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Research Article

Evaluation of Pakistani Medicinal Plants used traditionally to treat Leishmaniasis

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Abstract

Cutaneous leishmaniasis (CL) is caused by various species of the protozoal parasite Leishmania, affecting approximately ten million individuals globally. CL, primarily caused by *Leishmania tropica*, is most common in Pakistan and has spread throughout the country due to the migration of several million people. Approximately 90% of CL cases have been reported from all provinces of Pakistan. Pentavalent antimonials are the conventional treatment, although they are quite expensive and have several side effects when administered parenterally. A literature review showed that no natural compound has been approved against leishmaniasis. Therefore, the objective of the present study was to screen the traditionally used medicinal plants for activities against leishmaniasis. Medicinal plants traditionally used to cure Leishmania were collected from various parts of Pakistan. Methanolic extracts and fractions were prepared according to the standard methods and tested for important secondary metabolites. *Citrullus colocynthis* (L.) Schrad (IC_{50} 12.25 µg/mL), *Juniperus M. Bieb* (IC_{50} 11.97 µg/mL), and *Asparagus gracilis* L (IC_{50} 10.68 µg/mL) showed strong antileishmanial activity against *Leishmania tropica* promastigotes. These extracts will be subsequently separated and fractionated. Furthermore, mechanistic research on the isolated compounds from these plants will be carried out to discover cost-effective and safe therapies for cutaneous leishmaniasis.

Keywords: Medicinal Plants, Crude Extract, Phytochemical Analysis, Antileishmanial Activity

1. Introduction

Leishmaniasis is one of the most common diseases caused by an infection with the Leishmania genus. The disease is transmitted through the bite of infected female sandflies, making it a major concern in underdeveloped countries. Leishmaniasis is strongly associated with poverty and accounts for forty-four lac disability-adjusted life years. Leishmania infections are prevalent, affecting 98 countries, with 600 million to 1 billion people at risk of infection (Mann et al. 2021, Kaye et al. 2023). Cutaneous leishmaniasis (CL) is commonly recognized as the major type of human leishmaniasis. It is characterized by the presence of skin ulcers and subsequent scar formation. The global prevalence of CL is predicted to range between 60,000 and 1 million new infections each year. This ailment typically exhibits the potential to repair itself, yet it can seldom manifest as persistent or long-lasting. Injuries frequently result in the production of disfiguring scars, which can contribute to the sensation of social isolation and psychological discomfort (Surur et al. 2020).

There are many cases of Cl in the Eastern Mediterranean region. Moreover, Pakistan also has a high incidence of CL, especially in its rural areas. The word "kaal daana" is used locally in Pakistan to describe this disease. There are

between 21,000 and 35,000 cases of CL in Pakistan each year. The occurrence rate of the disease is contingent upon several factors, such as the existence of appropriate habitats for sandflies, the presence of animal breeding grounds, and the influx of Afghan migrants within the nation's boundaries. The pathogenic organisms in Pakistan responsible for this illness are either *Leishmania tropica* or *Leishmania major*. Female sandflies from the Phlebotomus and Lutzomyia genera spread the disease among vertebrate hosts. This specific disease is influenced by some variables, including migration patterns, socioeconomic conditions, and climatic change (Kayani et al. 2021, Ullah, Shah, and Khan 2020).

The two main antimonial medications used to treat cutaneous leishmaniasis are sodium stibogluconate and meglumine antimonite. These medications are famous for their high cost, potential safety risks, and ability to create resistance with continuous use. As a result, these components may eventually cause therapeutic failure. As a result, continued research developing new and improved drugs is essential (Ahmed et al. 2019, Hendrickx, Caljon, and Maes 2019). The key element leading to the existing health gap and the ongoing existence of neglected illnesses is the limited ability to generate novel therapies, along with a reliance on outside aid (Kaye et al. 2023, Chakravarty and Sundar 2019).

Natural products have played a crucial role in promoting human well-being because of various pharmacological effects and complex chemical structures. Natural products have a significant role as the primary source of pharmaceuticals. Globally, there exists a range of 250,000 to 500,000 plant species, of which a mere six percent have been subjected to investigation so far (Mantravadi, Parthasarathy, and Kalesh 2021, Cortes et al. 2020). It is commonly known that medicinal plants are a great source of bioactive compounds. For example, saponins have notable insecticidal, cytotoxic, and anti-diabetic effects. Strong anti-inflammatory, antibacterial, antiviral, and anti-cancer effects are exhibited by a variety of flavonoids and terpenoids. Previous research has shown that saponins have anti-inflammatory qualities. Tannins have antibacterial activity via numerous mechanisms, including hydrogen bonding, iron deficiency, and interactions with vital proteins, such as enzymes, in microbial cells. Many benefits are exhibited by phenols, such as anti-inflammatory, anti-mutagenic, anti-carcinogenic, anti-oxidative, and anti-diabetic effects. Steroids are known to have strong cardiotonic effects in addition to their insecticidal and antibacterial properties (Calixto 2019).

Plants may be a great source for the development of novel therapies targeting cutaneous leishmaniasis, as demonstrated by the safety and effectiveness of several plant extracts against antimonial-resistant Leishmania parasites as well as their cost-effectiveness. The underdeveloped countries affected by CL have a wide range of plant species, which provides significant opportunities for the development of novel medications aimed at lowering the prevalence of CL (Hussein and El-Anssary 2019, Passero et al. 2021, Tariq et al. 2016, Ullah et al. 2016). However, searching through the literature showed that no natural compound has been approved against leishmaniasis. Therefore, the objective of the present study was to screen the traditionally used medicinal plants for activities against leishmaniasis.

2. Materials & Methods

2.1 General Experimental

Methanol was used as an extracting solvent, while *n*-hexane, chloroform, and ethyl acetate were the organic solvents used for fractionation. The cultures of *L. tropica* were kept alive in Medium 199 (M199) (Gibco, Invitrogen, Carlsbad, CA, USA), which was enhanced with 10% heat-inactivated fetal bovine serum (PAA Laboratories, GmbH, Austria), 100 mg/mL streptomycin (Bio Basic Inc., Markham, ON, Canada), and 100 U/mL penicillin (Sigma, Milwaukee, WI, USA). For MTT experiments, an ELISA reader (ELx800 BioTek) was utilized.

2.2 Plants Collection

The medicinal plants traditionally employed for the treatment of leishmaniasis were collected from March 2022 to May 2022 from various regions of Pakistan. The botanical identity of these plants was verified by Dr. Ghazala, Assistant Professor, Department of Botany, KUST. The voucher specimen has been appropriately deposited inside the Botany Department herbarium, accompanied by the corresponding Catalogue number for each plant. This study was approved by the Department of Pharmacy, Kohat University of Science & Technology, Kohat, Pakistan.

2.3 Preparation of Crude Extract

The air-dried plants were chopped, pounded into a coarse powder, and macerated in methanol at room temperature for 14 days. The methanol soluble residues were filtered and concentrated using a vacuum rotary evaporator at 40°C. The filtrate yielded crude methanol extracts. All extracts were preserved in the refrigerator for future project studies (Hussain et al. 2019).

2.4 Qualitative Phytochemistry of Methanolic Extracts

Standard protocols were employed to conduct preliminary phytochemical analyses of crude extracts to identify significant secondary metabolites The phytoconstituents included in the crude extracts were identified using standard techniques with minor adjustments (Shah, Ullah, Ayaz, Wahab, et al. 2019, Ayaz et al. 2014). The presence of saponins in the entire extracts was indicated by the persistent production of froth following the vigorous shaking of the extracts with 5 mL of distilled water in a test tube. The crude extracts were mixed with a 2 mL solution of NaOH, which had a concentration of 2%. The presence of flavonoids was indicated by the observation of a deep yellow color. "Keller Killiani" Test detected glycosides. Glacial acetic acid and ferric chloride were combined into a test solution. Two layers formed after adding a few drops of concentrated H_2SO_4. A lower reddish-brown layer and a top acetic acid layer that turns blue-green suggest glycoside positivity. The presence of terpenoids was verified by observing the development of a greyish color upon combining a crude extract with 2 mL of chloroform and 2 mL of saturated H2SO4. The presence of alkaloids was determined by observing the formation of a precipitate upon the addition of 2 mL of 1% hydrochloric acid, followed by the subsequent addition of Mayer's and Wagner's reagents to the extract. The presence of phenolic compounds was indicated by the development of a dark green color when the extract was dispersed in 5 mL of distilled water and a few drops of a 5% ferric chloride solution were added. A volume of 5mL of extract was obtained and thereafter subjected to boiling in the presence of 20mL of CHCl3. The observation of a reddish color upon the addition of a 0.1% $FeCl_3$ solution to the filtrate indicates the presence of tannin.

2.5. Fractionation Process

The crude extracts (500 g) were dispersed in distilled water (500 mL) and sequentially extracted with *n*-hexane, chloroform, ethyl acetate, acetone, and butanol organic solvents to obtain polarity-based fractions (Shinwari et al. 2019).

2.6 Growth of Promastigotes

Promastigotes were cultured in RPMI-1640 medium at a density of 1× 106 /mL in a 25 mL non-vented culture flask. The medium was supplemented with 10 % heat-inactivated fetal calf serum, 100 µg/mL streptomycin, 100 IU penicillin, and L-glutamine. For a week, the growth was counted every day using a Neubauer hemocytometer (Shah, Ullah, Ayaz, Sadiq, et al. 2019, Ayaz et al. 2019).

2.7 Antileishmanial Screening of Fractions and Sub-fractions

A preliminary anti-leishmanial screening against promastigotes of *L. tropica* was performed on all fractions. Dimethyl sulfoxide (DMSO) was used to dissolve the extracts at a concentration of 1 mg/mL for stock solutions, which were then progressively diluted. The wells of a microtiter plate were filled

Table 1: Medicinal plants with catalog number

1	***Aloe vero* (L.) Burm. F**	**BOT-KHS-1**
2	*Asparagus gracilis* L	BOT-KHS-2
3	*Asparagus asiaticus* L	BOT-KHS-3
4	*Trachyspermum ammi* (L.)	BOT-KHS-4
5	*Citrulluscolocynthis* L. Schrad	BOT-KHS-5
6	*Juniperus* M. Bieb	BOT-KHS-6
7	*Jurinea dolomiaea* Boiss	BOT-KHS-7
8	*Melia azedarach* L	BOT-KHS-8
9	*Onosma griffithii* Vatke	BOT-KHS-9
10	*Perotis hordeiformis*	BOT-KHS-10
11	*Physalis minima* L	BOT-KHS-11
12	*Sarcococca hookeriana* Baill	BOT-KHS-12
13	*Stellaria media* L. Vill	BOT-KHS-13
14	*Thuspeinanta brahuica* (Boiss)	BOT-KHS-14
15	*Tylophora hirsute* Wight	BOT-KHS-15
16	*Swertiachirata* Roxb ex	BOT-KHS-16
17	*Nepeta praetervisa*	BOT-KHS-17
18	*Sida cordata* L.	BOT-KHS-18

with approximately 180 µL of M199, 100 µL of *L. tropica* log phase culture, and 20 µL of each drug. The plates were then incubated for 72 hours at 24 ◦C. By using the mitochondrial dehydrogenase enzyme to generate purple formazan crystals in living cells, the viability of the *L. tropica* promastigotes was demonstrated. A microplate ELISA reader was used to quantitatively evaluate the promastigote survival percentage at 540 nm, using Glucantime as a positive control (Shah, Ullah, Ayaz, Sadiq, et al. 2019).

2.8 Statistical Analysis

Data was analyzed as Mean ± SEM, n=3, and two-way ANOVA followed by the Bonferroni test for the statistical analysis of the standard and testes samples was performed. Comparisons were made and notations (*P < 0.05, **P < 0.01, ***P < 0.001, ns; not significant) compared to the standard drug were used.

3. Results

3.1 Preparation of Crude Extract and Qualitative Phytochemical Investigation

The phytochemical screening results showed that selected plant species possess all the major secondary metabolites (Table 2) which explains how these plants have traditionally been used to treat leishmaniasis.

3.2 Fractionation Process and Antileishmanial Screening

The crude extracts of 18 medicinal plants were dispersed in water and 6 fractions of each plant namely n-hexane, chloroform, ethyl acetate, acetone, and butanol were obtained. In this regard, 108 fractions were screened for antileishmanial evaluations. The antileishmanial activity results were divided into four categories i.e. extracts with no activity, weak activity, moderate activity, and significant activity. The extracts of three medicinal

Table 2: Qualitative Phytochemistry of Selected Medicinal Plants

S. No	Medicinal Plants	Phytochemicals Class Present	Phytochemicals Class Absent
1	*Aloe vera* (L.) Burm. F	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	……..
2	*Asparagus gracilis* L	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	'……
3	*Asparagus asiaticus* L	Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	Tannins
4	*Trachyspermum ammi* (L.)	Tannins, Saponins, Flavonoids, Anthraquinones, Terpenoids, Steroids, Alkaloids, Glycosides, Phenolic Compounds	-------
5	*Citrulluscolocynthis* L. Schrad	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	------
6	*Juniperus* M. Bieb	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	-------
7	*Jurinea dolomiaea* Boiss	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	-------
8	*Melia azedarach* L	Tannins, Saponins, Flavonoids, Terpenoids, Phenolic Compounds	Alkaloids, Glycosides
9	*Onosma griffithii* Vatke	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides	-------
10	*Perotis hordeiformis*	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	-------
11	*Physalis minima* L	Tannins, Saponins, Flavonoids, Glycosides	Alkaloids
12	*Sarcococca hookeriana*	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	……..
13	*Stellaria media* L. Vill	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	----------
14	*Thuspeina nta brahuica*	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	----------
15	*Tylophora hirsute* Wight	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides	----------
16	*Swertiachirata* Roxb ex	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	Glycosides
17	*Nepetapraetervisa* Rech.f	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	----------
18	*Sida cordata* L.	Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, Phenolic Compounds	----------

plants *Citrullus colocynthis* (L.) Schrad, *Juniperus M. Bieb,* and *Asparagus gracilis* L show significant activity and thus are recommended for further isolation of compounds.

4. Discussion

Medicinal plants have shown beneficial pharmacological activities over the decades (Imran et al. 2012, Hussain et al. 2010, Gul et al. 2011). Traditional plant therapy is still practiced in

Table 3: Significant Antileishmanial Activity of Selected Medicinal Plants.

S. NO	Concentration (µg/ml)	% inhibition	IC50(µg/ml)
Citrullus colocynthis (L.) Schrad Chloroform extract	100 50 10 1	82.03 ± 0.71*** 78.75 ± 0.99*** 49.44 ± 0.42*** 8.45 ±0.47ns	**12.25**
Juniperus M. Bieb Crude extract	100 50 10 1	90.58 ± 0.52ns 79.25 ± 0.23*** 24.16 ± 0.12** 21.06 ± 0.20**	**11.97**
Asparagus gracilis L Ethyl acetate extract	100 50 10 1	87.68 ± 0.60ns 79.33 ± 0.11ns 30.43 ± 0.41** 22.84 ± 0.22**	**10.68**
Glucantime Standard drug	100 50 10 1	90.21 ± 0.19 83.00 ± 0.66 45.97 ± 0.91 31.06 ± 0.04	**5.56**

Data were analyzed as Mean ± SEM, n=3, TWO-way ANOVA followed by the Bonferroni test for the statistical analysis of the standard and testes samples. While *P < 0.05, **P < 0.01, ***P < 0.001, ns; not significant to that of the standard drug used in this assay.

many rural areas of underdeveloped countries, including Pakistan, and it serves as a starting point for the investigation of pharmacologically effective drugs (Shah, Ullah, Ayaz, Wahab, et al. 2019). The importance of Phyto-investigations has increased because of the excessive side effects of the synthetic medicines. Natural products can be utilized for beneficial effects because they are non-toxic, inexpensive, and widely accessible. To make it easier to find new phytomedicines that may be utilized to treat a variety of ailments, phytochemical screenings, isolations, and pharmacological investigations must be carried out (Ayaz et al. 2014). Due to its distinctive soil composition and varying climatic conditions, Pakistan is a habitat for a wide variety of important medicinal plants. By carefully examining these plant samples, it is possible to identify natural medicines that are both safe and effective (Hussain et al. 2019). The presence of bioactive phytocomponents in medicinal plants is thought to be the cause of their pharmacological effects. The phytochemical screening results showed that selected plant species possess all the major secondary metabolites (Table 2) which explains how these plants have traditionally been used to treat leishmaniasis.

The findings show that different plant extracts and the standard therapy, Glucantime, have variable inhibitory effects at different concentrations. *Citrullus colocynthis* (L.) Schrad and *Juniperus M. Bieb* extracts had considerable inhibition at higher concentrations but less effectiveness at lower levels, while *C. colocynthis s*howing the sharpest drop. *Asparagus gracilis* exhibits consistent inhibition with a moderate IC_{50} of 10.68 µg/mL, demonstrating superior efficacy over other plant extracts. Glucantime remains the most powerful with the lowest IC_{50} of 5.56 µg/mL, exhibiting superior inhibitory action at all doses. Glucantime is employed as a positive control in antileishmanial activity studies since it is a well-established, effective therapy for leishmaniasis. It acts as a standard for comparison, allowing researchers to assess novel compounds against a reliable and approved medicine. Its method of

action gives it an excellent reference for this research. These findings suggest that mentioned plant extracts are more effective and thus recommended for further study.

Secondary metabolites of plants are an ideal source for the manufacture of pharmaceuticals to treat human ailments since they have a wide range of physiological properties. Primary metabolites, which include carbohydrates, proteins, lipids, vitamins, and nucleic acid components, are biomolecules required for an organism's development, growth, and metabolism. They are found in all plants and are consumed as food by humans. The industrialized world now has a genuine interest in therapeutic plants (Hussain et al. 2019). In fact, research academics place a high value on using plants as traditional medicines to treat a wide range of illnesses. Scholars and business professionals are concentrating on plants that originate from natural sources in order to discover novel chemical compounds. They intend to create pharmacologically active entities by extracting and purifying various sorts of compounds from different plant sources. Conventional therapy is frequently less expensive than modern medications, which are typically out of reach for many people in impoverished or developing countries. Approximately 40% of newly marketed medications during the last 20 years have been derived from natural sources, with plants playing a particularly significant role (Fulda and Efferth 2015, Kamil Hussain, Saquib, and Faheem Khan 2019).

Several natural substances from medicinal plants have been identified and studied for anti-leishmanial properties. Licochalcone A, luteolin, quercetin, isoorientin, Podocarpus flavone, sulfuretin (flavonoids), diphyllin (lignan), methyl 3-4 dihydroxy benzoic acid, decanoic acid, and lauric acid (lipids) are examples of bioactive antileishmanial natural compounds. Triterpenes include mimengoside A and sergeolide, saponin beta-hedrine, quinones such as disopyrin and jacaranone, alkaloids such as camptothecine, acivicine, berberine, cusparin, and obaberin, and lactones such as argentilactone, annonacin, and mycophenolic acid. Vernolide, Neurolenin B, Kudtriol, Artemether (a Sesquiterpene), Amarogentin, Jatrophone, Picroliv (an Iridoid), Curcumin, and Piperogalin (Benzenoid) all have leishmanicidal properties (Ullah et al. 2016, Salem and Werbovetz 2006). Anti-leshmanial effects shown in the present study may also be due to the anti-inflammatory effects of the phytocompounds present in these plants. Several natural and synthetic compounds have shown anti-inflammatory activities in the past (Aslam et al. 2008, Ahmed et al. 2014).

CL reflects the primary type of leishmaniasis, which is common throughout Pakistan. Approximately 90% of reported CL cases have been documented in northern, southern, and southwestern areas of Pakistan due to the favorable climatic conditions for the growth and development of vector sandflies. The said regions in Pakistan predominantly consist of rural areas that are characterized by a lack of contemporary health and education infrastructure. The residents of these regions have low economic status because of limited sources of income. However, those residing in rural areas depend on their extensive traditional knowledge as a means of primary healthcare, primarily due to low socioeconomic status (Passero et al. 2021, Ayaz et al. 2014). The plant groups Lamiaceae, Liliaceae, and Asclepiadaceae are extensively utilized in Pakistan for the management of leishmaniasis. The literature survey suggests that the Lamiaceae family possesses a diverse range of secondary metabolites with anti-parasitic properties, thereby warranting further investigation in future studies (Shah, Ullah, Ayaz, Wahab, et al. 2019, Ayaz et al. 2014). The anti-leishmanial action has been observed in nearly all plant components, with leaves, fruits, roots, and aerial portions being the most utilized sections in Pakistan. Leaves have been shown to be the primary focus of plant-based in vitro screening for leishmaniasis, not just in Pakistan but also in other nations worldwide (Passero et al. 2021).

5. Conclusions

It was the first report on the phytohemical and pharmacological profile of extracts which are traditionally used in Pakistan for the therapeutic management of leishmaniasis. The percent inhibitory effects of glucantime and plant extracts were variable. *Citrullus colocynthis* and *Juniperus M. Bieb* showed significant activity at higher concentrations while *Asparagus gracilis* consistently inhibits *Leishmania tropica* with an IC_{50} of 10.68 µg/mL. Based on a thorough review of the current literature, it is evident that there is a lack of documented evidence supporting the approval of any plant-derived molecule for the therapeutic management of leishmaniasis. All these bioactive extracts are recommended for the isolation of pure compounds responsible for the above-mentioned experimental effects for the discovery of various pure chemicals as possible natural leishmanicidal agents.

Data Availability

All the relevant data of this manuscript is available with the authors.

Conflicts of Interest

The authors declare no conflicts of interest.

Consent Forms

NA

Approval of Study

This study was approved by the Department of Pharmacy, Kohat University of Science & Technology, Kohat, Pakistan.

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Author Contributions

MT and RB performed experimental work, data collection and evaluation, and literature search. SMS, AK, and SH supervised the research work, compiled results, and prepared and refined the manuscript for publication. The authors read and approved the final manuscript for publication.

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