. B. & Philpott, K. L. (2011). Principles of early drug discovery. *British J. pharmacology*, *162*(6): 1239-1249.

Hwang, H. J., Kim, P., Kim, C. J., Lee, H. J., Shim, I., Yin, C. S. & Hahm, D. H. (2008). Antinociceptive effect of amygdalin isolated from Prunus armeniaca on formalin-induced pain in rats. *Biolog Pharmac Bullet*, 31(8): 1559-1564.

Hyson, D. A. (2011). A comprehensive review of apples and apple components and their relationship to human health. *Advances in nutrition*, *2*(5): 408-420.

Ibrahim, M.E. & Barker, D.C. (2001). The origin and evolution of the Leishmania donovani complex as inferred from a mitochondrial cytochrome oxidase II gene sequence. Infection, *Genetics and Evolution,* 1: 61-68.

Iddawela, D., Vithana, S. M. P., Atapattu, D. & Wijekoon, L. (2018). Clinical and epidemiological characteristics of cutaneous leishmaniasis in Sri Lanka. *BMC infecti dise*, 18(1): 1-9.

Iftikhar, N., Bari, I. & Ejaz, A. (2003). Rare variants of cutaneous leishmaniasis: whitlow, paronychia and sporotrichoid. *Int. J. Dermatol,* 42: 807-9.

Iqbal, H. (2012). Comparative efficacy of *Aloe vera* and *Tamarix aphylla* against cutaneous leishmaniasis. *IJBMSP*, 2(2): 42-45.

Iqbal, M., Raja, N. I., Hussain, M., Ejaz, M. & Yasmeen, F. (2019). Effect of silver nanoparticles on growth of wheat under heat stress. *Iranian J. Sci Technology, Transactions A: Sci*, 43(2): 387-395.

Jaabir, M. M., Vigneshwaran, R. & Hassan, T. M. E. U. (2015). Study on the antimicrobial activity of ethanolic extract of the fruits of Solanum torvum and its phytochemical analysis by GC-MS. *Biomedical and Pharmacology Journal*, *3*(1): 117-121.

Jaabir, M.M., Vigneshwaran, R. & Hassan, T.M.E.U. (2010). Study on the antimicrobial activity of ethanolic extract of the fruits of *Solanum torvum* and its phytochemical analysis by GC-MS. *Biomedi. Pharmacolo J*, *3*(1): 117-121.

Jabir, M.S.M. Vigneshwaran, R. Hassan, E. Ul T., Md, T. & Kumar, S.S. (2010). Study on the Antimicrobial Activity of Ethanolic Extract of the Fruits of *Solanum torvum* and its Phytochemical Analysis by GC-MS, *Biomed Pharmacol J,* 3(1): 1192.

Jacob, R.B., Andersen, T. & McDougal, O.M. (2012). Accessible High-Throughput Virtual Screening Molecular Docking Software for Students and Educators. *PLoS Comput. Biol,* 8: e1002499.

Jaffarany, M. & Haroon, T.S. (1992). Cutaneous Leishmaniasis in Pakistan. *Biomedica,* 8: 39-44.

Jafri, L., Saleem, S., Ihsan ul, H., Ullah, N. & Mirza, B. (2017). In vitro assessment of antioxidant potential and determination of polyphenolic compounds of Hedera nepalensis. *Arabian J. Chem,* 10(2): 3699-3707.

Jafri, L., Saleem, S., Ullah, N. & Mirza, B. (2014). In vitro assessment of antioxidant potential and determination of polyphenolic compounds of Hedera nepalensis. *Arab. J. Chem*, 10: 3699-3706.

Jagtap Supriya, G., Shelar Rohan, S., Munot Neha, M., Ghante Minal, R. & Sawant Sanjay, D. (2012). Antimicrobial activity of Ficus glomerata Linn bark. *Internati. Resea. J. Pharmacy*, *3*(5): 281-284.

Jain, A.K., Sharma, S., Vaidya, A., Ravichandran, V. & Agrawal, R.K. (2013). 1, 3, 4 thiadiazole and its derivatives: a review on recent progress in biological activities. Chem. Biol. Drug Des, 81(5): 557-576.

Jain, K. & Jain, N. K. (2013). Novel therapeutic strategies for treatment of visceral leishmaniasis. *Drug discovery today*, 18(23-24): 1272-1281.

Jain, P. K., Soni, A., Jain, P. & Bhawsar, J. (2016). Phytochemical analysis of Mentha spicata plant extract using UV-VIS, FTIR and GC/MS technique. *J. Chemi Pharm Research*, 8(2): 1-6.

Jain, P. K., Soni, A., Jain, P. & Bhawsar, J. (2016). Phytochemical analysis of Mentha spicata plant extract using UV-VIS, FTIR and GC/MS technique. *J. Chem. Pharma. Res*, *8*(2): 1-6.

Jakhar, R., Dodd, C., Salotra, P. & Satoskar, A.R. (2013). Clinco Epidemiologic study of cutaneous leishmaniasis in Bikaner, Rajasthan, India Naushin. *Am. J. Trop. Med. Hyg,* 89(1): 111-115.

Jalouk, L., Al Ahmed, M., Gradoni, L. & Maroli, M. (2007). Insecticide-treated bednets to prevent anthroponotic cutaneous leishmaniasis in Aleppo Governorate, Syria: results from two trials. *Transact. Royal Socie. Tropical Medici. Hyg*, *101*(4): 360-367.

Jamal, Q., Shah, A., Ali, N., Ashraf, M., Awan, M.M., Clarence, M. & Lee, C.M. (2013). Prevalence and Comparative Analysis of Cutaneous Leishmaniasis in Dargai Region in Pakistan. *Pakistan J. Zool,* 45(2): 537-541.

Jameel, M., Islamuddin, M., Ali, A., Afrin, F. & Ali, M. (2014). Isolation, characterization and antimicrobial evaluation of a novel compound N-octacosan 7 β ol, from *Fumaria parviflora Lam*. *BMC comple altern medi*, *14*(1): 1-9.

James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J. & Vilgalys, R. (2006). Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature*, 443(7113): 818-822.

Jamjoom, M. B., Ashford, R. W., Bates, P. A., Kemp, S. J. & Noyes, H. A. (2002). Polymorphic microsatellite repeats are not conserved between *Leishmania donovani* and *Leishmania major*. *Molecular Ecology Not, 2*(2): 104-106.

Johnston, W. H., Karchesy, J. J., Constantine, G. H. & Craig, A. M. (2001). Antimicrobial activity of some Pacific Northwest woods against anaerobic bacteria and yeast. *Phytothe. Resea*, *15*(7): 586-588.

Jones, G., Willett, P., Glen, R. C., Leach, A. R. & Taylor, R. (1997). Development and validation of a genetic algorithm for flexible docking. *J. molecular biology*, 267(3): 727-748.

Kahime K., Bounoua, L., Fouad O., Messouli, M. & Boumezzough, A. (2014). Leishmaniasis in Morocco: diseases and vectors. *Asian Pacific J Tropical Disease,* 4(2): 530-534.

Kakarsulemankhel J.K. (2004). Cutaneous leishmaniasis in South West Pakistan: a preliminary study. *Tarkiye Parazitoloji Degisis,* 28: 5-11.

Kala, C.P. (2002) Medicinal plants of Indian trans-Himalaya. Bishen Singh Mahendra Pal Singh Dehradun, India.

Kalaichelvi, K. & Dhivya, S.M. (2017). Screening of phytocnstituents, UV-VIS Spectrum and FTIR analysis of *Micrococca mercurialis* (L.) Benth. *Int. J. Herbal Med,* 5(6): 40-44.

Kambli, J., Patil, A., Shree, C. & Keshava R. (2014). Phytochemical screening, and evaluation of antibacterial, antioxidant and cytotoxic activity of Ficus racemosa Linn. 2014. *Int J Pharm Pharm Sci*, 6(4): 464-468.

Kamhawi, S., Modi, G. B., Pimenta, P. F. P., Rowton, E. & Sacks, D. L. (2000). The vectorial competence of Phlebotomus sergenti is specific for *Leishmania tropica* and is controlled by species-specific, lipophosphoglycan-mediated midgut attachment. *Parasitology, 121*(1): 25-33.

Kamink, S., Abdi, A., Kamau, C., Ashraf, S., Ansari, M.A., Qureshi, N.A., Schallig, H., Grobusch, M.P., Fernhout, J. & Ritmeijer, K. (2019). Failure of an Innovative Low-Cost, Noninvasive Thermotherapy Device for Treating Cutaneous Leishmaniasis Caused by *Leishmania tropica* in Pakistan. *Am J Trop Med Hyg,* 101(6): 1373-1379.

Kaneria, M., Kanani, B. & Chanda, S. (2012). Assessment of effect of hydroalcoholic and decoction methods on extraction of antioxidants from selected Indian medicinal plants. *Asian Pacific J. tropical biomedicine*, *2*(3): 195-202.

Karami, M., Doudi, M. & Setorki, M. (2013). Assessing epidemiology of cutaneous leishmaniasis in Isfahan, Iran. *J. Vector Borne Dis,* 50(1): 30.

Karapetyan, T.D.1. Mirzoyan, V.S.1. Hanisyan, R.M.1. & Sahakyan, N.Zh. (2011). In vitro antimicrobial activity of dried and fresh leaf extracts of old and young apricot trees *(Prunus armeniaca*) the new *armen medi j*, 5(4): 44-49.

Karpagasundari, C. & Kulothungan, S. (2014). *J. Pharmacognosy Phytochemistry*, 3(4): 196.

Kashyap, D., Kumar, S., & Dhatwalia, V. (2015). Review on phytochemical and pharmacological properties of *Prunus persica* Linn. *Asian Paci. J. Pharmace.l Appli. Sci*, *2*: 5-11.

Kassi, E., Papoutsi, Z., Fokialakis, N., Messari, I., Mitakou, S. & Moutsatsou, P. (2004). Greek plant extracts exhibit selective estrogen receptor modulator (SERM)-like properties. J. *agricultural and food chemistry*, 52(23): 6956-6961.

Kassi M. & Kasi P. M. (2005). Diagnosis of cutaneous leishmaniasis by fine needle aspiration cytology: a report of 66 cases," Tropical Doctor, 35(1): 50-51.

Kassi, M., Kasi, P. M., Marri, S. M., Tareen, I. & Khawar, T. (2008). Vector control in cutaneous leishmaniasis of the old world: a review of literature. *Dermatology online J.* 14(6).

Katakura, K. (2009). Molecular epidemiology of leishmaniasis in Asia (focus on cutaneous infections). *Current opinion infecti disea*, *22*(2): 126-130.

Kaufman, S. (1993). New tetrahydrobiopterin-dependent systems. *Ann revi nutrit*, 13(1): 261-286.

Kaur, K., Coons, T., Emmett, K. & Ullman, B. (1988). Methotrexate-resistant *Leishmania* donovani genetically deficient in the folate-methotrexate transporter. *J. Biological Chemistry*, 263(15): 7020-7028.

Kellenberger, E., Rodrigo, J., Muller, P. & Rognan, D. (2004). Comparative evaluation of eight docking tools for docking and virtual screening accuracy. *Proteins: Structure, Funct, Bioinformat*, 57(2): 225-242.

Kemp, A., Rawlings, E. I. & Green, B. L. (1991). Post-traumatic stress disorder (PTSD) in battered women: A shelter sample. *Journal of Traumatic Stress*, 4(1): 137-148.

Kessler, M., Ubeaud, G. & Jung, L. (2003). Anti- and pro-oxidant activity of rutin and quercetin derivatives. *J. Pharm. Pharmacol*, 55(1): 131-142.

Khalid, W., Badshah, A., Khan, A. U., Nadeem, H. & Ahmed, S. (2018). Synthesis, characterization, molecular docking evaluation, antiplatelet and anticoagulant actions of 1, 2, 4 triazole hydrazone and sulphonamide novel derivatives. *Chemistry Central Journal*, *12*(1): 1-16.

Khamesipour, A. & Rath, B. (2016). Refugee health and the risk of cutaneous leishmaniasis in Europe. *Inter. J. Inf.* 53: 95-96.

Khan, A.M., Ali, M., Khilji, F.M., Janjua, R.A., Imran, M. & Altintas, K.H. (2019). Hepatitis B and C related knowledge, attitudes and practices of health care workers in Azad Kashmir. Pakistan –Potentially Disastrous Area, 71: 40–46.

Khan, N.H., Bari, A., Hashim, R., Khan, I., Muneer, A., Shah, A., Wahid, S., Yardley, V., O'Neil, B. & Colin, J. (2016). Cutaneous Leishmaniasis in Khyber Pakhtunkhwa Province of Pakistan: Clinical Diversity and Species-Level Diagnosis. *Am. J. Trop. Med. Hyg,* 95(5): 1106-1114.

Khanra, S., Bandopadhyay, S., Chakraborty, P., Datta, S., Mondal, D. & Chatterjee, M. (2011). Characterization of the recent clinical isolates of Indian Kala-azar patients by RAPD-PCR method. *J. Parasit. Dis,* 35(2): 116-122.

Kheirandish, F., Delfan, B., Farhadi, S., Ezatpour, B., Khamesipour, A., Kazemi, B. & Rashidipour, M. (2011). The effect of *Satureja khuzestanica* essential oil on the lesions induced by *Leishmania major* in BALB/c mice. *Afr J Pharm Pharmacol*, *5*(5): 648-653.

Khou, L. B., Mohebali, M., Lahiji, M. N. & Tavana, A. M. (2007). The therapeutic effect of Eucalyptus, Myrtus, Ferula, Aretmisia, Allium and Urtica extracts against cutaneous leishmaniasis caused by *Leishmanaia major* in small white mice (out-bred). *Hakim*, 10: 21-27.

Klaus, S. & Frankenburg, S. (1999). Cutaneous Leishmaniasis in the Middle East. *Clin. Dermatol*. 17: 137-141.

Knighton, D. R., Kan, C. C., Howland, E., Janson, C. A., Hostomska, Z., Welsh, K. M. & Matthews, D. A. (1994). Structure of and kinetic channelling in bifunctional dihydrofolate reductase–thymidylate synthase. *Nat. struct. biolo*, *1*(3): 186-194.

Kobets, T., Havelková, H., Grekov, I., Volkova, V., Vojtíšková, J., Slapničková, M. & Lipoldova, M. (2012). Genetics of host response to *Leishmania tropica* in mice–different control of skin pathology, chemokine reaction, and invasion into spleen and liver. *PLoS Negl Trop Dis*, 6(6): e1667.

Kolaczinski, J., Brooker, S., Reyburn, H. & Rowland, M. (2004). Epidemiology of anthroponotic cutaneous leishmaniasis in Afghan refugee camps in northwest Pakistan. *Trans R Soc Trop Med Hyg*, 98(6): 373-378.

Koltas, I.S., Eroglu, F., Alabaz, D. & Uzun, S. (2014). The emergence of *Leishmania major* and *Leishmania donovani* in southern Turkey. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *108*(3): 154-158.

Krishnaiah, D., Sarbatly, R. & Nithyanandam, R. (2011). A review of the antioxidant potential of medicinal plant species. *Food Bioprod. Process*, 89(3): 217-233.

Krishnakumari, V. & Nagaraj, R. (2012). Binding of peptides corresponding to the carboxy-terminal region of human-β-defensins-1–3 with model membranes investigated by isothermal titration calorimetry. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1818(5): 1386-1394.

Kritikar, K.R. & Basu, B.D. (1984). Indian Medicinal Plants, Bishen Singh Mahendra Pal Singh, Dehradun 1: 954.

Ksouri, R., Megdiche, W., Falleh, H., Trabelsi, N., Boulaaba, M., Smaoui, A. & Abdelly, C. (2008). Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes. *Comptes Rendus Biologi*, 331(11): 865-873.

Kubba R, El-Hassan A, Al-Gindan Y, Omer, A. H., Kutty, M. K. & Saeed, M.B. (1987). Dissemination in cutaneous leishmaniasis. I. Subcutaneous nodules. *Int. J. Dermatol*, 26: 300-4.

Kumalo, H. M., Bhakat, S. & Soliman, M. E. (2015). Theory and applications of covalent docking in drug discovery: merits and pitfalls. *Molecul*, 20(2): 1984-2000.

Kumar, J. K., & Prasad, A. D. (2011). Identification and comparison of biomolecules in medicinal plants of *Tephrosia tinctoria* and *Atylosia albicans* by using FTIR. *Rom. J. Biophys*, *21*(1), 63-71.

Kumar, P., Sundar, S. & Singh, N. (2007). Degradation of pteridine reductase 1 (PTR1) enzyme during growth phase in the protozoan parasite *Leishmania donovani*. Exp Parasitol. 116: 182-189.

Kumar, S. A., Lo, P. H. & Chen, S. M. (2008). Electrochemical selective determination of ascorbic acid at redox active polymer modified electrode derived from direct blue 71. *Biosensors Bioelectronics*, *24*(4): 518-523.

Kumar, T. & Jain, V. (2015). Appraisal of Total Phenol, Flavonoid Contents, and Antioxidant Potential of Folkloric *Lannea Coromandelica* Using in Vitro and in Vivo Assays. *Scientifica,* 1-15.

Kurus, M., Taslidere, E. & Elbe, H. (2014). Protective Effects of *Prunus armeniaca* L.(Apricot) on Low dose radiation-induced kidney damage in rats. *Turk. Neph. Dial. Transpl*, 23(2): 105-111.

Labake, F. Haruna, Kaita, A. & Ilyas, M. (2014). Antibacterial Activity of 1,2-Benzenediccarboxylic Acid, Dioctyl Ester Isolated from the Ethyl Acetate Soluble Sub-portion of the unripe Fruits of *Nauclea latifolia. Int. J. Pure App. Biosci.* 2 (1): 223-230.

Lage, P. S., de Andrade, P. H. R., Lopes, A. D. S., Chavez Fumagalli, M. A., Valadares, D. G., Duarte, M. C. & Coelho, E. A. F. (2013). *Strychnos pseudoquina* and its purified compounds present an effective in vitro antileishmanial activity. *Evidence-Based Complementary Alternative Medi*, *2013*.

Lage, S., Burian, A., Rasmussen, U., Costa, P. R., Annadotter, H., Godhe, A. & Rydberg, S. (2016). BMAA extraction of cyanobacteria samples: which method to choose?. *Environmental Science and Pollution Research*, *23*(1): 338-350.

Lahlou, G., Rodallec, M., Nguyen, Y., Sterkers, O. & Kalamarides, M. (2019). How to radiologically identify a spontaneous regression of sporadic vestibular schwannoma?. *Plos one*, *14*(6): e0217752.

Lainson, R. & Shaw, J.J. (1987). Evolution, classification and geographical distribution. In: The Leishmaniases in Biology and Medicine, 1: 1-120. London: Academic Press.

Lavinas, F. C., Macedo, E. H. B., Sá, G. B., Amaral, A. C. F., Silva, J. R., Azevedo, M. M. & Rodrigues, I. A. (2019). Brazilian stingless bee propolis and geopropolis: promising sources of biologically active compounds. *Revista Brasileira de Farmacognosia*, 29(3): 389-399.

Layegh, P., Rahsepar, S. & Rahsepar, A.A. (2011). Systemic meglumine antimoniate in acute cutaneous leishmaniasis: children versus adults. *Ameri Soci Trop Medic Hyg*, 84(4): 539-42.

Leelavathi, V. & Udayasri, P. (2018). Qualitative and Quantitative Analytical Studies for the Screening of Phytochemicals from the Leaf Extracts of *Senna alexandrina M*ill. *In. J. Pharmaceutical Clin Res,* 10(8): 210-215.

Li, H.Y., Hao, Z.B., Wanga, X.L., Huang, L. & Li JP. (2009). Antioxidant activities of extracts and fractions from *Lysimachia foenum-graecum* Hance. *Bioresource Tech,* 100: 970-974.

Liew, F. Y., Parkinson, C., Millott, S., Severn, A. & Carrier, M. (1990). Tumour necrosis factor (TNF alpha) in leishmaniasis. I. TNF alpha mediates host protection against cutaneous leishmaniasis. *Immunology*, 69(4): 570.

Liew, F. Y., Parkinson, C., Millott, S., Severn, A. & Carrier, M. (1990). Tumour necrosis factor (TNF alpha) in leishmaniasis. I. TNF alpha mediates host protection against cutaneous leishmaniasis. *Immunology*, *69*(4): 570.

Lindoso, J. A. L., Costa, J. M. L., Queiroz, I. T. & Goto, H. (2012). Review of the current treatments for leishmaniases. *Research and reports in tropical medicine*, *3*: 69.

Lingegowda, D. C., Kumar, J. K., Prasad, A. D., Zarei, M. & Gopal, S. (2012). FTIR spectroscopic studies on Cleome gynandra–comparative analysis of functional group before and after extraction. *Romanian Journal of Biophysics*, *22*(3-4): 137-143.

Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A., Saraiva, E. & Sacks, D. (1999). Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of *Leishmania donovani*. *The Journal of infectious diseases*, *180*(2): 564-567.

Loza-Mejia, M. A., Salazar, J. R. & Sánchez-Tejeda, J. F. (2018). In Silico studies on compounds derived from calceolaria: phenylethanoid glycosides as potential multitarget inhibitors for the development of pesticides. *Biomolecules*, *8*(4), 121.

Lu, Y. & Foo, L.Y. (2001). Antioxidant activities of polyphenols from sage (Salvia officinalis). *Food Chem,* 75(2): 197-202.

M. Kassi, I. Tareen, A. Qazi, and P. M. Kasi, "Fine-needle aspiration cytology in the diagnosis of cutaneous leishmaniasis," Annals of Saudi Medicine, vol. 24, no. 2, pp. 93–97, 2004.

Machado, V. S., Oikonomou, G., Bicalho, M. L. S., Knauer, W. A., Gilbert, R. & Bicalho, R. C. (2012). Investigation of postpartum dairy cows' uterine microbial diversity using metagenomic pyrosequencing of the 16S rRNA gene. *Veterinary microbiology*, 159(3-4): 460-469.

Madrau, M. A., Piscopo, A., Sanguinetti, A. M., Del Caro, A., Poiana, M., Romeo, F. V. & Piga, A. (2009). Effect of drying temperature on polyphenolic content and antioxidant activity of apricots. *European Food Research and Technology*, 228(3): 441-448.

Maga, J.A. (1978) Simple phenol and phenolic compounds in food flavour. *CRC Crit Rev Food Sci Nutr*, 10: 323-372. pmid: 363364

Mahmoudvand, H., Sharifi, I., Harandi, M. F. & Shakibaie, M. (2014). Anti-leishmania effects of methotrexate (MTX) alone and in combination with meglumine antimoniate (MA) against Iranian. *Asi Pacifi J. Tropi Medic*, 412: 420.

Mahmoudvand, H., Tavakoli, R., Sharififar, F., Minaie, K., Ezatpour, B., Jahanbakhsh, S. & Sharifi, I. (2015). Leishmanicidal and cytotoxic activities of Nigella sativa and its active principle, thymoquinone. *Pharmaceutical Biolo*, *53*(7): 1052-1057.

Makowski, G.S., Davis, E.L., Aslanzadeh, J. & Hopfer, S.M. (1995). Enhanced direct amplification of Guthrie card DNA following selective elution of PCR inhibitors. *Nucleic Acids Res,* 23: 3788-3789.

Mamta, S. & Jyoti, S. (2012). Phytochemical screening of *Acorus calamus* and *Lantana camara*. *Inte. Res J.Pharmacy*, 3(5): 324-326.

Marco, J. D., Bhutto, A. M., Soomro, F. R., Baloch, J. H., Barroso, P. A., Kato, H. & Hashiguchi, Y. (2006). Multilocus enzyme electrophoresis and cytochrome b gene sequencing–based identification of Leishmania isolates from different foci of

cutaneous leishmaniasis in Pakistan. *The American journal of tropical medicine and hygiene*, *75*(2): 261-266.

Marfurt, J., Niederwieser, I., Makia, N. D., Beck, H. P. & Felger, I. (2003). Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP. *Diagnostic microbiolo infectious disease*, *46*(2): 115-124.

Masmoudi, K., Gras-Champel, V., Masson, H. & Andrejak, M. (2006). Parkinsonism and/or cognitive impairment with valproic acid therapy: a report of ten cases. *Pharmacopsychiatry, 39*(01): 9-12.

Maspi, N., Ghafarifar, F., Bahrami, A. M., Bastaminejad, S. & Shamsi, M. (2010). Evaluation of leishmanicidal effect of watery & ethanolic flowers *Calendula officinalis* extract on promastigotes of leishmania major (MRHO/IR/75/ER) in vitro. *J Ilam Univ Med Sci*, 18: 28-33.

Matoussi, N., Ameur, H. B., Amor, S. B., Fitouri, Z. & Becher, S. B. (2007). Cardiotoxicity of n-methyl-glucamine antimoniate (Glucantime). A case report. *Medecine et maladies infectieuses*, 37: 257-9.

Mauricio, I. L., Gaunt, M. W., Stothard, J. R. & Miles, M. A. (2001). Genetic typing and phylogeny of the *Leishmania donovani* complex by restriction analysis of PCR amplified gp63 intergenic regions. *Parasitology*, *122*(4): 393-403.

Mbaebie, B. O., Edeoga, H. O. & Afolayan, A. J. (2012). Phytochemical analysis and antioxidants activities of aqueous stem bark extract of Schotia latifolia Jacq. *Asian Pacific J.Tropi. Biomedi*, *2*(2): 118-124.

McDade, T.W. (2014). Development and validation of assay protocols for use with dried blood spot samples. *Am. J. Hum. Biol,* 26: 1-9.

McDade, T.W. (2014). Development and validation of assay protocols for use with dried blood spot samples. *Am. J. Hum. Biol,* 26: 1-9.

McGwire, B.S. & Satoskar, A.R. (2014). Leishmaniasis: clinical syndromes and treatment. *QJM,* 107(1): 7-14.

Menezes, C., Kamath, J.V., Satyanarayana, D., Mishra, A. & Sandeep Mishra, A. (2013). Antibacterial activity of fruits of *Ficus glomerata* cylma. *J. Harmonized Rese Pharmacy*, 2(4): 256-259.

Mengesha, B., Endris, M., Takele, Y., Mekonnen, K., Tadesse, T., Feleke, A. & Diro, E. (2014). Prevalence of malnutrition and associated risk factors among adult visceral leishmaniasis patients in northwest Ethiopia: a cross sectional study. BMC Res Notes, 7: 75.

Miller, R. L., Gerster, J. F., Owens, M. L., Slade, H. B. & Tomai, M. A. (1999). Review article imiquimod applied topically: a novel immune response modifier and new class of drug. *Inter J. immunopharmaco*, 21(1): 1-14.

Minaiyan, M., Ghannadi, A., Asadi, M., Etemad, M. & Mahzouni, P. (2014). Anti-inflammatory effect of *Prunus armeniaca* L. (Apricot) extracts ameliorates TNBS-induced ulcerative colitis in rats. *Resea. Pharmaceut. Sci*, 9(4): 225-231.

Moein, M. R., Pawar, R. S., Khan, S. I., Tekwani, B. L. & Khan, I. A. (2008). Antileishmanial, antiplasmodial and cytotoxic activities of 12, 16-dideoxy aegyptinone B from Zhumeria majdae Rech. f. & Wendelbo. *Phytotherapy Research: Internat. J. Devoted Pharmacolo. Toxicol.l Evalua. Natur. Produ. Derivat*, *22*(3), 283-285.

Mohapatra, S. (2014). Drug resistance in leishmaniasis: Newer developments. *Tropi parasitolo*, *4*(1): 4-9.

Moine, E., Denevault-Sabourin, C., Debierre-Grockiego, F., Silpa, L., Gorgette, O., Barale, J. C. & Enguehard-Gueiffier, C. (2015). A small-molecule cell-based screen led to the identification of biphenylimidazoazines with highly potent and broad-spectrum anti-apicomplexan activity. *Euro. J. medi. Chem*, 89: 386-400.

Momeni, A.l. & Aminjavaheri, M. (1994). Clinical picture of cutaneous leishmaniasis in Isfahan, Iran. *Int J Dermatol*, 33(4): 260-5.

Mondal, D., Alam, M.S., Karim, Z., Haque, R., Boelaert, M. & Kroeger, A. (2008). Present situation of vector-control management in Bangladesh: a wake up call. *Health Policy*, 87(3): 369-76.

Monzote, L. (2011). Antileishmanial patents antileishmanial current drugs and relevant patents. *Recent patents on anti-infecti drug discovery*, *6*(1): 1-26.

Monzote, L., García, M., Pastor, J., Gil, L., Scull, R., Maes, L. & Gille, L. (2014). Essential oil from Chenopodium ambrosioides and main components: activity against *Leishmania*, their mitochondria and other microorganisms. *Experimental Parasitolo*, 136: 20-26.

Morizot, G., Kendjo, E., Mouri, O., Thellier, M., Pérignon, A., Foulet, F. & Rebauder, S. (2013). Travelers with cutaneous leishmaniasis cured without systemic therapy. *Cli. Infec. Dise, 57*(3): 370-380.

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S. & Olson, A. J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. computa. Chemi*, 30(16): 2785-2791.

Mughal, T.M., Ul Haq, E., Ali, A. & Ilyas T. (2014). Cutaneous Leishmaniasis and Assessment of Environmental Risk Factors in Mirpur AJK. *JRMC*, 18(1):110-113.

Mumtaz, S., Munir, A. H., Asghar, M. & Naheed, N. (2016). Frequency and types of leishmaniasis in Khyber Pakhtunkhwa (KPK). *J. Medi. Sci*, 24(3): 136-140.

Mura, C. & McAnany, C. E. (2014). An introduction to biomolecular simulations and docking. *Molecular Simulation*, 40(10-11): 732-764.

Murray, H. W., Berman, J. D., Davies, C. R. & Saravia, N. G. (2005). Advances in leishmaniasis. *The Lancet, 366*(9496): 1561-1577.

Murugan, S. V., Ramasubramaniyan, M. R. & Vaseekaran, M. K. S. (2014). Water management policies in India: current trends and future policy options. *JAIR*, *3*(2): 73.

Myint, C. K., Asato, Y., Yamamoto, Y. I., Kato, H., Bhutto, A. M., Soomro, F. R. & Uezato, H. (2008). Polymorphisms of cytochrome b gene in Leishmania parasites

and their relation to types of cutaneous leishmaniasis lesions in Pakistan. *J. dermatology*, *35*(2): 76-85.

Nare, B., Luba, J., Hardy, L. W. & Beverley, S. (1997). New approaches to Leishmania chemotherapy: pteridine reductase 1 (PTR1) as a target and modulator of antifolate sensitivity. *Parasito*, *114*(7): 101-110.

Nasir, E., Ali, S. & Stewart, R.R. (1972). Flora of West Pakistan: an annotated catalogue of the 560 vascular plants of West Pakistan and Kashmir: *Fakhri,* 561: 56.

Nasir, YJ. & Ali, S. Flora of Pakistan. Department of Botany, University of Karachi; 562 National Herbarium, 1994-2010.

Nawaz, M., Din, M., Khan, A., Khan, A., Ali, M., Din, S. U. & Aslam, K. (2020). Epidemiological features of cutaneous leishmaniasis endemic in hilly areas of district Karak, Khyber-Pakhtunkhwa province of Pakistan. *Journal of Parasitic Diseases*, 44(4): 725-729.

Nawaz, R., Khan, A.M., Khan, S.U. & Rauf A. (2010). Frequency of Cutaneous leishmaniasis in an Afghan refugee Camp at Peshawar, Gomal. *J. Medical Sci,* 8(1): 16-19.

Negera E, Gadisa E, Yamuah L, et al. Outbreak of cutaneous leishmaniasis in Silti woreda, Ethiopia: risk factor assessment and causative agent identification. Trans R Soc Trop Med Hyg. 2008; 102(9):883–890. doi: 10.1016/j.trstmh.2008.03.021.

Nei, M. & Kumar, S. (2000). Molecular Evolution and Phylogenetics. *Oxford University Press*, *New York.*

Néris, P. L., Caldas, J. P., Rodrigues, Y. K., Amorim, F. M., Leite, J. A., Rodrigues-Mascarenhas, S.& Oliveira, M. R. (2013). Neolignan Licarin A presents effect against Leishmania (Leishmania) major associated with immunomodulation in vitro. *Experimental Parasitology*, *135*(2): 307-313.

Neves, L. O., Talhari, A. C., Gadelha, E. P., Silva Júnior, R. D., Guerra, J. A., Ferreira, L. C. & Talhari, S. (2011). A randomized clinical trial comparing meglumine antimoniate, pentamidine and amphotericin B for the treatment of cutaneous leishmaniasis by Leishmania guyanensis. *An Bras Dermatol*, *86*(6): 1092-101.

Newman, D. J. & Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. natur. Produ*, *75*(3): 311-335.

Nguta, J. M., Mbaria, J. M., Gakuya, D. W., Gathumbi, P. K., Kabasa, J. D. & Kiama, S. G. (2012). Evaluation of acute toxicity of crude plant extracts from Kenyan biodiversity using brine shrimp, artemia salina l. (artemiidae). *Open Conf Proc J,* 3: 30-34.

Nichol, C.A., Smith, G.K. & Duch, D.S. (1985). Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Annual review of biochemistry*, 54(1): 729-764.

Nithiyanantham, S., Selvakumar, S. & Siddhuraju, P. (2012). Total phenolic content and antioxidant activity of two different solvent extracts from raw and processed legumes, *Cicer arietinum* L. and *Pisum sativum* L. *J. food Composi. Analy*, 27(1): 52-60.

Nogueira, R. G., Lutsep, H. L., Gupta, R., Jovin, T. G., Albers, G. W., Walker, G. A. & Smith, W. S. (2012). Trevo versus Merci retrievers for thrombectomy revascularisation of large vessel occlusions in acute ischaemic stroke (TREVO 2): a randomised trial. *The Lancet*, *380*(9849): 1231-1240.

Noor, S.M. & Hussain, D. (2004). Cutaneous leishmaniasis in Sadda, Kurram agency, Pakistan. *J Pak Assoc Dermatol*, 14: 114-117.

Nour, A. M., Khalid, S. A., Kaiser, M., Brun, R., Wai'l, E. A. & Schmidt, T. J. (2010). The antiprotozoal activity of methylated flavonoids from *Ageratum conyzoides* L. *J. Ethnopharmacology*, 129(1): 127-130.

Noyes, H.A., Reyburn, H., Bailey, J.W. & Smith, D. (1998). A nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. Clin Microbiol. 36(10): 2877-81.

Odiwuor, S., De Doncker, S., Maes, I., Dujardin, J.C. & Van der Auwera, G. (2011). Natural *Leishmania donovani*/*Leishmania aethiopica* hybrids identified from Ethiopia. *Infect, Genet. Evol.* 11(8): 2113-2118.

Ogeto, T.K., Odhiambo, R.A., Shivairo, R.S., Muleke, C.I., Osero, B.O., Anjili, C. & Osuga, I.M. (2013). Antileishmanial activity of *Aloe secundi* flora plant extracts against *Leishmania major*. *Adv. Life Sci. Technol*, 13: 2224-81.

Olila, D. & Opuda-Asibo, J. (2001). Antibacterial and antifungal activities of extracts of *Zanthoxylum chalybeum* and *Warburgia ugandensis,* Ugandan medicinal plants. *Afric. Hea. Sci*, *1*(2): 66-72.

Oliveira, L.F., Schubach, A.O., Martins, M.M., Passos, S.L., Oliveira, R.V., Marzochi, M .C. & Andrade, C.A. (2011). Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. *Acta Trop*, 118: 87-96.

Onwukaeme, D.N., Ikuegbvweha, T.B. & Asonye, C.C. (2007). Evaluation of phytochemical constituents, antibacterial activities and effect of exudate of *Pycanthus angolensis* Weld Warb (Myristicaceae) on corneal Ulcers in Rabbits. *Trop. Pharm. Rese,* 6(2): 725-730.

Oryan, A. & Akbari, M. (2016). Worldwide risk factors in leishmaniasis. *Asian Pac J Trop Med*, 9:925-32.

Oryan, A. (2015). Plant-derived compounds in treatment of leishmaniasis. *IJVR*, 16 (1): 1-19.

Oryan, A., Alidadi, S., Moshiri, A. & Maffulli, N. (2014). Bone regenerative medicine: classic options, novel strategies, and future directions. *J. orthopaedic surgery resea*, *9*(1): 1-27.

Osman, O. F, Oskam, L., Kroon, N. C., Schoone, G. J., Khalil, E. T., El Hassan, A. M., Zijlstra, E. E. & Kager, P. A. (1998). Use of PCR for diagnosis of post kala-azar dermal leishmaniasis (PKDL*). J. Clin. Microbiol,* 36(6): 1621-1624.

Ostler, M.W., Porter, J.H. & Buxton, O.M. (2014). Dried blood spot collection of health biomarkers to maximize participation in population studies. *J. Vis. Exp,* 28(83): 1-9.

Ostyn, B., Vanlerberghe, V., Picado, A., Dinesh, D.S., Sundar, S., Chappuis, F., Rijal, S., Dujardin, J.C., Coosemans, M., Boelaert, M. & Davies C. (2008). Vector control by insecticide-treated nets in the fight against visceral leishmaniasis in the Indian subcontinent, what is the evidence? *Trop Med Int Health,* 13(8): 1073-1085.

Ozen T., Demirats, I. & Aksit, H. (2011). Determination of antioxidant activities of various extracts and essential oil compositions of *Thymus praecox* susp. skorpilii var. skorpilii. *Food Chem,* 124: 58-64.

Paolini, V., Fouraste, I. & Hoste, H. (2004). In vitro effects of three woody plant and sainfoin extracts on third-stage larvae and adult worms of three gastrointestinal nematodes. *Parasitology*, 129: 69-77.

Parekh, J. & Chanda, S.V. (2007). *In vitro* antimicrobial activity and phytochemical analysis of some Indian medicinal plants.*Turkish J. Biology,* 31: 53-58.

Parlakpinar, H., Olmez, E., Acet, A., Ozturk, F., Tasdemir, S. & Ates, B. (2009). Beneficial effects of apricot-feeding on myocardial ischemia reperfusion injury in rats. *Food Chem Toxicol*, 47: 802-809.

Parle, M. & Arzoo. (2016). Honey: a sweet way to cure psychosis. *Eur. J. Biomed. Pharma. Sci,* 7: 479-86

Pedrosa Fde, A. & Ximenes, R.A. (2009). Sociodemographic and environmental risk factors for American cutaneous leishmaniasis (ACL) in the state of Alagoas, Brazil. *Am J Trop Med Hyg,* 81:195-201.

Picado, A., Singh, S. P., Rijal, S., Sundar, S., Ostyn, B., Chappuis, F. & Boelaert, M. (2010). Longlasting insecticidal nets for prevention of Leishmania donovani infection in India and Nepal: paired cluster randomised trial. *Bmj*, *341*: 6760-6760.

Pohanka, M., Hrabinova, M., Kuca, K. & Simonato, J. P. (2011). Assessment of acetylcholinesterase activity using indoxylacetate and comparison with the standard Ellman's method. *Internat. J. mole. Scie*, *12*(4): 2631-2640.

Portu`s, M., Rioux, J.A., Ga`llego, J., Lanotte, G., Pratlong, F. & Moreno, G. (1986). Les leishmaniosesenCatalogne (Espagne): a propos de l'identificationenzymatique de 9 souchesd'originehumaine et canine. In: *Leishmania*: Taxonomie et phyloge´ne`se: Applications e´co-e´pide´miologiques. Montpellier, France: *IMEEE,* 14: 433-438

Pourmohammadi, B., Motazedian, M.H. & Kalantari, M. (2008). Rodent infection with *leishmania*in a new focus of human cutaneous leishmaniasis, in northern Iran. *Ann Trop Med Parasitol.*102 (2): 127-33.

Pozio, E., Gramiccia, M., Gradoni, L. & Amerio, P. (1985). Isolation of the agent causing cutaneous leishmaniasis in Italy and its visceralisation in inbred hamsters. *Trans R Soc Trop Med Hyg,* 79: 260-261.

Prajapati, V. K., Awasthi, K., Gautam, S., Yadav, T. P., Rai, M., Srivastava, O. N. & Sundar, S. (2011). Targeted killing of Leishmania donovani in vivo and in vitro with amphotericin B attached to functionalized carbon nanotubes. *J. antimicrobial chemotherapy*, *66*(4): 874-879.

Prasad, K.N., Yang, B., Yang, S., Chen, Y., Zhao, M., Ashraf, M. & Jiang, Y. (2009). Identification of phenolic compounds and appraisal of antioxidant and

antityrosinase activities from litchi (Litchi sinensis Sonn.) seeds. *Food Chem,* 116(1): 1-7.

QuispeTintaya, K.W., Ying, X., Dedet, J.P., Rijal, S., De Bolle, X. & Dujardin, J.C. (2004). Antigen genes for molecular epidemiology of leishmaniasis: polymorphism of cysteine proteinase B and surface metalloprotease glycoprotein 63 in the *Leishmania donovani* complex. *J. Infect. Dis,* 189: 1035-1043.

Qureshi, N. A., Rahman, H. U., Ali, A., Naseer Ullah, S. A. A. S. & Khan, I. (2014). In-vitro evaluation of the anti-leishmanial activity of Euphorbia helioscopia stem extract in comparison with synthetic drug amphotericin B. *Asian J Nat Appl Sci*, 3(3): 12-17.

Qureshi, N.A., Alia, A., Rashid, U., Ur-Rehman, T. & Alida N. (2016). Prevalence of *Leishmania tropica* in school boys of khyber agency, FATA near Pak-Afghan border Departm. *Acta Trop.* 164: 90-94.

Rab, M. A., Al Rustamani, L., Bhutta, R. A., Mahmood, M. T. & Evans, D. A. (1997). Cutaneous leishmaniasis: iso-enzyme characterisation of *Leishmania tropica*. *J.Pak. Medi Association*, *47*: 270-273.

Rab, M.A., Iqbal, J., Azmi, F.H., Munir, M.A. & Saleem, M. (1989). Visceral leishmaniasis: a seroepidemiological study of 289 children from endemic foci in Azad Jammu and Kashmir by indirect fluorescent antibody technique. *J Pak Med Assoc.* 39: 225-228.

Rahman, H.U. & Rehman, A.U. (2018). Cutaneous leishmaniasis in Dir Lower District, North-West Pakistan: epidemiology and treatment response. *J Pak Assoc Derma,* 27(4): 353-362.

Rahman, S. B. & ul Bari, A. (2006). Comparative cellular immune host response in acute vs healed lesions of cutaneous leishmaniasis. *J Ayub Medical College Abbottabad*, *18*(3).

Raj, V., Jain, A. & Chaudhary, J. (2012). *Prunus armeniaca* (Apricot): An Overview. *J Pharm Res*, 8:3964-6.

Rajeshkumar, R. & Jeyaprakash, K. (2016). Screening of UV-VIS, TLC and FTIR spectroscopic studies on selected red seaweed (*Acanthophora specifera*) collected from Gulf of Mannar, Tamilnadu, India. *World J. Pharmaceutical Sci*, 4(10): 28-33.

Ramdane, F., Mahammed, M. H., Hadj, M. D. O., Chanai, A., Hammoudi, R., Hillali, N. & Bahaz, C. (2015). Ethnobotanical study of some medicinal plants from Hoggar, Algeria. *J. Medici. Plan. Res*, *9*(30): 820-827.

Ramezany, M., Sharifi, I., Babaei, Z., Ghasemi Nejad Almani, P., Heshmatkhah, A., Keyhani, A. & Bamorovat, M. (2018). Geographical distribution and molecular characterization for cutaneous leishmaniasis species by sequencing and phylogenetic analyses of kDNA and ITS1 loci markers in south-eastern Iran. *Pathogens and global health*, *112*(3): 132-141.

Ramírez, D. & Caballero, J. (2016). Is It Reliable to Use Common Molecular Docking Methods for Comparing the Binding Affinities of Enantiomer Pairs for Their Protein Target? *Int. J. Mol. Sci, 17*(4): 1-15.

Ranalli, A., Contento, S., Lucera, L., Di Febo, M., Marchegiani, D. & Di Fonzo V. (2006). Factors affecting the contents of iridoid oleuropein in olive leaves (*Olea europaea* L.). *J. Agricultural Food Chemi*, *54*: 434-440.

Rani, N., Sharma, S. & Sharma, M. (2016). Phytochemical Analysis of *Meizotropis pellita* by FTIR and UV-VIS Spectrophotometer. *Indian J. Sci. Technolo*, 9(31): 1-4.

Rarey, M., Kramer, B., Lengauer, T. & Klebe, G. (1996). A fast flexible docking method using an incremental construction algorithm. *J. molecular biology*, 261(3): 470-489.

Rashid, F., Ahmed, R., Bibi, N., Kazmi, S.U. & Ansar, N. (2005). Triterpene acid and its glycoside from Prunus armeniaca and antibacterial and antioxidant activities of fruit extracts. *J. Trop. Med. Plants*, 6(1): 31-35.

Rashid, F., Ahmed, R., Mahmood, A., Ahmad, Z., Bibi, N. & Kazmi, S. U. (2007). Flavonoid glycosides from Prunus armeniaca and the antibacterial activity of a crude extract. *Archi. pharmacal resear*, 30(8): 932-937.

Rashid, F., Ahmed, R., Mahmood, A., Ahmad, Z., Bibi, N. & Kazmi, S. U. (2007). Flavonoid glycosides from Prunus armeniaca and the antibacterial activity of a crude extract. *Archi pharma resea*, 30(8): 932-937.

Rashid, U., Sultana, R., Shaheen, N., Hassan, S. F., Yaqoob, F., Ahmad, M. J. & Qureshi, N. A. (2016). Structure based medicinal chemistry-driven strategy to design substituted dihydropyrimidines as potential antileishmanial agents. *European journal of medicinal chemistry*, *115*: 230-244.

Ravel, C., Dubessay, P., Britto, C., Blaineau, C., Bastien, P. & Page`s, M. (1999). High conservation of the fine-scale organisation of chromosome 5 between two pathogenic *Leishmania* species. *Nucleic Acids Resea*, 27: 2473-2477.

Razack, S., Kumar, K. H., Nallamuthu, I., Naika, M. & Khanum, F. (2015). Antioxidant, biomolecule oxidation protective activities of Nardostachys jatamansi DC and its phytochemical analysis by RP-HPLC and GC-MS. *Antioxidants*, 4(1): 185-203.

Reed, S.G. (1996) Diagnosis of Leishmaniasis. *Clini. Dermatolo*, 24: 471-478.

Reithinger, R., Dujardin, J.C., Louzir, H., Pirmez, C., Alexander, B., Brooker, S. (2007). Cutaneous leishmaniasis. *Lancet Infect Dis*, 7(9): 581-596.

Reithinger, R., Mohsen, M., Aadil, K., Sidiqi, M., Erasmus, P. & Coleman, P.G. (2003). Anthroponotic cutaneous leishmaniasis, Kabul, Afghanistan. *Emerg. Infect. Dis,* 9: 727-729.

Reyburn, H., Ashford, R., Mohsen, M., Hewitt, S. & Rowland, M.A. (2000). Randomized controlled trial of insecticide-treated bednets and chaddars or top sheets, and residual spraying of interior rooms for the prevention of cutaneous leishmaniasis in Kabul, Afghanistan. *Trans R Soc Trop Med Hyg,* 94: 361-66.

Riera, E., Golas, Y., Blanco, A., Gallego, J. A., Blasco, M. & Mulet, A. (2004). Mass transfer enhancement in supercritical fluids extraction by means of power ultrasound. *Ultrasonics sonochemistry*, 11(3-4): 241-244.

Rioux, J.A., Lanotte, G., Serres, E., Pratlong, F., Bastien, P. & Perie`res, J. (1990). Taxonomy of Leishmania. Use of isoenzymes. Suggestions for a new classification. *Annales de Parasitologie Humaine et Comparee*, 65: 111-125.

Robert, N.D., Margriet, D.B. & Koert, R. (2009). Paromomycin. *Trans R Soc Trop Med Hyg.* 103: 653-60.

Rocha, L. G., Almeida, J. R. G. S., Macedo, R. O. & Barbosa-Filho, J. M. (2005). A review of natural products with antileishmanial activity. *Phytomedicine*, *12*(6-7): 514-535.

Rodrigues, I. A., Azevedo, M. M., Chaves, F. C., Bizzo, H. R., Corte-Real, S., Alviano, D. S. & Vermelho, A. B. (2013). In vitro cytocidal effects of the essential oil from Croton cajucara (red sacaca) and its major constituent 7-hydroxycalamenene against Leishmania chagasi. *BMC complementary and alternative medicine*, *13*(1): 1-9.

Roelfsema, J.H., Nozari, N., Herremans, T., Kortbeek, L.M. & Pinelli, E. (2011). Evaluation and improvement of two PCR targets in molecular typing of clinical samples of *Leishmania* patients. *Exp. Parasitol.* 127(1): 36-41.

Rotureau, B., Ravel, C., Couppié, P., Pratlong, F., Nacher, M., Dedet, J. P. & Carme, B. (2006). Use of PCR-restriction fragment length polymorphism analysis to identify the main new world Leishmania species and analyze their taxonomic properties and polymorphism by application of the assay to clinical samples. *J cli microbiolo*, 44(2): 459-467.

Rowland, M., Munir, A., Durrani, N., Noyes, H. & Reyburn, H. (1999). An outbreak ofcutaneous leishmaniasis in an Afghan refugee settlement in north-westPakistan. *Trans. R. Soc. Trop. Med. Hyg.* 3: 133-136.

Sachdeva, H., Sehgal, R. & Kaur, S. (2013). Studies on the protective and immunomodulatory efficacy of Withania somnifera along with cisplatin against experimental visceral leishmaniasis. *Parasitol resea*, *112*(6): 2269-2280.

Sacks, D. & Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nature Reviews Immunology*, *2*(11): 845-858.

Sacks, D. L., Kenny, R. T., Kreutzer, R. D., Jaffe, C. L., Gupta, A. K., Sharma, M. C., Sinha, S. P., Neva, F. A. & Saran, R. (1995) Indian kala-azar caused by *Leishmania tropica*. The Lancet, 345.

Sacks, D.L., Hieny, S. & Sher, A. (2001). Identification of cell surface carbohydrate and antigenic changes between noninfective and infective developmental stages of *Leishmania major* promastigotes. *J. Immunol,* 135(1): 564-9.

Sadeghi-Nejad, B. (2000). Prevalence of cutaneous leishmaniasis in patients referring to health centers Khuzestan province from 1998-1999. *Third Congr Med Parasitol*, 292.

Saebi, E. (2005). Parasitic diseases in Iran (protozoa). 9th ed. Tehran: Aeizh.

Saeed, N., Khan, M. R. & Shabbir, M. (2012). Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts Torilis leptophylla L. *BMC Complem. alternative medi*, 12(1): 1-12.

Sahreen, S., Khan, M.R. & Khan, R.A. (2010). Evaluation of antioxidant activities of various solvent extracts of Carissa opaca fruits. *Food Chem*, 122(4): 1205-1211.

Sairafianpour, M., Christensen, J., Stærk, D., Budnik, B. A., Kharazmi, A., Bagherzadeh, K. & Jaroszewski, J. W. (2001). Leishmanicidal, antiplasmodial, and cytotoxic

activity of novel diterpenoid 1, 2-quinones from Perovskia abrotanoides: new source of tanshinones. *J. nat. produ*, 64(11): 1398-1403.

Salam, N., Al-Shaqha, W. M. & Azzi, A. (2014). Leishmaniasis in the Middle East: incidence and epidemiology. *PLoS Negl Trop Dis*, *8*(10): e3208.

Salam, N., Mohammed, W. & Azzi, A. (2014). Leishmaniasis in the Middle East: Incidence and Epidemiology. *PLoS Negl Trop Dis,* 8(10)-1-8.

Salehabadi, A., Karamian, M., Farzad, M. H. & Namaei, M. H. (2014). Effect of root bark extract of Berberis vulgaris L. on Leishmania major on BALB/c mice. *Parasitology research*, 113(3): 953-957.

Sallusto, F., Lanzavecchia, A. & Mackay, C. R. (1998). Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunology today*, *19*(12): 568-574.

Samoylenko, V., Ashfaq, M. K., Jacob, M. R., Tekwani, B. L., Khan, S. I., Manly, S. P. & Muhammad, I. (2009). Indolizidine, antiinfective and antiparasitic compounds from Prosopis glandulosa var. glandulosa. *J. nat. products*, 72(1): 92-98.

Sandanayaka, R., Kahawita, I., Gamage, A., Siribaddana, S. & Agampodi, S. (2014). Emergence of cutaneous leishmaniasis in Polonnaruwa, Sri Lanka 2008-2011. *Tropical Med Int Health.* 19(2): 140-145.

Santos-Buelga, C., Francia-Aricha, E. M. & Escribano-Bailón, M. T. (1995). Comparative flavan-3-ol composition of seeds from different grape varieties. *Food Chemi*, 53(2): 197-201.

Sarala, V., Radhakrishnan, M., & Balagurunathan, R. (2011). Inhibitory activity of terpenoid from the medicinal plant Andrographis paniculata against biofouling bacteria. *Int J Chem Tech Res*, *3*(3), 1225-1231.

Saravia, N. G., Segura, I., Labrada, L. A., Weigle, K., Giannini, S. H., Pacheco, R. & Goncalves, A. (1990). Recurrent lesions in human Leishmania braziliensis infection—reactivation or reinfection?. *The Lancet*, 336(8712): 398-402.

Sarkar, J., Pal, S., Bhattacharya, S. & Biswas, M. (2013). In vitro antileishmanial activity of Pleumeria pudica leaf extracts on Leishmania donovani promastigotes. *American-Eurasian J. Scientif. Research*, *8*(2): 68-71.

Scebba, F., Sebastiani, L. & Vitalgiano, C. (2001) Activity of antioxidant enzymes during senescence of *Prunus armeniaca* leaves. Biol Plant, 44: 41-46.

Schnur, L. F., Zuckerman, A. & Greenblatt, C. L. (1972). Leishmanial serotypes as distinguished by the gel diffusion of factors excreted in vitro and in vivo. Israel. *J. Med. Sci*, 8: 932-942.

Schonian, G., Nasereddin, A., Dinse, N., Schweynoch, C., Schallig, H.D., Presber, W. & Jaffe, C.L. (2003). PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis,* 47(1): 349-58.

Schönian, G., Schnur, L., El Fari, M., Oskam, L., Kolesnikov, A. A., Sokolowska-Köhler, W. & Presber, W. (2001). Genetic heterogeneity in the species Leishmania tropica revealed by different PCR-based methods. *Transactions Royal Society Tropical Medicine Hygi*, *95*(2): 217-224.

Schor, J. & White, K. E. (2010). *Plenitude: The new economics of true wealth*. New York: Penguin Press.

Selvapandiyan, A., Dey, R., Nylen, S., Duncan, R., Sacks, D. & Nakhasi, H. L. (2009). Intracellular replication-deficient Leishmania donovani induces long lasting protective immunity against visceral leishmaniasis. *J. Immunology*, 183(3): 1813-1820.

Sen, R. & Chatterjee, M. (2011). Plant derived therapeutics for the treatment of Leishmaniasis. *Phytomedic*, 18(12): 1056-1069.

Shah, N.A., Khan, M.R. & Nadhman, A. (2014). Antileishmanial, toxicity, and phytochemical evaluation of medicinal plants collected from Pakistan. *Bio Med Res. Int,* 1-7.

Shahbazi, Y. (2017). Antibacterial and Antioxidant Properties of Methanolic Extracts of Apple (*Malus pumila*), Grape (*Vitis vinifera*), Pomegranate (*Punica granatum L.)* and Common Fig (*Ficus carica L.)* Fruits. *Phar Sci*. 23: 308-315.

Shaheen, N., Qureshi, N. A., Qureshi, M. Z., Fatima, H., Afzal, M. & Alhewairini, S. S. (2020). Molecular epidemiological survey of cutaneous leishmaniasis from Azad Jammu and Kashmir, Pakistan. *Acta tropica*, *206*: 105434.

Shakila, A., Bilqees, F. M., Salim, A. & Moinuddin, M. (2006). Geographical distribution of cutaneous leishmaniasis and sand flies in Pakistan. *Turkiye parazitolojii dergisi*, 30(1): 1-6.

Shamsedini, S. R. A. S., Rajab Alian, S. A. E. I. D., Mirzaei, M. A. H. B. O. U. B. E. H. & Brofei, M. (2006). Efficacy of Mimosa tenuiflora extract on growth of Leishmania protozoa in vitro. *Iranian J. Dermatology*, 9(2): 173-178.

Sharma, S., Satpathy, G. & Gupta, R. K. (2014). Nutritional, phytochemical, antioxidant and antimicrobial activity of Prunus armenicus. *J. Pharmaco. Phytoche*, *3*(3): 23-28.

Sharquie, K. E., Hassan, A. S., Hassan, S. A & Al-Hamamil, A. (2002) Evaluation of diagnosis of cutaneous leishmaniasis by direct smear, culture and histopathology. *Saudi. Med. J,* 23: 925-8.

Shinwari, M.I. & Shinwari, M. I. (2010). Botanical Diversity in Pakistan; Past, Present and Future. Wor. Environ. Day. 7: 85-104.

Shinwari, Z. K. (2010). Medicinal plants research in Pakistan. *J. medicin. plants research*, 4(3): 161-176.

Shioji, T., Ueda-Nakamura, T., Aparício, D., Prado, B., Morgado-Díaz, J.A. & de Souza, W. (2005). Antileishmanial activity of parthenolide, a sesquiterpene lactone isolated from *Tanacetum parthenium*, Antimicrob Agent Chemother. 49: 176-182.

Shokri, A., Sharifi, I., Khamesipour, A., Nakhaee, N., Harandi, M. F., Nosratabadi, J. & Barati, M. (2012). The effect of verapamil on in vitro susceptibility of promastigote and amastigote stages of *Leishmania tropica* to meglumine antimoniate. *Parasitology research*, 110(3): 1113-1117.

Shon, M.Y., Choi, S.D., Kahng, G.G., Nam, S.H. & Sung, N.J. (2004). Antimutagenic, antioxidant and free radical scavenging activity of ethyl acetate extracts from white, yellow and red onions. *Food Chem. Toxicol*, 42(4): 659-666.

Shui, G. & Leong, L.P. (2002) Separation and determination of organic acids and phenolic compounds in fruit juices and drinks by HPLC. *J Chromatograp* A 977: 89-96.

Sienkiewicz, N., Ong, H. B. & Fairlamb, A. H. (2010). Trypanosoma brucei pteridine reductase 1 is essential for survival in vitro and for virulence in mice. *Molecul microbiolo*, 77(3): 658-671.

Sifaoui, I., López-Arencibia, A., Martín-Navarro, C. M., Chammem, N., Reyes-Batlle, M., Mejri, M. & Piñero, J. E. (2014). Activity of olive leaf extracts against the promastigote stage of Leishmania species and their correlation with the antioxidant activity. *Experimental parasitology*, *141*: 106-111.

Sifaoui, M. (2014). Histoire secrète de l'Algérie indépendante: Édition mise à jour. Nouveau Monde Editions.

Sithara, N. V., Komathi, S. & Rajalakshmi, G. (2017). Identification of bioactive compounds using different solvents through FTIR studies and GCMS analysis. *J. Medici. Plant Stud*, 5(2): 192-194.

Skraba, C.M., Mello, T.F.P.D., Pedroso, R.B., Ferreira, É.C., Demarchi, I. G., Aristides, S. M. A. & Silveira, T.G.V. (2015). Evaluation of the reference value for the Montenegro skin test. *Revi. da Soci. Brasil. de Medici. Trop*, 48(4): 437-444.

Sladowski, D., Steer, S. J., Clothier, R. H. & Balls, M. (1993). An improved MIT assay. *J. immunolol meth*, 157(1-2): 203-207.

Sliwoski, G., Kothiwale, S., Meiler, J. & Lowe, E. W. (2014). Computational methods in drug discovery. *Pharmacological reviews*, *66*(1): 334-395.

Sochor, J., Zitka, O., Skutkova, H., Pavlik, D., Babula, P., Krska, B. & Kizek, R. (2010). Content of phenolic compounds and antioxidant capacity in fruits of apricot genotypes. *Molecules*, 15(9): 6285-6305.

Sofowora, A. (1993). Screening plants for bioactive agents. *Medi. pla. traditi. medic. Africa*, 2: 134-156.

Sorensen, K.M., Jespersgaard, C., Vuust, J., Hougaard, D., Nørgaard-Pedersen, B. & Andersen, P.S. (2007). Whole genome amplification on DNA from filter paper blood spot samples: an evaluation of selected systems. *Genet Test.* 11: 65-71.

Sorensen, K.M., Jespersgaard, C., Vuust, J., Hougaard, D., Nørgaard-Pedersen, B. & Andersen, P.S. (2007). Whole genome amplification on DNA from filter paper blood spot samples: an evaluation of selected systems. *Genet Test,* 11: 65-71.

Souza, A., Marins, D.S.S., Mathias, S.L., Monteiro, L.M., Yukuyama, M.N., Scarim, C.B., Lobenbergd, R. & Bou-Chacra, N.A. (2018). Promising nanotherapy in treating leishmaniasis. *Int. J. Pharm*, 547: 421-431.

Sozangar, N., Jeddi, F., Reaghi, S., Khorrami, S. & Arzemani, K. (2012). Abulkhalsa and yarrow plant effect on *Leishmania major* in vitro. *J. North Khorasan Uni. Medical Sci*, 4(3): 329-333.

Sozangar, N., Jeddi, F., Reaghi, S., Khorrami, S. & Arzemani, K. (2012). Abulkhalsa and yarrow plant effect on Leishmania major in vitro. *J. North Kho University MediSci*, *4*(3): 329-333.

Srinivasan, M., Padmaja, B. & Nair, S. (2014). GC-MS profiling and in vitro radical scavenging effect of Adhatoda beddomei. *Journal of Pharmacognosy and Phytochemistry*, *2*(5).

Srinivasan, M., Padmaja, B. & Nair, S. (2014). GC-MS profiling and in vitro radical scavenging effect of Adhatoda beddomei. *J. Phar. Phyto*, *2*(5).

Stenger, S., Thuring, H., Rollinghoff, M. & Bogdan, C. (1994). Tissue expression of inducible nitric oxide synthase is closely associated with resistance to Leishmania major. *J. experimental medicine*, *180*(3): 783-793.

Sticher, O. (2008). Natural product isolation. *Nat. Prod. Rep,* 25: 517-554.

Sundar, S. & Chakravarty, J. (2015) An update on pharmacotherapy for leishmaniasis, Expert Opinion on Pharmacotherapy, 16(2): 237-252.

Sundar, S. & Chakravarty, J. (2012). Recent advances in the diagnosis and treatment of kala-azar. *Natl Med J India,* 25: 85-9.

Sundar, S. & Chakravarty. (2010). J Antimony toxicity**.** *Int J Environ Res Public Health,* **7**: 4267-4277.

Sundar, S., Jha, T. K., Thakur, C. P., Sinha, P. K. & Bhattacharya, S. K. (2007). Injectable paromomycin for visceral leishmaniasis in India. *New Engl J. Medi*, 356(25): 2571-2581.

Sunder, M. (2001). Cultural dissent. *Stanford Law Review*, 495-567.

Tajima, F. (1989). Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. *Genetics,* 123: 585-595.

Talari, S.A., Talaei, R., Shajari, G., Vakili, Z. & Taghaviardakani, A. (2006). Childhood cutaneous leishmaniasis: report of 117 cases from Iran. *Korean J Parasitol,* 44(4): 355-360.

Talat, H., Attarwala, S. & Saleem, M. (2014). Cutaneous leishmaniasis with HIV - Case report. *J Coll Physicians Surg Pak,* 24(Special Suppl 2): 93-95.

Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. *Natl. Acad. Sci,* 101: 11030-11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (**2013)**. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol,* 30: 2725-2729.

Tang, B., Bi, W., Tian, M. & Row, K. H. (2012). Application of ionic liquid for extraction and separation of bioactive compounds from plants. *J. Chromatography B*, 904: 1-21.

Tantar, A. A., Conilleau, S., Parent, B., Melab, N., Brillet, L., Roy, S. & Horvath, D. (2008). Docking and Biomolecular Simulations on Computer Grids. *Current Computer Aided-Drug Design*, 4(3): 235-249.

Tezuka, M., Takahashi, C., Kuroyanagi, M., Satake, M., Yoshihira K. & Natori, S. (1973). Phytochemistry, (12): 175-183.

Thakur, B. R., Singh, R. K. & Nelson, P. E. (1996). Quality attributes of processed tomato products: A review. *Food Reviews International*, *12*(3): 375-401.

Thomaidou, E., Horev, L., Jotkowitz, D., Zamir, M., Ingber, A., Enk, C. D. & Molho-Pessach, V. (2015). Lymphatic dissemination in cutaneous leishmaniasis following local treatment. *Ameri tropi medicin hyg*, *93*(4): 770-773.

Thony, B., Auerbach, G. & Blau, N. (2000). Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem J,* 347: 1-16.

Tiuman, T. S., Brenzan, M. A., Ueda-Nakamura, T., Dias Filho, B. P., Cortez, D. A. G. & Nakamura, C. V. (2012). Intramuscular and topical treatment of cutaneous leishmaniasis lesions in mice infected with Leishmania amazonensis using coumarin (−) mammea A/BB. *Phytomedicine*, 19(13): 1196-1199.

Tiuman, T.S., Santos, A.O., Ueda-Nakamura, T., Filho, B.P.D., Nakamura, C.V. (2011). Recent advances in leishmaniasis treatment. *Int. J. Infect. Dis.* 15.

Torres-Guerrero, E., Quintanilla-Cedillo, M.R., Ruiz-Esmenjaud, J. & Arenas, R. (**2017).** Leishmaniasis, Review, 6: 750.

Torres-Santos, E. C., Lopes, D., Oliveira, R. R., Carauta, J. P. P., Falcao, C. B., Kaplan, M. A. C. & Rossi-Bergmann, B. (2004). Antileishmanial activity of isolated triterpenoids from *Pourouma guianensis*. *Phytomedicine*, 11(2-3): 114-120.

Trott, O. & Olson, A.J. (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem. 31*: 455-461.

Uddin, G., Rauf, A., Siddiqui, B. S. & Shah, S. Q. (2011). Preliminary comparative phytochemical screening of Diospyros lotus Stewart. *Middle-East J. Scienti. Res*, 10(1): 78-81.

Uddin, G., Rauf, A., Siddiqui, B. S., Muhammad, N., Khan, A. & Shah, S. U. A. (2014). Anti-nociceptive, anti-inflammatory and sedative activities of the extracts and chemical constituents of *Diospyros lotus* L. *Phytomedicine*, 21(7): 954-959.

Uddin, R., Wahid, M. I., Jasmeen, T., Huda, N. H. & Sutradhar, K. B. (2011). Detection of formalin in fish samples collected from Dhaka city, Bangladesh. *Stamford J. Pharmaceutical Sciences*, *4*(1): 49-52.

Ullah, S., Jan, A.H., Wazir, S.M. & Ali, N. (2009). Prevalence of cutaneous leishmaniasisin lower dir district (N.W.F.P). *J. Pak. Assoc. Dermatol,* 9: 212-215.

Uzun, S., Gürel, M.S, Durdu, M., Akyol, M., Karaman F., B., Aksoy, M., Aytekin, S., Borlu, M., Dogan, E.I., Dogramacı, C.A., Kapıcıoglu, Y., Karakas, T.K., Mulayim, K.M., Ozbel, Y., Toz, S.O., Ozgoztaşı, O., Yeşilova, Y. & Harman, M. (2018). Clinical practice guidelines for the diagnosis and treatment of cutaneous leishmaniasis in Turkey. *Int J Dermatol*, 57(8): 973-82.

Valgas, C., Souza, S. M. D., Smânia, E. F. & Smânia Jr, A. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian journal of microbiology*, *38*(2): 369-380.

Vardy, D., Frankenburg, S., Goldenhersh M, Leibovici, V. & Klaus, S. N. (1993). Unusually extensive disease caused by *Leishmania major* parasites. *Clin Exp. Dermatol,* 18: 36-40.

Velmurugan, C., Bhargava, A. (2014). Total phenolic, flavonoids and tannin content of various extracts from *Pyrus communis* fruit. *IJPAR*, 3(4): 384- 390.

Venkatachalam, U. & Muthukrishnan, S. (2012). Free radical scavenging activity of ethanolic extract of Desmodium gangeticum. *J. Acute medici*, 2(2): 36-42.

Victoir, K., Arevalo, J., De Doncker, S., Barker, D.C., Laurent, T., Godfroid, E., Bollen, A., Le Ray, D. & Dujardin, J.C. (2005). Complexity of the major surface protease (msp) gene organization in *Leishmania* (Viannia) braziliensis: evolutionary and functional implications. *Parasitology,* 131: 207-214.

Victoir, K., De Doncker, S., Cabrera, L., Alvarez, E., Arevalo, J., Llanos-Cuentas, A., Le Ray, D. & Dujardin, J.C. (2003). Direct identification of *Leishmania* species in biopsies from patients with American tegumentary leishmaniasis. *Trans R Soc Trop Med Hyg,* 97(1): 80-7.

Vimalavady, A. & Kadavul, K. (2013). Phytocomponents identified on the various extracts of stem of *Hugonia mystax* L. (Linaceae). *Eur J Exp Biol*, *3*(1): 73-80.

Vinodh, S., Devadasan, S. R., Vimal, K. E. K. & Kumar, D. (2013). Design of agile supply chain assessment model and its case study in an Indian automotive components manufacturing organization. *J. Manufacturing Systems*, 32(4): 620-631.

Voi, A. L., Impembo, M., Fasanaro, G. & Castaldo, D. (1995). Chemical characterization of apricot puree. *J. Food Composit Analy*, *8*(1): 78-85.

Wall, M. E., Wani, M. C., Brown, D. M., Fullas, F., Olwald, J. B., Josephson, F. F. & Kinghorn, A. D. (1996). Effect of tannins on screening of plant extracts for enzyme inhibitory activity and techniques for their removal. *Phytomedi*, 3(3): 281-285.

Wang, J., Leblanc, É., Chang, C. F., Papadopoulou, B., Bray, T., Whiteley, J. M. & Ouellette, M. (1997). Pterin and Folate Reduction by theLeishmania tarentolaeH Locus Short-Chain Dehydrogenase/Reductase PTR1. *Archives biochemistry biophysics*, 342(2):197-202.

Wang, J.Y., Cui, G., Chen, H.T., Zhou, X.N., Gao, C.H. & Yang, Y.T. (2012). Current epidemiological profile and features of visceral leishmaniasis in people's republic of China. *Parasites & Vectors.* 5(31): 1-11.

Weigel, M.M., Armijos, R.X., Moosa, Kazemi, S.H. & Zahrai Ramazani, A.R. (1994). Cutaneous leishmaniasis in subtropical Ecuador: popular perceptions, knowledge, and treatment. *Bull Pan Am Health Org.* 28(2): 142-55.

Weina, P. J., Neafie, R. C., Wortmann, G., Polhemus, M., Aronson, N. E. & Strausbaugh, L. J. (2004). Old world leishmaniasis: an emerging infection among deployed US military and civilian workers. *Clini infecti disea*, 39(11): 1674-1680.

WHO. (1991) Information on the epidemiology and control of the leishmaniases by country or territory. DrP.Desjeux *Division of Control of Tropical Diseases*, 91: 30.

Wijngaard, H., Hossain, M. B., Rai, D. K. & Brunton, N. (2012). Techniques to extract bioactive compounds from food by-products of plant origin. *Food Resea. Internati*, 46(2): 505-513.

Wink, M. (2012). Medicinal plants: a source of anti-parasitic secondary metabolites. Molecul, 17: 12771-12791.

Wiwanitkit, V. (2012). Interest in paromomycin for the treatment of visceral leishmaniasis (kala-azar). *Therapeuti clini. risk manage*, *8*: 323.

Woolhouse, M. E., Taylor, L. H. & Haydon, D. T. (2001). Population biology of multihost pathogens. *Science, 292*(5519): 1109-1112.

World Health Organization (WHO) 2010. Control of the Leishmaniases. Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases. Geneva (WHO/DOC/949).

World Health Organization. (2014). *Global status report on noncommunicable diseases 2014* (No. WHO/NMH/NVI/15.1). World Health Organization.

World Health Organization. (2015). Investing to Overcome the Global Impact of Neglected Tropical Diseases. Third WHO Report on Neglected Diseases, (accessed 13.03.15).

World Health Organization. (2016). Weekly Epidemiological Record (WER). 91(22): 285-296.

World Health Organization. (2018). Noncommunicable diseases country profiles 2018.

Yadon, Z.E., Rodrigues, L.C. & Davies, CR. (2013). Indoor and peri-domestic transmission of American cutaneous leishmaniasis in Northwestern Argentina: a retrospective case-control study. *Am J Trop Med Hyg,* 68(5): 519-26.

Yadon, Z.E., Rodrigues, L.C., Davies, C.R. & Quigley, M.A. (2003). Indoor and peridomestic transmission of American cutaneous leishmaniasis in northwestern Argentina: a retrospective case-control study. *Am J Trop Med Hyg*, 68(5): 519-26.

Yaghoobi Ershadi, M. R., Akhavan, A. A., Zahraei Ramazani, A. V., Abai, M. R., Ebrahimi, B., Vafaei Nezhad, R. & Jafari, R. (2003). Epidemiological study in a

new focus of cutaneous leishmaniasis in the Islamic Republic of Iran. *EMHJ-East. Medi. Heal J, 9(4): 816-826.*

Yaghoobi-Ershadi, M.R., Moosa-Kazemi, S.H. & Zahraei-Ramazani, A.R. (2006). Evaluation of deltamethrin-impregnated bed nets and curtains for control of zoonotic cutaneous leishmaniasis in a hyperendemic area of Iran. *Bull Soc Pathol Exot,* 99: 43-48.

Ye, H., Zhou, C., Sun, Y., Zhang, X., Liu, J., Hu, Q. & Zeng, X. (2009). Antioxidant activities in vitro of ethanol extract from brown seaweed Sargassum pallidum. *Euro. Food Rese. Technology*, 230(1): 101-109.

Yehia, L., Adib-Houreih, M., Raslan, W.F., Kibbi, A.G., Loya, A. & Firooz, A. (2012). Molecular diagnosis of cutaneous leishmaniasis and species identification: analysis of 122 biopsies with varied parasite index. *J. Cutan. Pathol*, 39(3): 347-355.

Yektaeian, N., Rafeian, M., Khalili-Dehkordi, B., Hejazi, S. H., Shirani-Bidabadi, L. & Hosseini, S. A. (2012). Effect of combination of Achillea millefolium, Artemisia absinthium & Juglans regia leaves extracts on Leishmania major (MRHO/IR/75/ER), in vitro. *J. Medi. Plan*, 2(42): 197-204.

Yeung, H.C. (1985). Handbook of Chinese Herbs and Formulas. Institute of Chinese Medicine. *Los Angeles*, 2-6.

Yigit, D., Yiğit, N. & Mavi, A. (2009). Antioxidant and antimicrobial activities of bitter and sweet apricot (Prunus armeniaca L.) kernels. *Brazil. J. Medi. Biologi. Resea*, 42(4): 346-352.

Yilmaz I. & Carotenoids. J. (2010).*Turgut Ozal Med Cent*,17: 223-31.

Yurchenko, V., Kolesnikov, A. A. & Lukes, J. (2000). Phylogenetic analysis of Trypanosomatina (Protozoa: Kinetoplastida) based on minicircle conserved regions. *Folia Parasitologica*, 47(1): 1-5.

Yuriev, E. & Ramsland, P. A. (2013). Latest developments in molecular docking: 2010-2011 in review. *J. Molecular Recognition*, 26(5): 215-239.

Yuriev, E., Agostino, M. & Ramsland, P. A. (2011). Challenges and advances in computational docking: 2009 in review. *J. Mole. Recogni*, *24*(2): 149-164.

Yusufzai, S. K., Khan, M. S., Hanry, E. L., Rafatullah, M. & Elison, B. B. (2019). GC-MS Analysis of Chemical Constituents and in vitro Antioxidant Activity of the Organic Extracts from the Stem of Bridelia stipularis. *Sains Malaysiana*, 48(5): 999-1009.

Zemanova, E., Jirku, M., Mauricio, I.L., Miles, M.A. & Lukes, J. (2004). Genetic polymorphism within the Leishmania donovani complex: correlation with geographic origin. *American J. Tropical Medicine Hygiene*, 70: 613-617.

Zhao, P.H., Fan, W., Dong, J., Chen, L., Shan, L., Lin, Y. & Kong, W. (2008). Evaluation of antioxidant activities and total phenolic contents of typical malting barley varieties. Food Chemistry, 107(1): 296-304.

Zijlstra, E.E. (2016). Visceral leishmaniasis: a forgotten epidemic. Arch. Dis. Child, 101(6): 561-567.

Zijlstra, E.E., El-Hassan, A.M., Ismael, A. & Ghalib, H.W. (1994). Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis. *American J. Tropical Medicine Hygiene*, 51: 826-836.

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Molecular epidemiological survey of cutaneous leishmaniasis from Azad Jammu and Kashmir, Pakistan.

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ABSTRACT

Cutaneous leishmaniasis (CL) is an emerging neglected tropical disease in Azad Jammu and Kashmir which is an underdeveloped area. Prevalence and parasite species identification are the key factors to control disease in a particular population, which were the objectives of the present study. Due to a lack of previous data, we performed a district-based active CL surveillance in 2018. The data of CL, suspected (n = 20,000) cases were analyzed statistically and identified the parasite species in microscopic positive cases by ITS1-PCR RFLP and also obtained accession numbers MN891719-28 from gene Bank. The phylogenetic tree was constructed using MEGA6 software. Out of 20,000 CL, suspected cases the highest rate of 4.02% (135/3360) of CL in Mirpur and the lowest 1.58% (8/505) in Neelum was reported. The slide positivity rate, annual parasite incidence rate and annual blood examination rate were 2.27 per 1000 population, 0.08 and 0.34%. The males were more infected 58.12% (297/511) than females 41.88% (214/511) and the age group of 1–20 years were found highly infected 82.78% (423/511) than 21–40 years 13.89% (71/511) and 41–60 years 3.33% (17/511) in the studied population. The patients 56.36% (288/511) had a single lesion whereas 29.35% (150/511) had two, only 10.76% (31/288) and 8% (12/150) were using bed nets. The patients 14.29% (73/511) had three or more lesions were not using bed nets. Only 27.98% (143/511) patients had received treatment, while 72.02% (368/511) didn't. Microscopically positive cases were found to be 2.56% (511/20,000) and ITS1-PCR positive cases were found to be 91.39% (467/511). The RFLP assay confirmed the presence of *Leishmania tropica* in 467 samples.

1. Introduction

The leishmaniasis is a vector born infection caused by parasitic protozoa (Kinetoplastida, Tripanosomatidae) known as *Leishmania* (genus). It is transmitted through the female sand fly vector of genera *Lutzomya* (zoonotic) and *Phelobotomus* (anthroponotic) in different endemic areas of New and Old world accordingly (Reithinger et al., 2007; Karami et al., 2013; Hailu et al., 2016). About thirty species of *Leishmania* parasites are reported, out of which twenty species are pathogenic for humans(Sacks, 2001). The leishmaniasis comprises three clinical forms that are categorized as cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) to visceral leishmaniasis (VL) form with the uppermost prevalence of CL in the Middle East (Blum and Hatz, 2009). The leishmaniasis is a fast-growing neglected infectious threat in more than 98 countries around the world (Souza et al., 2018), million new cases are reported annually (Alvar et al., 2012). About 75% of CL cases are reported from Colombia, Ethiopia, Iran, Peru, Sudan, Brazil, Algeria, Syrian Arab Republic, Costa Rice and Afghanistan (Alvar et al., 2012; Pigott et al., 2014). Devastating epidemics of the CL have also been described from all provinces of Pakistan i.e. Khyber Pakhtunkhwa, Punjab, Balochistan, and Sindh along with Azad Jammu and Kashmir (Noor and Hussain, 2017; Kakarsulemankhel, 2004; Talat et al., 2014). In Pakistan, about 21,000 to 35,000 cases of both anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL) forms of CL are reported. The migration of infected refugees and the local inhabitants are the main source for spreading the infection in non-endemic areas (Alvar et al., 2012). In 1935, the first case of leishmaniasis was reported from Baluchistan following an earthquake. For earthquake disasters, Azad Jammu and Kashmir is a potentially dangerous region (Khan et al., 2019). The zones of Azad

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Molecular characterization and genetic diversity of cutaneous leishmaniasis from North Eastern Pakistan

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ARTICLE INFO

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ABSTRACT

The genetic diversity of *Leishmania spp.* in North Eastern Pakistan remains undetermined despite increased cases of cutaneous leishmaniasis (CL). This study was designed to decipher the molecular characterization and genetic diversity of *Leishmania spp.* in North Eastern Pakistan. Out of 13761 CL suspected cases, 567 cases were microscopically positive and confirmed as *Leishmania spp.* by internal transcribed spacer (ITS) gene amplification through the PCR- RFLP technique. Further, isolates were directly sequenced to conduct phylogenetic analysis for genetic diversity. Among suspected CL cases, Mirpur showed the highest proportion of CL infection with 4.85% (118/2431) of the cases, while the Neelum district showed the lowest percentage at 3.29% (9/273). The slide positivity rate, annual blood examination rate, and annual parasite incidence rate were 3.84, 0.27, and 0.01% respectively, and the incidence of CL in the age group 1–20 years old was higher in males (50.92%) than females (25.75%). The RFLP analysis and sequencing confirmed the occurrence of *Leishmania tropica, Leishmania major,* and *Leishmania infantum. Leishmania tropica* ($p = 0.02$) confirmed significantly higher nucleotides variation than *L. major* ($p = 0.05$). Current findings confirmed the prior assumption that anthroponotic CL is the primary CL form present in North Eastern Pakistan. Moreover, this is the first report based on molecular identification of *L. major*, and *L. infantum* from North Eastern Pakistan. This remarkable heterogeneity in the *Leishmania spp.* is the leading cause of treatment failure and emergence of new haplotypes. Therefore more extensive investigations are recommended from all geographical regions of North Eastern Pakistan, especially those using a large sample size.

1. Introduction

Leishmaniasis is one of the most neglected health problems in tropical and subtropical regions. Protozoan parasites from the *Leishmania* (trypanosomatida and order Kinetoplastids) family are the causative agents of different forms of leishmaniasis (Ramos et al., 2013; Calee Chakravarty, 2015; Salam et al., 2014). Leishmaniasis is widespread across 102 nations (WHO, 2016; Uzun et al., 2018). In 2016, 2500 cases of cutaneous leishmaniasis (CL) were reported in Brazil, Morocco, Syria, Saudia Arabia, Algeria, Iran, Tunisia, Peru, Afghanistan, Turkey, Colombia, and Pakistan annually (WHO, 2016). *Leishmania tropica, Leishmania major, Leishmania braziliensis, and Leishmania amazonensis are*

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RESEARCH ARTICLE

Evaluation of Safety, Antileishmanial, and Chemistry of Ethanolic Leaves Extracts of Seven Medicinal Plants: An *In-vitro* Study

Nargis Shaheen[1], Naveeda Akhter Qureshi[1,*], Attiya Iqbal[1], Asma Ashraf[1] and Huma Fatima[1]

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Abstract:

Background:

Cutaneous leishmaniasis is a neglected tropical disease that currently affects people among 98 countries and causes significant morbidity and mortality. Current chemotherapeutic intervention is unsatisfactory and has various limitations that highlight the necessity to develop safe and effective therapeutic approaches from natural products.

Objective:

The main objective of current study was the evaluation of the antileishmanial activity along with toxicity assessment of selected plant extracts.

Methods:

The ethanolic leaves extracts of selected plants were evaluated for their qualitative and quantitative phytochemical screening by standard protocols. The antioxidant potential of plant extracts was determined by total antioxidant capacity, ferric reducing power and DPPH radical scavenging assays. The cytotoxicity analysis using brine shrimp lethality assay and *in-vitro* antileishmanial activity against promastigotes of L. tropica (Accession# MN891719) were also evaluated.

Results:

The preliminary examination of crude extracts revealed that P. armeniaca showed the highest total phenolic and flavonoid content (279.62±5.40µgGAE/mgDW and 205.70 ±2.41µgQA/mgDW, respectively), among others. P. armeniaca showed strongest antioxidants (120.37±4.90 µgAAE/mgDW) and FRP values (278.71±1.03µgAAE/mgDW). All the plant extracts showed cytotoxicity in safety range >1000µg/ml except F. glomerata having LC50 values of 454.34 µg/ml. In the present study, P. communis and P. pashia showed some level of activity (LC50 56.68 and 60.95µg/ml respectively) while P. armeniaca demonstrated the highest antileishmanial activity (LC50 16.18µg/ml).

Conclusion:

The findings are highly encouraging so, further and extensive investigations of P. arminica should be carried out; especially bio guided fractionation to identify the active fraction and further chemical characterization of structure.

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In vitro anti-leishmanial activity of *Prunus armeniaca* fractions on *Leishmania tropica* and molecular docking studies

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ARTICLE INFO

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ABSTRACT

Prunus armeniaca (L.) is a member of the Rosaceae, subfamily Prunoideae, shows anticancer, antitubercular, antimutagenic, antimicrobial, antioxidant, and cardioprotective activities. Here we fractionated the leaves extract of this highly medicinally important plant for antileishmanial activity. In the current study, the leaves extract was fractionated and characterized using column and thin layer chromatography by n-hexane, ethyl acetate, and methanol solvents. Twelve fractions were isolated and subjected for evaluation of their cytotoxicity and in vitro antileishmanial activity against promastigotes and amastigotes of *Leishmania tropica*. Among all fractions used, the fraction (F7) exhibited the strongest antileishmanial activity. The bioactive fraction was further characterized by spectroscopy (FTIR, UV–Vis), and GC-MS analysis. The in silico docking was carried out to find the active site of PTR1. All derived fractions exhibited toxicity in the safety range $IC_{50} > 100$ µg/ml. The fraction (F7) showed significantly the highest antipromastigotes activity with IC_{50}11.48 ± 0.82 µg/ml and antiamastigotes activity with IC_{50} 21.03 ± 0.98 µg/ml compared with control i.e. 11.60 ± 0.70 and 22.03 ± 1.02 µg/ml respectively. The UV–Vis spectroscopic analysis revealed the presence of six absorption peaks and the FTIR spectrum revealed the presence of alkane, aldehyde, carboxylic acid, thiols, alkynes, and carbonyls compounds The GC–MS chromatogram exhibited the presence of nine compounds: (a) benzeneethanol, alpha, beta dimethyl, (b)carbazic acid, 3-(1 propylbutylidene)-, ethyl ester, (c)1, 2-benzenedicarboxylic acid, diisooctyl ester, (d) benzeneethanamine a-methyl, (e)2aminononadecane, (f)2-heptanamine-5-methyl, (g)cyclobutanol, (h)cyclopropyl carbine, and (i)nitric acid, nonyl ester. Among all compounds, the 1, 2-benzenedicarboxylic acid, diisooctyl ester bound well to the PTR1 receptor. Fraction (F7) showed acceptable results with no cytotoxicity. However, in vivo studies are required in the future.

1. Introduction

Leishmaniasis is the third most important vector born public health problem worldwide [1]. The bite of female's sandfly of genera *Lutzomyia* (zoonotic) and *Phlebotomus* (anthroponotic) transmitted leishmaniasis in different endemic regions of the New and Old world respectively [2,3]. About 30 *Leishmania* species are reported for *Leishmania* infection, out of which only 20 species are pathogenic for human beings [4]. Approxi-

Syrian, Turkey, Saudia Arabia, Afghanistan, Sudan, Peru, Coasta Rice, Colombia, Ethiopia, Algeria, Morocco, Tunisia, and Pakistan [7]. In Pakistan, approximately, twenty-one thousand to thirty-five thousand cases of both zoonotic cutaneous leishmaniasis (ZCL) (*Leishmania major*) and anthroponotic cutaneous leishmaniasis (ACL) by *Leishmania tropica* of CL are reported [7–11]. The CL incidence is raising in Pakistan especially Punjab, Baluchistan, and Nothern areas [9–13]. The migration of people is the main source of spreading the infection in disease-

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December 09, 2021, London

Dear Dr. Shaheen,

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Your chapter will appear in the Open Access book, "Leishmania - One of the Most Diverse Anthroponotic Parasitic Diseases" edited by Dr. Leonardo de Azevedo Calderon

Congratulations on your achievement! I would like to thank you for your important contribution to the scientific community, and for ensuring your research is freely available to readers all over the world.

We wish you every success with your publication.

Sincerely,

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Plant-based alternative treatment for Leishmanaisis-A neglected tropical disease

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Abstract

Leishmanaisis is a 3rd most important vector born disease caused by intracellular parasite belongs to genus *Leishmania*. The leishmaniasis is prevalent in 102 countries/areas worldwide. Approximately, it effected 350 million people worldwide. Leishmanaisis effects developing and undeveloped countries globally. Antileishmanial drugs (pentavalent antimonials, stibogluconate, miltefosin, paramycin, and amphotericin) are