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RESEARCH ARTICLE

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

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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.

Keywords: *Leishmania tropica*, Antileishmanial activity, Antioxidant potential, Medicinal plants, Cutaneous leishmaniasis, Cytotoxicity.

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1. INTRODUCTION

Leishmaniasis is a life-threatening vector born infection caused by a parasite of the genus *Leishmania* (family Trypanosomatidea) [1]. The female sandfly (by bite) belonging to genera *Phlebotomus* and *Lutzomyia* is responsible for *Leishmania* transmission [2]. The leishmaniasis includes a wide range of clinical manifestations that is determined by the immune response of the human host, and affecting species are divided into three groups: (i) Visceral leishmaniasis, (ii) Muco-cutaneous leishmaniasis, and (iii) Cutaneous leishmaniasis [3]. The causative agents of CL belong to various *Leishmania* spp. *i.e.*, *Leishmania major, Leishmania tropica, Leishmania braziliensis,* and *Leishmania amazonensis*. In Pakistan, the most prevalent form of CL is *L. tropica* [4], and it has a huge impact on public health [5]. About 1.5-2 million individuals are affected by this infection in 98 countries, while 350 billion

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people are breathing in those areas which are prone to leishmaniasis [6]. Approximately 70,000 deaths are recorded due to CL infection annually [7]. CL is endemic in the following eight countries; Afghanistan, Iran, Pakistan, Saudi Arabia, Algeria, Peru, Brazil, and Syria [8]. In Pakistan, devastating epidemics of CL has been described in Khyber Pakhtunkhwa, Punjab, Baluchistan, Sindh, and Azad Jammu Kashmir [9 - 12]. The spread of CL increase to non-endemic areas of Pakistan is due to the migration of millions of refugees toward North Western Pakistan [13]. It is now well documented that CL is one of the important neglected tropical diseases with the utmost disease burden [14, 15].

Up to now, there is no effective vaccine available for the treatment of CL [16]. Chemotherapy is the main approach for the treatment of CL infection. The 1st line treatment of CL, including sodium stibogluconate (pentostam) and meglumine antimoniate (glucantime) is usually recommended since1959 [17]. Treatment of CL with the pentavalent antimonials compound has some limitations because it needs intramuscular injection administration, side effects, resistance, and prolonged treatment periods [17, 18]. Moreover, the 2nd line drugs, *i.e.*, pentamidine and amphotericin b are toxic and expensive [17]. The pentavalent antimony compound glucantime is the most common commercially available medication used for the treatment of CL in Pakistan, Iran, Afghanistan, and other parts of the world [19 - 22]. The amphotericin b deoxycholate is used as 1st choice drug to cure the pregnant women and 2nd choice when pentavalent antimonials response is failed [23]. However, in Brazil, amphotericin b has been associated with severe side effects [24]. Liposomal amphotericin b also reported adverse effects [25]. The safety profile of various amphotericin formulations has not been evaluated. Even though liposomal amphotericin b has not yet been approved against leishmaniasis in few countries such as Brazil, the medicine indicated in a case in which other options (amphotericin b deoxycholate, pentamidine, and pentavalent antimonials) are unsuccessful [23]. All synthetic medicines have side effects that enhance the enzyme secretions such as glucantime, which creates kidney and liver problems [26, 27]. There are many other therapeutic regimens used, but no agreement exists over which is the best one [28]. 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 free of side effects on the liver, kidney and renal artery failures [29 - 31]. A new alternative effective drug is urgently needed to treat CL infection because new cases of leishmaniasis are increasing now a day [32]. The importance of medicinal plants due to the presence of various medical agents gains more interest worldwide [33]. Therefore, 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 [34]. 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 [18, 35]. Approximately 35% of standard drugs developed from semi-synthetic derivatives, while about 30% were based on pharmacophore or natural products. However, it is notable that about 65% of parasitic medications [15] isolated from natural products have been accepted by health authorities from 1981-2006 [36]. 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 [37].

Kashmir is well known as a global center for the diversity of plants [38]. The wide topographical variations in plant species ranging from alpine subtropical flora and higher altitude flora plains [39]. About 80% of Pakistan's plant species are confined to Kashmir and the Western Mountains [40]. Due to vastness and inaccessibility with climate variation, numerous regions of Azad Jammu and Kashmir 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. Therefore, it deserves special consideration for the preservation of the environment and also for the suitable development of natural products [41]. In Azad Jammu and Kashmir, there is no report of the test conducted on medicinal plants for antileishmanial activity [40]. Therefore, this study aims to explore the phytochemical properties, antioxidant activities, and *in-vitro* antileishmanial activity of *Pyrus pashia*, *Malus pumila*, *Prunus persica, Pyrus communis*, *Prunus armeniaca*, *Ficus glomerata.* and *Diospyros lotus* extracts. If the promising activity against promastigotes 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.

2. MATERIALS AND METHODS

2.1. 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 Azad Jammu and Kashmir. 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. **1**).

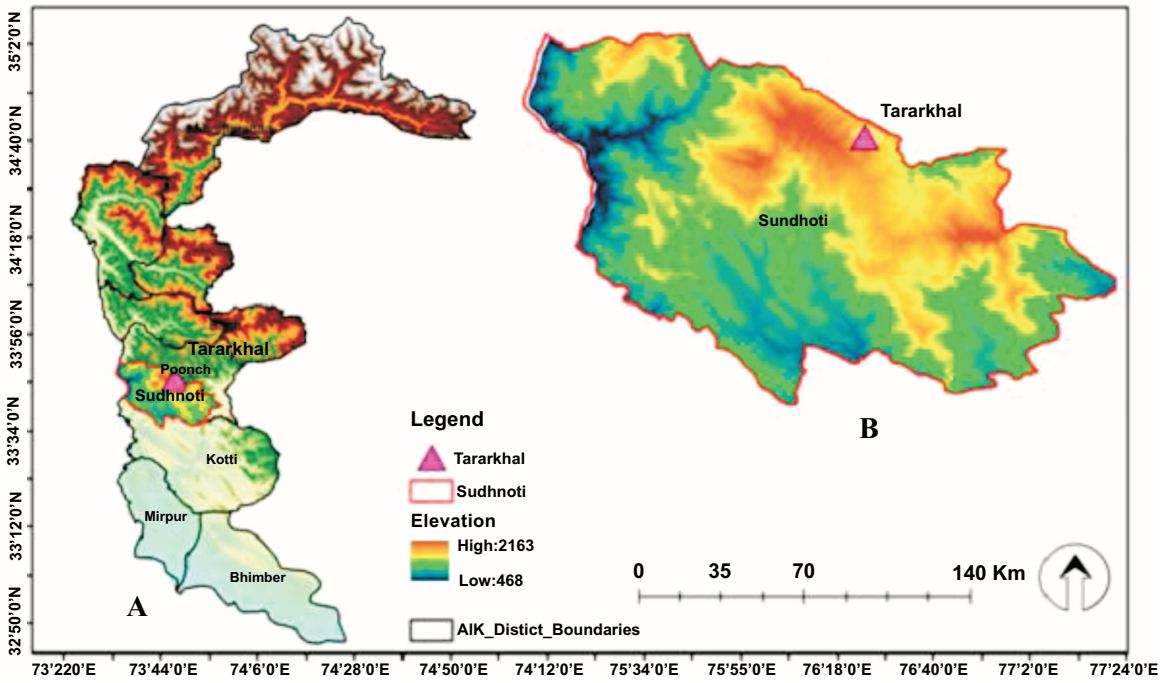

Fig. (1). Map shows the study area of plant collection in Azad Jammu and Kashmir. A: Showing ten districts of Azad Jammu and Kashmir B: showing study area Trarkhel of district Sudhnoti Azad Jammu and Kashmir.

2.2. 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 [42, 43], 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 **1**. After collection, the leaves of plants were washed with tap water and shadow dried for three weeks at room temperature (27-37ºC).

2.3. Extracts Preparation

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 40-60°C (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 4°C for further analysis.

2.4. Phytochemical Analysis

A standard qualitative phytochemical test was conducted in the current study for the determination of saponins, terpenoids, flavonoids, alkaloids [44], phenols [45], tannins [46], and coumarins [47] in selected ethanolic leaves extracts.

2.4.1. Total Phenolic Content

For quantitative phytochemical analysis, the Folin-Ciocalteu (FC) method was used to determine total phenolic content (TPC) in the plant extracts [48]. 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).

Table 1. Plants scientific names, family names, common names and voucher number.

Plant name	Family	Common name	Voucher number
Pyrus pashia	Rosaceae	Tangi, wild pear	130871
Malus pumila	Rosaceae	Sab, apple	130869
Prunus persica	Rosaceae	Arwari, peach	130866
Pyrus communis	Rosaceae	Pear	130870
Prunus armeniaca	Rosaceae	Khubani, apricot	130872
Ficus glomerata	Moraceae	Toshi, wild fig	130867
Diospyros lotus	Ebenacea	Date plum	130868

2.4.2. Total Flavonoid Content

Total flavonoid content (TPC) was determined using the method of aluminum chloride colorimetric method previously reported by Bouyahya *et al.* 2016, 2017 [49, 50]. 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.

2.5. Antioxidant Activities Determination

2.5.1. Total Antioxidant Capacity

The antioxidant capacities (TAC) of extracts were determined by phosphomolybdenum assay spectrophotometrically using the process reported by Jafri *et al.* (2014) [51]. This assay is used daily to estimate the TAC of plant extracts, based on acidic molybdenum (VI) reduction to molybdenum (V) by the complex natural extract (antioxidant compound) and green phosphomolybdenum (V) absorption at 695nm [52]. 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).

2.5.2. Ferric Reducing Power Test

The ferric reducing power (FRP) assay of crude extract was conducted as reported by Zhao *et al.* (2008) [53]. 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).

2.5.3. 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) [54]. 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 = $[A_\circ - A1/A_\circ] X 100$

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.

2.6. 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±1°C 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. The stock solutions of samples were prepared by dissolving the required amount of extracts in a specific volume of 0.5% dimethyl sulfoxide and seawater. Then specific volumes of the samples were shifted from the stock solution to the test tubes to get final sample concentrations of 10, 100, and 1000 ppm (µg/mL) [55]. 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.

2.7. 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 [56].

2.8. 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 [57].

2.9. Evaluation of Anti Promastigotes Activity

The antileishmanial activity assays were done by the methods already described Ogeto *et al.* (2013) [58] 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 were dispensed to various wells of microtiter plates. Along with amphotericin b as the drug standard, dimethyl sulfoxide as negative control were used. Afterword, the micropipette 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).

2.10. Statistical Analysis

All triplicate experiments were conducted, 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 considerd as significant.

3. RESULTS ANS DISCUSSION

3.1. Phytochemical Screening

The natural products are rich sources of selective and new agents for curing imperative tropical diseases caused by protozoan and many other parasites [33]. 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 [38]. 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 [59]. 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 phyto-chemical screening showed all leaf extracts were rich in phenolics and flavonoids constituents. Similar observations were reported by Saeed *et al*. (2012) [60] for *Fagonia olivieri* and *Torilis leptophylla* extracts. 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 [61, 62]. 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 **2**).

3.2. Total Phenolic and Flavanoid Content

The therapeutic and biological advantages are due to the presence of flavonoids and phenolics constituents in all tested leaf extracts. In Table **3**, 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±5.40µg GAE/mg DW), followed by *P. pashia* (241.71±4.27µgGAE/mg DW) and *P. communis(*180.52 ±4.22 µg GAE/mg DW). *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 µg GAE/mg DW) 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±9.81µg QA/mg DW, respectively). The *P. communis, D. lotus,* and *F. glomerata* exhibited 136.42±2.10, 98.62±1.50, and 106.90±2.90µg QA/mg DW respectively. The current investigations showed that the tested plants contained a distinctive but significant amount of flavonoid and phenolic contents (Table **3**). The existence of such compounds explains the use of these plants in folk medicine [63].

Table 2. 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	-	-	-	-	+	-	-

Table 3. 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.

3.3. Antioxidant Activities Determination

In Table **4,** 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.*, AAE µg/ 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±3.21 µg AAE/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**).

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.* 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 **5**). 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 [63]. The antioxidant activity of the hydroxyl group number and location to the carboxyl group and as high as the hydrolation increases [64]. Furthermore, the flavonoid mechanism of action was correlated with free radical scavenging or metal ion chelation [65]. This power to scavenge free radicals gives them antioxidant ability [66]. In the current investigations, a parallel correlation was observed between TAC and FRP. This parallel correlation was also reported by Kumar and Jain [67] 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+}) [68]. The present study claimed that the higher the TPC in plant extracts, the more the reduction capacity it will have [69]. 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 [70]. The DPPH (517nm absorption) is a stable radical capable of antioxidant scavenging [71]. 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 [72]. In the reaction, DPPH absorbs radical hydrogen or electron from antioxidants and changes its color, which can be calculated by spectrophotometer at 517nm [73].

Table 4. 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.

Table 5. 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

(Table 5) contd.....

Plant name	Concentrations (µg/ml) % inhibition by plants and the Vitamin C					IC50
	0.5	5	25	50	100	µg/ml
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

Table 6. *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)	*P* Value
Concentration (µg/ml)	0.5	5	25	50	100		
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	0.001
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

3.4. Cytotoxicity Assays

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 [74]. 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 [75]. 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. The *F. glomerata* extract showed moderate toxicity having LC_{50} values of 454.34µg/ml following the statement of Nguta *et al.* [75]. The crude extract of *F. glomerata* showed moderate activity and is need to not be left as irrelevant because Bussmann *et al.* (2011) [76] and Nguta *et al.,* (2012) [75] 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 had 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 [77, 78].

3.5. Antileishmanial Activity

About more than 100 plant extracts showed promising activity against various forms of *Leishmania* parasite [79]. The medical plants Wormwood (*Artmisia aucheri Bioss*), Purple coneflower (Echinacea purpura), and Marigold (Calendula officinalis) belong to the Asteraceae family showed promising antileishmanial activity against promastigotes of CL *in-vitro* [80, 81]. The *Aloe emodin* of the family Aloaceae showed decrease promastigotes growth, having IC_{50} of 52.8µg/ml. The garlic (*Allium sativum*) inhibits promastigotes growth in 48hrs at a dose of 37µg/ml [82]. The extracts of Henna (*Lawsonia inermis*) and Thyme (*Zajuria multiflora Bioss*) were used for wound healing in CL infection [83]. The crude extract of *Aloe vera* leaf was assed to test against CL showed a significant effect [84]. Hamarsheh *et al.* in (2017) [85] stated that *Malva sylves tris* and *Artemisia inculta* showed strong antileishmanial activity against CL infection. The grand wormwood (*Artemisia absinthium*), Yarrow (*Achillea millefolium,* and Walnut (*Jaglana regia*) arrest promastigotes growth high as compared to control [86]. The Royle (*Arnebia euchroma*), Sweet wormwood (*Artemisia annua*), and Jurema (*Mimosa tenuiflorw*) showed significant antileishmanial activity against promastigotes of *L. major* as compared to control [87 - 89]. The *Eucalyptus camaldulensis* (Blue gum) showed an inhibitory effect on the parasitic growth of CL, and it also showed a reduction of the lesion size [90]. Similarly, many other medicinal plants have been screened against *Leishmania* parasites by conducting in-vitro bioassays.

In Table **6,** 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. Biological activates of *P.*

pashia were also reported by Guven *et al.* (2006) [91]. *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 [92], *D. lotus* used as antiseptic, antitumor, and antidiabetic [93, 94], and *F. glomerata* reported for bacterial infection [95]. 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 antioxidants [96], antimicrobial activities [97], and anti-asthmatic [98]. Minaiyan *et al.* in 2014 [99] reported that *P. ameniaca* have been used in many parasitic diseases. 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±0.00 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.

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 has been already purified from *Calophyllum brasiliense* (Calophyllaceae) reported antileishmanial activity against *L. amazonesis* [100]. The alkaloids from the genus *Prosopis* showed significant *in-vitro* parasitic activity against *L. donovania* with compared to the control drug [101]. Some other investigations reported the presence of phenolic [102] and flavonoids [103] from *Ageratum conyzoides* (Asteraceae) possess antileishmanial activities against promastigotes of *L. donovania*. Torres Santos *et al.* (2004) [104] 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) [105] and *Perovskia abrotanoides* (Lamiaceae) [106]. Paolini *et al.* (2004) [107] 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. Azad Jammu and Kashmir 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.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present study was conducted with the approval and assigned a protocol (#BEC-FBS-QAU-152) by the bioethical committee of Quaid-i-Azam University Islamabad, Pakistan.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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