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Phytochemical, Antioxidant and Anti-Leishmania Activity of Selected Pakistani Plants

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Abstract

Back ground: Leishmaniasis is a disease caused by protozoal parasite of genus Leishmania and transmitted by sand fly which causes significant mortality and morbidity in different countries. Herbal medicines are source of different leads to anti-leishmania drugs which can offer potential of therapeutic switch chemotherapy. The aim of this study was to evaluate the phytochemical, antioxidant and anti-leishmania activity of five selected Pakistani medicinal plants.

Methods: Extract was prepared by soaking the plant powder for 72 hours in crude methanol and in vitro technique was used to determine the anti-promastigote activity. Radical scavenging activity, Brine-Shrimp assay and Aluminum chloride colorimetric assay was used to determine anti-oxidative, cytotoxicity and presence of flavonoids, respectively.

Results: IC_{50} values for cytotoxicity were found to be 1209±2.0 µg/ml for *Celtis anstracis*; 0.00µg/ml for *Phytanthar embalics*, 1030±4.5 µg/ml for *Cassia gloca*, 1903±3.5 µg/ml for *Eryobotraya japonica* and 1755±3.5µg/ml for *Citrus sinensis*. Anti-leishmania activity and flavonoids of *Celitis anstracis*, *Phytanthar embalics*, *Cassia gloca* and *Eryobotraya japonica* showed significant relationship giving 0.87 R2 value.

Conclusion: Medicinal plants described in this study have potent anti-leishmanial activity. Therefore, further isolation of compounds and *in vivo* study is required.

Keywords: Antioxidant; Anti-leishmania; Medicinal plants.

Introduction

Leishmaniasis is a major health risk throughout the world [1,2]. In Pakistan's rural areas, it is a distinct problem. This disease may vary in its presentation; it can be self-healing or fatal. Various infectious types of leishmaniasis can be categorized as: a) cutaneous leishmaniasis; b) diffuse cutaneous leishmaniasis; c) mucocutaneous leishmaniasis; and d) visceral leishmaniasis [3]. Leishmaniasis is highly endemic and spreads over 87 countries of almost 5 continents and it is endangering about 348 million of people all around the world. In Pakistan leishmaniasis was first accounted in northern areas in 1960. In the beginning it was confined to northern sphere but now it is widely spreading throughout the country [4]. All body parts especially the exposed ones are mainly targeted by cutaneous leishmaniasis. The nascent lesion may give rise to an ulcer. Systemic leishmaniasis affects internal organs of a body i.e. spleen and liver. Although it is rare in Pakistan but can prove to be fatal. Leishmaniasis is a vector borne disease and is transmitted by an infected female sand fly. The multiple ulcers resulting from multiple bites of sand fly are not a rare case in Pakistan. The causative agents of leishmaniasis were discovered about 100 years ago but unfortunately it is still not completely uprooted. On the other hand, it is rapidly spreading out creating an alarming situation. Cutaneous and Visceral leishmaniasis are the major threats to Pakistan despite of, muco cutaneous leishmaniasis which is rarely reported [5]. Many naturally existing compounds in different plants have multi potent effect. A crucial role is played by these multi potent antioxidants against disease. Free radicals are involved in pathogenesis and mostly, more than one pathogenic factor contributes to a disease. Thus, discovery of multi effective compounds instead of, single targeting molecules is essential. In diseased organisms, these anti-oxidants reduce the injuries and loss produced by the free radicals [2]. Flavonoids belong to a vast group of naturally occurring substances varying in their poly phenolic structures

having a basic nucleus and two aromatic ring structures inter linked by a heterocyclic three carbon atom ring. Being secondary metabolites of plants, in nature they are abundantly found in vegetables, grains, roots, leaves, fruits and tea. Flavonoids are involved in scavenging of free radicals disposed by reactive oxygen species. They are segregated from medicinal plants and are the major constituent of traditionally used medicines [2].

Material and Methods

Collection of plants

Plant were randomly selected and collected from different region of Islamabad, Pakistan in their flowering seasons from May 2011 to June 2012. Move over, these plants were identified and were assigned voucher numbers.

Plant extracts preparation

About 200g of each plant part was air dried and was crushed into a fine powder form by using an electric grinder and was stored at 4°C. Thereafter, extraction was performed three times by use of crude methanol at room temperature. The resultant filtrate was further dried by using a rotary evaporator. To carry out experimentation 1 mg of each plant extract was dissolved in 1 ml of DMSO, vortexed and then sonicated for 10 min at 30°C. In each assay, the concentration of DMSO was adjusted to less than 1.0% of the complete culture medium, concentration that showed no growth inhibitory effect on the parasite and non-toxic to brine shrimp eggs. Extracts were serially diluted to obtain the concentration from 333 to 1.3 µg/ml using DMSO and were filtered by using a 0.2µm filter.

Parasites

Following Zhai's methodology [6], pre isolated *Leishmania tropica's* promastigotes from a patient were used and cultured on triple N- media over layered by 199 media. Agar (2g) was dissolved in 50 ml of deionized water and autoclaved to prepare triple N media. Followed by, addition of defibernated rabbit blood and gentamycin for anti-bacterial activity in a sterilized pattern. Solidified triple N media slants were over layered by 199 media maintained at pH 7.4 at 37°C. FCS was also added to meet the demand of *in vitro* cultivation.

Anti-Leishmania assay

Anti-leishmanial assay was done based on methods described previously with slight modification [7,8]. Three days old log phase promastigotes at $1x10^6$/100µl were used for this assay. About 90µl 199 media, 50 µl of *Leishmania tropica* log phase culture and 10µl of each plant dilution were dispensed to different wells of micro-titter plate. Along with, negative and positive controls. Here, DMSO was used as a negative control, while amphotericin B as positive control. Afterwards, micro-titter plate was incubated at 22-26°C for 72 hours. After incubation about 15µl of each dilution was pipetted on a neubar counting chamber and were counted under an electron microscope. Prism Pad software was used to calculate IC_{50} values of each dilution.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The radical scavenging activity free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was performed according to the method described by Amarowicza R, et al., Bondet V, et al. [9,10] with slight modification. This assay is based on reduction of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) which is a stable free radical. DPPH free radicals exhibit maximum value of optical density at 517 nm. When an antioxidant reacts with DPPH its reduced form i.e. DPPHH is generated due to the acceptance of H+ which decreases the optical density of the solution [11]. The degree to which the absorbance decreases is directly proportional to the extent of reduction of DPPH. Here, 100 µl of each methanolic extract having concentrations from 500-31.5 µg/ml and 1ml of DPPH solution (at absorbance 0.9 at 517 nm) were incubated for 30 min a 37°C covered by aluminum foil and their absorbance was taken at 517 nm by using a UV-visible range spectrophotometer. Ascorbic acid was run along as a standard. Free radical scavenging activity of DPPH was calculated by formula:

% SA = (abs. C – abs. S / abs. C) x 100

Here

Abs.C is absorbance of control

Abs. S is absorbance of sample

%SA is % scavenging activity

Brine-Shrimp assay

To find out the general toxicity cytotoxicity assay was performed by carrying out the hatching of brine shrimp eggs in sea salt water (28 g/L) and incubating them at room temperature for n over-night. Three dilutions of plant extract were prepared in 5 ml of sea salt water of concentration 2500, 500 and 50µg/ml. To each vial about 20 larvae were added and left for 24 hours to determine cytotoxic activity. Number of live shrimps was counted after 24 hours and the IC_{50} values were calculated by using prism pad software. Assays were done based on methods described by José Luis Carballo et al. and BN Meyer [12,13].

Determination of Flavonoids:

Flavonoids determination by aluminum chloride colorimetric assay was done based on methods described previously [14]. Various phytochemicals have therapeutic effects that play an important part in prevention and cure of diseases and helps in maintenance of healthy state of a being. Aluminum chloride colorimetric assay was used to find out total Flavonoids. 250 µl of

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plant extract sample of concentration 500 µg/ml was taken and equal volume of deionized water was added to it followed with addition of 75 µl of 5% $NaNO_2$. After 6 minutes of incubation, 150 µl of 10% $AlCl_3$ and 500 µl of 1 M NaOH were added. Here, Rutin was run as standard. Absorbance was measured at 510nm after 5 minutes of incubation at 37°C.

Results and Discussion

In vitro assay was performed to assess safety of the extracts. Plant extracts were screened for cytotoxicity assay using Brine-shrimp assay. The present study shows *Phytanthar Emblics* has no toxicity to brine shrimp eggs while *Celtis Anstracis* and *Eryobotraya Japonica* are more toxic than others (Table 1). DPPH is a free stable radical and is often used to determine the anti-oxidant activity of different compounds. When a donor molecule donates a hydrogen atom, the dark purple color of DPPH disappears [15] (Table 2). The IC50 in µg/ml of *Celtis anstracis* was found to be 1.02±0.02, *Phytanthar embalics's* was 7.06±0.01µg/ml, *Cassia gloca's* IC_{50} was 34.29±0.02µg/ml, IC_{50} of *Eryobotraya japonica* and *Citrus sinensis* was 0.39±0.01 & 168±1.5 in µg/ml, respectively. Thus, the lowest IC50 calculated was of *Eryobotraya japonica* and the negative control (DMSO) was devoid of any antileishmanial activity. In brine shrimp hatching assay extent of inhibition was observed against different concentrations of a plant extract dilutions. Brine shrimp cytotoxicity assay is a low cost and an easy bioassay. Usually, carried out to figure out the lethal activity of a plant at different concentrations and this biological effect can be the consequence of a single constituent or multiple compounds comprising a plant [16]. IC_{50} values for cytotoxicity were found to be 1209±2.0 for *Celtis anstracis* and in case of *Phytanthar embalics*, *Cassia gloca*, *Eryobotraya japonica*, *Citrus sinensis* was 0.00, 1030±4.5, 1903±3.5, 1755±1.5 µg/ml, respectively. The lowest cytotoxicity was shown by *Phytanthar embalics* while the highest was exhibited by *Eryobotraya japonica*. At the lowest IC_{50} all brine shrimps were observed alive, revealing that *Phytanthar embalics* showed no toxicity against brine shrimps at 2500 µg/ml concentration. While, discussing other plants we can say that they also have negligible cytotoxic effect on brine shrimps.

Flavonoids quantity was determined in mg Rutin equivalent/g dry extract. *Celitis anstracis* Flavonoids quantity was determined to be 125 mg Rutin equivalent/g dry extract. *Phytanthar embalics*, *Cassia gloca*, *Eryobotraya japonica* and *Citrus sinensis* expressed flavonoids content as 81, 214, 170 and 281 mg Rutin equivalent/g dry extract, respectively. Here, we have correlated the Flavonoids and anti-leishmania activity as Flavonoids are the antioxidants playing their part against diseases. Here, we calculated the co-relation between the Flavonoids and anti-leishmania activity. It was ascertained that both show significant relationship. Anti-leishmania activity and flavonoids of *Celitis anstracis*, *Phytanthar embalics*, *Cassia gloca* and *Eryobotraya japonica* showed significant relationship giving 0.87 R^2 value. On the other hand, *Citrus sinensis* did not showed any significant relationship with the above mentioned plants. Characterizing, high flavonoid contents of these plants can be responsible for anti-leishmania activity. While in case of *Citrus sinensis* compounds other than flavonoids may be involved, on which further studies have to be accomplished.

Table 1: Anti-leishmania activity and cytotoxcity assay.

Plant	Anti-leishmania IC_{50} (µg/ml)	Cytotoxicity IC50 (µg/ml)
Celtis anstracis	69.13 ± 0.01	1209 ± 2.0
Phytanthar emblics	152.1 ± 0.03	0.00
Cassia gloca	9.62 ± 0.02	1030 ± 4.5
Eryobotraya japonica	10.59 ± 0.01	1903 ± 3.5
Citrus sinensis	12.27 ± 0.01	1755 ± 1.5
DMSO	-	-
- Means has no activity against parasite growth and brine shrimp eggs		

Table 2: DPPH and Flavonoids assay.

Plant	DPPH IC_{50} (µg/ml)	Flavonoids (mg Rutin Equivalent/g dry extract)
Celtis anstracis	1.02 ± 0.02	125
Phytanthar emblics	7.06 ± 0.01	81
Cassia gloca	34.29 ± 0.02	214
Eryobotraya japonica	0.39 ± 0.01	170
Citrus sinensis	168 ± 1.5	281

Conclusion

The results depict that the plants described above relatively have potent compounds involving in anti-leishmania activity when compared with amphotericin B and the present study may provide a proof for the potential use of plants described above for as anti-leishmania activity. Therefore, further studies are required for the isolation of these effective compounds and there *in vivo* implementation.

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