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Comprehensive investigations on anti-leishmanial potentials of *Euphorbia wallichii* root extract and its effects on membrane permeability and apoptosis

Bilal Ahmad[a], Arshad Islam[b,1], Arif Khan[a], Mubarak Ali Khan[a], Ihsan ul Haq[d], Laila Jafri[e], Mansoor Ahmad[a], Shaila Mehwish[a], Ajmal Khan[c], Nazif Ullah[a,*]

[a] Department of Biotechnology, faculty of Chemical and Life Sciences, Abdul Wali Khan University Mardan, Mardan, Pakistan
[b] Postgraduate Program in Physiology and Pharmacology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil
[c] Department of Biotechnology, Bacha Khan University, Charsada, Pakistan
[d] Department of Pharmacy, faculty of Biologica Sciences, Quaid-I-Azam University, Islamabad, Pakistan
[e] Department of Biochemistry, faculty of Biologica Sciences, Quaid-I-Azam University, Islamabad, Pakistan

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ABSTRACT

Clinically available synthetic chemotherapeutics to treat the vector-borne protozoan infection, leishmaniasis, are associated with serious complications such as toxicity and emergence of resistance. Natural products from plants consist of interesting biomolecules that may interfere with DNA or membrane integrity of the parasite and can possibly minimise the associated side effects. In the present study, various fractions of *Euphorbia wallichii* (EW) root extracts including n-hexane (EWNX), ethyl acetate (EWEA), chloroform (EWCH) and aqueous (EWAQ), were evaluated for their antileishmanial potential against *Leishmania tropica* followed by investigation of the possible mechanism of action via reactive oxygen species (ROS) quantification, membrane permeability (via sytox green dye) and apoptotic assay (via AO/EB method) using fluorescent microscopy. Two of the fractions i.e. EWEA and EWAQ inhibited the growth of promastigotes (IC_{50} 7.8 and 10.2 µg/mL, respectively) and amastigotes (IC_{50} 9.9 and 13.3 µg/mL, respectively) forms almost at similar concentrations as found for the standard antileishmanial drugs, tartar emetic (TA) and Glucantime (IC_{50} 9.4 and 21.5 µg/mL, respectively). Both the active fractions remained non-toxic towards human blood erythrocytes and were able to cause membrane permeability and apoptotic induction (using Triton X-100 as a positive control) leading to death of *Leishmania* parasites. However, both the fractions could not triger significant and persistent ROS generation, compared to hydrogen peroxide used as a positive control. Antilesihmanial activity of the two active fractions might be attributed to the presence of high quantity of tannins and saponins.

1. Introduction

Leishmaniasis is an infectious disease caused by more than 20 species of the parasites of the genus *Leishmania* and it is one of the most significant neglected tropical diseases (NTDs), with alarming hazards to human health worldwide like any other serious ailment [1,2]. The parasite is transmitted to the human host by the bite of sand-flies of the genera, *Phlebotomine* and *Leutzomia*. During infection, the parasite is phagocytized by the macrophages, where they subvert some important functions of macrophages by distorting some of their important signalling pathways and resides and divides there [3,4]. An escalating upsurge in the occurrence of leishmaniasis has been observed in the recent years around the globe, both in the developing as well underdevelping countries, especially in the poverty related areas including Pakistan, India, Bangladesh, Iran, Egypt, Brazil and some other North American countries. There are three major clinical manifestations of the disease including, cutaneous, mucocutaneous and visceral leishamiasis [5]. The currently available treatments for leishmaniasis include the pentavalent antimonials, stibogluconate and meglumine antimoniate (the first line antileismanial drug), liposomal amphotericin B (AmBisome®), meltifosine, pentamidine and paromomycin. However, these drugs are associated with many demerits like lake of availability due to high cost in the poverty-related areas, problems in oral administration (e.g., amphotericin B cannot be used orally), severe pain at injection's site, gastrointestinal troubles and nontheless, the cardiac and renal toxicities [6,7]. Furthermore, in some endemic areas the parasites

* Corresponding author at: Department of Biotechnolgoy, Faculty of Chemical and Life Sciences, Abdul Wali Khan University Mardan, Pakistan.
E-mail address: ullahnazif@awkum.edu.pk (N. Ullah).
[1] Present address: Sulaiman Bin Abdullah Aba Al Khail Centre for Interdisciplinary Research in Basic Sciences, International Islamic University, Islamabad 44000, Pakistan.

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have developed resistance towards the clinically avaible drugs due to the over-prescription and high-dose long term use of these drugs [7]. The mechanism behind resistance towards the clinically available drugs is often associated with rapid drug metabolism, lower drug uptake and over-expression of drug transporters [8,9]. Therefore, the current high prevalence and emergence of resistance presents a challeging task to find alternative means to develop new antileishmanial drugs which should be of low cost, highly effective, easy to access and more human friendly.

The advancements in natural products research are emerging to screen for the potent natural products, having health promoting attributes against many ailments [10]. Recently, a variety of plants extracts have been tested to expolre the potential drug candidates against leishmaniasis, however, none of these could be processed further to develop any antileishmanial drug [10]. Besides the importance of research studies on the evaluatiion of antileishmanial potentials, the mechanism of action of the potent natural products should also be elucidated through *invivo* testing and clinical trials [10].

Euphorbia wallichii Hook. f. of family *Euphorbiaceae,* an important medicinal plant, is endemic in Pakistan and has shown higher presence of biologically active phytochemicals including flavonoids, terpenoids, cardiac glucosides, tannins and saponins [11]. Plant secondary metabolites are ubiqutiously found in different parts of medicinal and aromatic plants [12]. Howevere, in many plants, the roots are reported for production of medicinally potent secondary metabolites in bulk. Roots further act as important repository of some rare natural products, which are not produced in any other part of the plant [13]. In our previous study [11], we have investigated the phytochemical contents in different parts of the *E. wallichii*, in which roots were found to accumulate substantial amount of important metabolites, including saponins and tanins those having reportedly higher potentail in apoptosis and memberane permeability. Therefore, in the current study, four different fractions of *E. wallichii* root extracts were selected for the evaluation of antileishmanial activity followed by investigation of their mechanism of action. For this purpose, different biological assays were performed including apoptotic analysis, membrane permeability, cytotoxicity analysis and reactive oxygen species quantification.

2. Methodology

2.1. Chemicals and reagents

Potassium antimonyl tartrate, 2′,7′-dichlorofluorescein diacetate (DCFDA), sytox green, potassium chloride, sodium bicarbonate, n-hexane, ethyl acetate, chloroform, dimethyl sulfoxide (DMSO), acridine orange (AO), Hank's balanced salt solution (HBSS), hydrogen peroxide (H_2O_2), RPMI-1640 culture medium, heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin solution and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, Mo. USA). Commercial Glucantime® and amphotericin B® were kindly provided by Adnan Traders® for research purpose only. All the solvents and chemicals were of reagent grade. Double-distilled deionized water was used throughout the experiments. Phosphate buffer saline (PBS) was prepared in double-distilled deionized water.

2.2. Extract preparation and fractionation

The four different fractions used in this study were prepared as previously reported by Ul-Haq et al. [11]. Briefly, the fresh roots of the *E. wallichii* were washed with tape water, cut into small pieces and shade-dried. The dried roots were then ground and extracted with methanol. The methanolic extract was then subjected to solvent-solvent extraction to obtain the required fractions namely ethyl acetate fraction (EWEA), chloroform fraction (EWCH), n-hexane fraction (EWHX) and aqueous fraction (EWAQ).

2.3. Biological activities

2.3.1. Parasite culture

Leishmania tropica (KWH23) isolates obtained from the Department of Biological Sciences, International Islamic University, Islamabad, Pakistan, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin solution at 25 ± 1 °C (in 25 cm^2 flasks – TPP® Sigma-Aldrich). After 4 days of incubation, the parasite culture was monitored using inverted microscope (Olympus®) and passaged for further growth.

2.3.2. Promastigote proliferation measurements by MTT assay

The promastigote forms of *L. tropica* (1×10^4 cells/well) were seeded in 96-well microtiter plates (Sigma-Aldrich) in RPMI-1640 (Gibco®) suplimented wit 10% FBS plus 1% penicillin and streptomycin solution and allowed to grow either in the presence of various concentrations (1, 10, 20, 50 and 100 µg/mL) of each fraction (EWEA and EWCH, EWHX, EWAQ) of *E. wallichii* root extract, or in the absence 0.1% DMSO (negative control) or potassium antimonyl(III) tartrate and amphotericin B (positive controls) for 24, 48 and 72 h at 24 ± 1 °C. The antileishmanial activity was evaluated using an MTT (3-(4,5- dimethylthiazol-2 yl)-2,5-diphenyltetrazolium bromide)-based microassay as a marker of cell viability [14]. After the incubation period, a 100 µL of MTT solution (5 mg/mL in PBS - Sigma Chemical Co., St. Louis, Mo.) was added to each well and incubated for 4 h at 37 °C. The enzymatic reaction was then stopped by the addition of 100 µL DMSO. Relative optical density (OD) was measured at 570 nm using a multi-well microtiter plate reader (Bio-Tek ELx-800). The background absorbance of multi-well plates was measured at 690 nm and subtracted from 570 nm measurements. The absorbance produced by the action of mitochondrial dehydrogenases of metabolically active cells was shown to correlate with the number of viable cells. The assay was performed in triplicate.

2.3.3. Anti-amastigote assay

Promastigote form of *L. tropica* were transformed into amastigote form by centrifuging the promastigotes culture and re-suspending the pellets in medium-199 (Sigma-Aldrich) containing Hank's balanced salts, supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/mL penicillin, 50 µg/mL streptomycin and the pH was adjusted to 5.5 using 1.0 N HCl. The cells were then incubated at 31 °C with 5% CO_2 in a humidified environment for 7-21 days.

A hundred microliter of *L. tropica* amastigotes from a culture density of 1×10^5 parasites/mL were added to each well of the 96-well microtiter plate and incubated with various concentrations (1, 10, 20, 50 and 100 µg/mL) of each fraction (EWEA and EWCH, EWHX, EWAQ) of *E. wallichii* root extract, 0.1% DMSO (negative control) or Glucantime and amphotericin B (positive controls) for 24, 48 and 72 h at 31 °C. After the incubation period, 20 µL of MTT solution was added to each well and incubated for 4 h at 37 °C. After incubation time, a 100 µL of solubilization solution (acidified isopropanol; 0.04 M HCl in absolute isopropanol) was added to solubilize the dye and the solubilized MTT formazan product was spectrophotometrically measured at 570 nm on microtiter plate reader (Bio-Tek ELx-800).

2.3.4. Cytotoxicity assays (Haemolytic activity)

Venous blood samples from healthy human volunteers with no current history of infectious diseases were collected by a trained health professional after obtaining informed written consent according to the Helsinki Declaration of 1975 (revised 1997). This study involving human subjects was approved from Bioethics Committee of Department of Biotechnology, Abdul Wali Khan University, Mardan, Pakistan.

The hemolytic activity was analyzed by incubating the different concentrations (1–100 µg/mL) of each root fraction of *E. wallichii* (EWEA and EWCH, EWHX, EWAQ) or TA with 5% human blood erythrocytes suspension (1 mL) at 37 °C for 3 h. The absence of hemolysis

Table 1
This table is representing the 50% inhibitory concentrations of each fraction of *E. wallichii* root extracts in *L. tropica* promastigotes and amastigotes at different incubation time compared to control.

	24 h	48 h	72 h
Fraction/drug	**[a]IC_{50} (μg/mL) [[b]CI 95%] in *L. tropica* promastigotes**		
EWNX	162.8 [134.7 – 196.8]	146.1 [118.7-179-8]	128.0 [101.4-161.6]
EWCH	140.7 [119.0 -166.3]	126.1 [102.2-155.6]	114.8 [92.0-143.1]
EWEA	10.26 [8.5-12.3]	9.0 [7.1-11.5]	7.8 [6.5-9.4]
EWAQ	12.28 [10.7-14.0]	11.21 [9.7-12.8]	10.2 [7.9-13.2]
TA	9.4 [8.2-10.8]	5.2 [3.2-8.2]	3.7 [2.4-5.8]
	IC_{50} (μg/mL) [CI 95%] in *L. tropica* amastigotes		
EWNX	175.4 [141.1 to 218.1]	167.1 [127.9 to 218.4]	140.2 [108.5 to 181.1]
EWCH	188.3 [144.7 to 245.1]	145.2 [121.4 to 173.7]	129.3 [102.7 to 163.0]
EWEA	13.34 [10.72 to 16.61	12.19 [10.06 to 14.78]	9.965 [7.910 to 12.56]
EWAQ	19.63 [16.52 to 23.31]	17.02 [14.44 to 20.06]	13.35 [10.25 to 17.39]
Glucantime®	21.56 [18.23 to 25.51]	–	–

[a]IC_{50}: 50% growth inhibitory concentrations as compared to non-treated control;
Values were calculated after 24, 48 and 72 h of treatment with each fraction of *E. wallichii* and are representative of three independent experiments;
[b]CI: confidence interval.
TA: tartar emetic.
- Not applicable.

or 100% hemolysis was determined by incubating the erythrocytes with an equal volume of 0.1% DMSO (blank negative control) or 0.5% Triton X-100 (positive control), respectively. The erythrocyte suspensions were centrifuged at 1000 ×g for 10 min and the cell lysis (haemoglobin release) was determined spectrophotometrically (576 nm) on spectrophotometer (Agilent-DAD, 8453, Agilent Tech., Germany) [15]. The results were determined by evaluating the percentage of haemolysis compared to the negative and positive controls using the following formula;

Haemolysis (%) = [(O.D. 576 nm in the sample solution – O.D. 576 nm in DMSO)/ (O.D. 576 nm in 0.5% Triton X-100 – O.D. 576 nm in DMSO)] × 100

2.3.5. Membrane permeability assay

For membrane permeability evaluation, the promastigotes form of *L. tropica* were treated with 100, 500 and 1000 μg/mL of each fraction of *E. wallichii* root extract for 24 h and washed with HBSS by using the method as previously described [16]. The samples were treated with RNase I (1 mg/mL), stained with 1 μM of Sytox green dye and incubated for 15 min. The treated cells were then mounted on a slide and the upsurge in fluorescence was checked by Leica fluorescent microscope with a Canon camera, using 485 and 530 nm filters for emission and excitation wavelength, respectively. Triton X-100 was taken as positive control for full permeability. The images were recorded and processed using ImageJ software (version ij150).

2.3.6. Apoptosis assay

Apoptosis was analyzed according to the ethidium bromide and acridine orange (EB/AO) staining assay as previously described [15], with slight modifications. *L. tropica* promastigotes were incubated with different fractions of *E. wallichii* root extract at a final concentration of 1000 μg/mL for 24 h. The cells were washed with phosphate buffer saline (PBS) by centrifugation (1000 × g, 5 min) and treated with RNase I (1 mg/mL) before staining it with ethidium bromide (EB) (100 μg/mL) mixed with acridine orange (AO) (100 μg/mL) in a 3:1 concentration. The variance in fluorescence was measured on a Leica fluorescent microscope with a Canon camera using 530 and 485 nm filters for emission and excitation wavelengths, respectively [15]. Triton X-100 (0.5%) was taken as positive control.

2.3.7. Reactive oxygen species quantification

Intracellular reactive oxygen species (ROS) [17] generation induced by *E. wallichii* root extract and H_2O_2 were measured using 2′,7′-dichlorodihydrofluorescein-diacetate (DCFDA– a cell permeable non-fluorescent probe) to stain the cell. Briefly, *L. tropica* promastigotes (1×10^7 cells/mL) were washed with PBS by centrifugation. The cells were then resuspended in PBS and incubated with DCFDA (100 μM) at 37 °C for 30 min to get the cells stained with DCFDA. The cells were then washed with and resuspended in 1X PBS and 100,000 stained cells/50 μL/well were seeded in a 96-well dark walled plate. Non-stained cells were used as blank. 50 μL of each fraction of *E. wallichii* root extract was added to each well such that the final concentration reached 1000 μg/mL and incubated for 10, 20, 30, 40, 50 and 60 min. A 20 μM H_2O_2 was used as positive control for ROS generation. Fluorescence intensity was subsequently measured using a fluorescence microplate reader.

3. Results

3.1. Time and dose-dependent antileishmanial activity

The fractions of the *E. wallichii* root extract (EWNX, EWCH, EWEA and EWAQ) were screened for their antileishmanial activity against *L. tropica* promastigote and amastigote forms. The parasite growth inhibition rates in the presence of growing concentration of each fraction and increasing incubation time (24, 48 and 72 h) were compared with those treated with 0.1% DMSO (negative control), TA (positive control in case of promastigote) and the commercial Glucantime® (positve controle in case of amastigotes). As shown in Table 1 and Fig. 1 A, B and C. Only the two fractions, EWEA and EWAQ, were found highly active against *L. tropica* promastigotes at fairly low concentrations with IC_{50} values of 9.02 μg/mL and 12.67 μg/mL after 24 h of treatment, respectively. Interestingly, a slight increase in their activity was also observed with increase in the time of incubation (IC_{50} 7.38 μg/mL and 12.18 μg/mL after 48 h while 6.6 μg/mL and 11.3 μg/mL after 72 h, respectively).

The growth of the axenic amastigote form of *L. tropica* was also inhibited by its incubation for different time periods (24, 48 and 72 h), wherin the two fractions EWEA and EWAQ were observed in similar fashion as in the case of promastigote form. As shown in Table 1 and Fig. 2(A, B & C), after 24 h of incubation with amastigote, the two fractions, EWEA and EWAQ, exhibited anti-amastigote activity with IC_{50} values as low as 13.34 and 19.62 μg/mL, respectively, which is almost similar at which they inhibited the growth of 50% of the promastigote form, when compared with the non-treated control.

In both cases, the activity of EWEA and EWAQ were comparable to the positive controls, TA (IC_{50} 9.4 μg/mL) and Glucantime®, (21.56 μg/

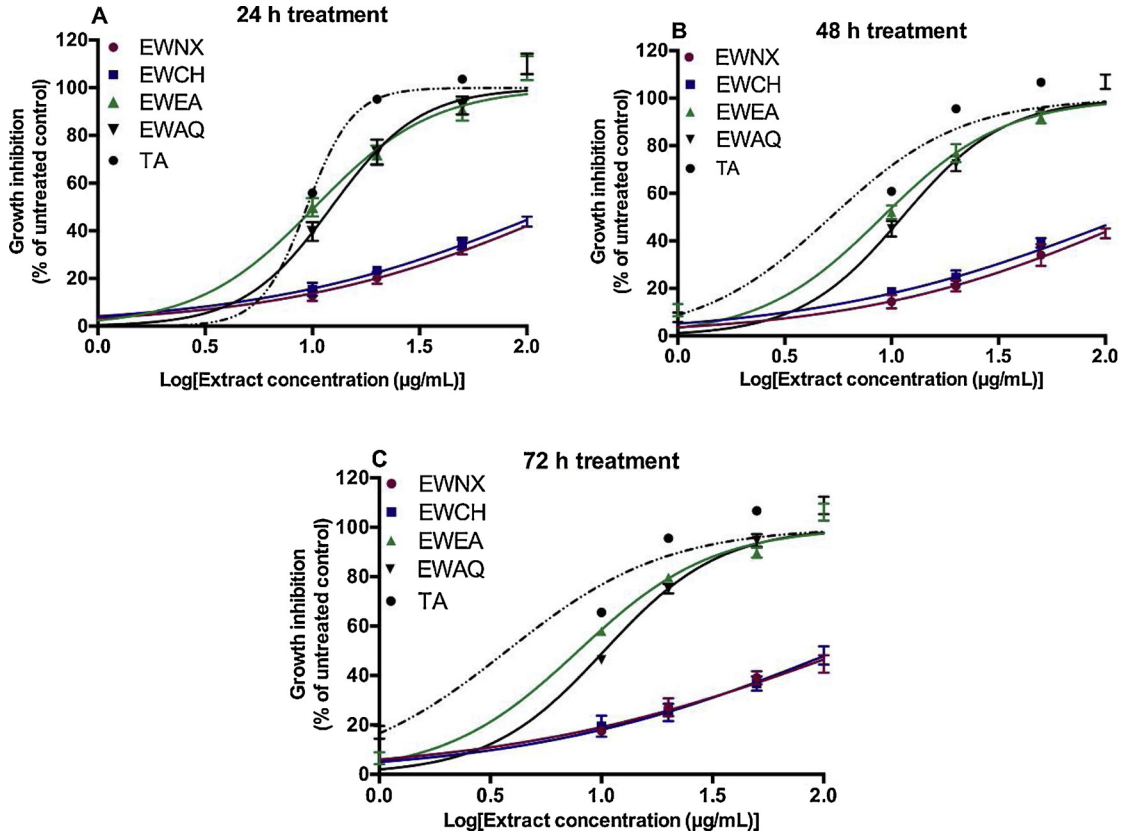

Fig. 1. *In vitro* growth inhibition curves of promastigote forms of *L. tropica* incubated at 24 ± 1 °C in the presence of various concentrations (1–100 µg/mL) of each fraction of *E. wallichii* root extract (EWNX, EWCH, EWEA and EWAQ), tartar emetic (TA; positive control) and 0.1% DMSO (negative control) for 24 h (**A**), 48 h (**B**) and 72 h (**C**). The growth inhibition of *L. tropica* promastigotes was assessed on Elisa plate reader as optical density as the result of tetrazolium salt (MTT) reduction into formazan crystals by the living promastigotes.

mL) after 24 h of incubation whereas the two fractions, EWNX and EWCH, were least active at concentration as high as 100 µg/mL compared to EWEA and EWAQ.

3.2. Cytotoxicity assay (haemolytic activity)

The percent haemolytic activity of the various fractions of root extracts to human blood erythrocytes showed that all the fractions tested were weakly haemolytic (haemolysis < 10% as compared to the positive control) at 50 and 100 µg/mL (Figs. 3 and 4). However, the percent haemolysis activity increased with an increase in the concentration of extract (> 100 µg/mL). Furthermore, EWNX resulted in the highest haemolytic activity (haemolysis 71.2%), while EWCH showed the lowest activity (haemolysis < 10%) at 500 µg/mL. Moreover, incubation of erythrocytes with EWNX and EWEA at 1000 µg/mL resulted in a higher haemolytic activities i.e 106% and 127%, respectively, when compared to that of 0.5% Triton X-100 (positive control).

3.3. Membrane permeability assay

After incubation with different concentrations (100, 500 and 1000 µg/mL) of each fraction of *E. wallichii* root extract, *L. tropica* promastigotes were stained with Sytox green (1 µM) for 15 min. The cells emitting the green fluorescence were visualized under the fluorescence microscope. Out of the four fractions, only two fractions, EWEA and EWAQ, showed membrane permeability as confirmed by emission of the green fluorescence (Figs. 5 and 6 respectively). While the remaining two fractions (EWCH and EWNX) did not show any fluorescence. The results were expressed relative to the fluorescence emitted by cells treated with triton X-100 which was taken as a control [16].

3.4. Apoptosis assay

Using AO/EB and fluorescent microscopy method, *L. tropica* cells treated with different fractions (EWNX, EWCH, EWEA and EWAQ) showed that among all the four fractions, only EWEA and EWAQ were capable to induce apoptotic activity at 1000 µg/mL after 24 h (Figs. 7 and 8). While the other two fractions, EWNX and EWCH, did not show any apoptotic activity. According to the results, EWEA was able to cause early apoptosis, characterized by chromatin condensation [17] and late apoptosis (LA), characterized by formation of orange to red nuclei containing extremely condensed or fragmented chromatin as well as apoptotic bodies as indicated in Fig. 7. The results were analyzed and compared with the method described by [18]. The results for EWAQ of *E. wallichii* root extract also showed apoptotic potential (Fig. 8). From the image late apoptosis (LA), early apoptosis (EA) and apoptotic bodies (AB) are clearly illustrated. AB are characterized by fragments of cells or dispersed bodies that gives bright green color but are smaller than rest of the cells.

3.5. Reactive oxygen species generation assay

Reactive oxygen species [17] generation in *L. tropica* promastigotes was determined by spectrofluorometric probe known as 2′,7′ –dichlorofluorescein diacetate (DCFDA). In principle DCFDA is first converted to DCFH by intracellular esterase and then to highly fluorescent form DCF by ROS. Therefore, intracellular ROS generation can be monitored directly by measuring the intensity of DCF [19]. The results showed the generation of low quantity of ROS even at higher concentration (1000 µg/mL) treatment at various intervals (1 min–60 min) of incubation as compared with H_2O_2 (control). All the fractions

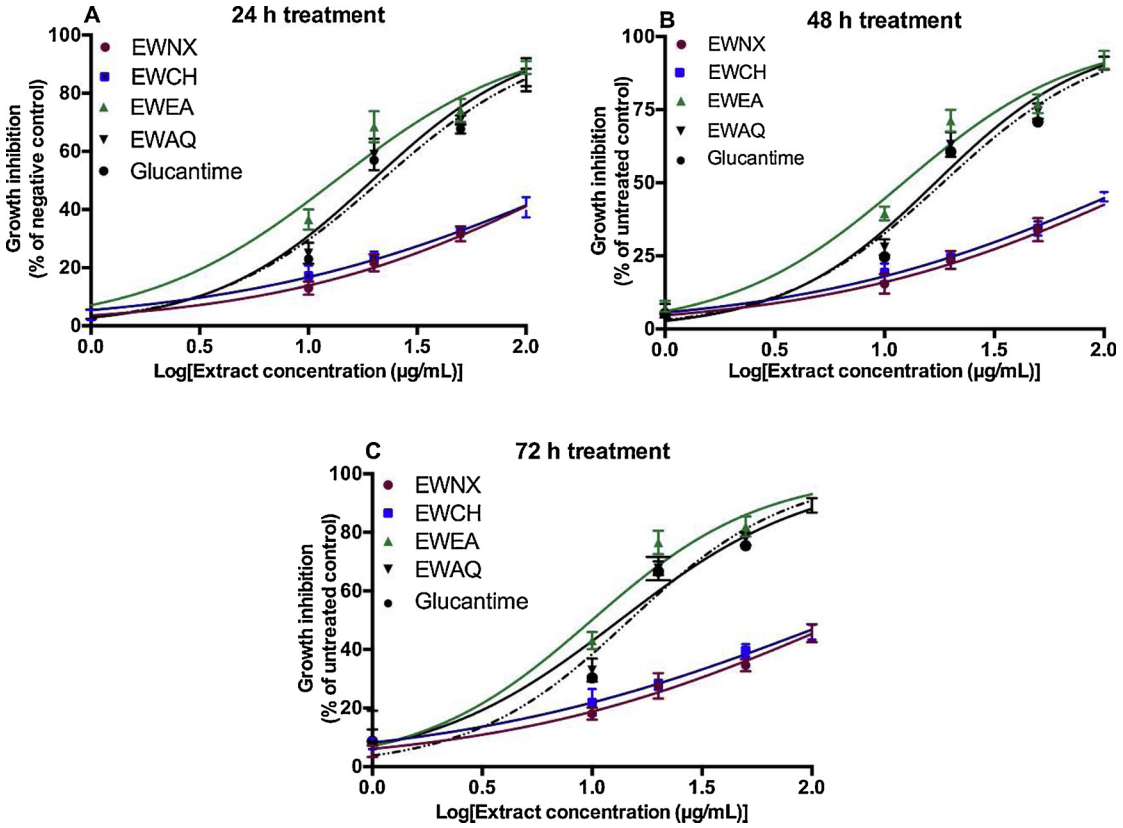

Fig. 2. *In vitro* growth inhibition curves of amastigote forms of *L. tropica* incubated in the presence of various concentrations (1–100 µg/mL) of each fraction of *E. wallichii* root extract, Glucantime (positive control) and 0.1% DMSO (negative control) for 24 h (**A**), 48 h (**B**) and 72 h (**C**) at 31 °C. The growth inhibition of *L. tropica* amastigotes was assessed on Elisa plate reader as optical density as the result of tetrazolium salt (MTT) reduction into formazan crystals by the living amastigotes.

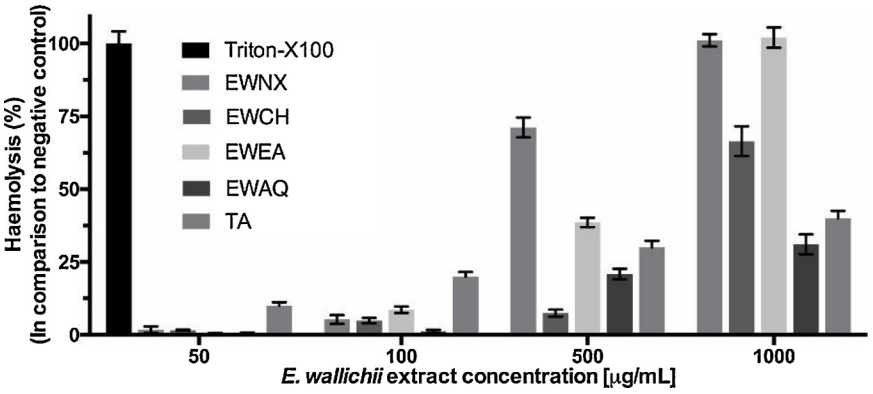

Fig. 3. Percent haemolytic activities of different fractions (EWNX, EWCH, EWEA and EWAQ) of *E. wallichii* root extracts, tartar emetic (TA) and Triton –X100 (positive control) at different concentrations over a suspension of human red blood cells (RBCs or erythrocytes), after 3 h of incubation at 37 °C, in comparison to the negative control (0.5% DMSO/water solution).

showed relatively low fluorescent intensity when compared with H_2O_2 except EWCH, which initially caused generation of higher quantity of ROS at early exposure (1–5 min), which suddenly droped to the lowest values after 5–10 min (Fig. 9).

4. Discussion

The major bottleneck in the available remedies against *Leishmania* are the absence of functional vaccine, and the several side effects associated with the clinically available antileishmanial drugs, it becomes crucial to search and explore for novel, effective but innocous antileishmanial drugs, which are highly demanding and challenging. As a rich source of bioactive compounds, medicinal plants, extracts or their products can play a pivotal role in providing a sustainable source for new antileishmanial drugs and thus a promising strategy for the elimination of this serious disease [20,21]. Natural products in the form of plants extracts and isolated bioactive compounds have been reported for their anti-leishmanial potentials and the research programs have focused with a higher magnitude in the field of isolation of natural products for treatment of many diseases in the recent years worldwide. However, the world is still far away from having a natural product as an antileishmanial drug. This may be due to the lack of interest of the deifferent research groups in indebt mechanistic studies followed by *in vivo* testing and clinical trials [10]. *E. wallichii* is a medicnially important plant endemic to Pakistan for which the antileishmnail potentials have not been reported yet. This plant, frequently available in Pakistan, has been reported for its high pharmacological properties due to the presence of biologically active phytochemicals like flavonoids, saponins, tannins, terpenoids and cardia glycoside [11]. Whereas, tannins, saponins, terpenoids and flavonoids are well known for their antileishmanial activities [2,22–24]. However, no report on the antileishmanial potentials of *E. wallichi* is available in literature.

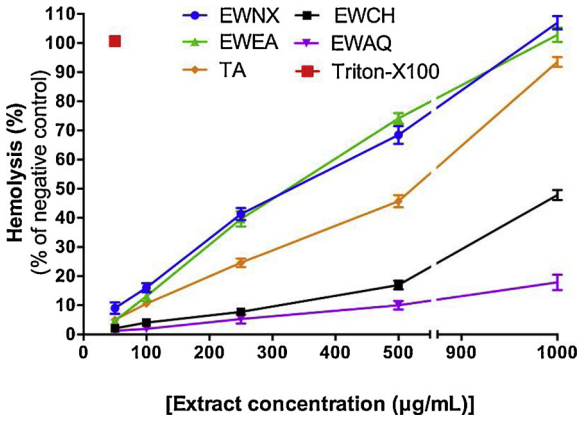

Fig. 4. Haemolytic activity of different fractions of *E. wallichii* root extract, tartar emetic and Triton –X100 (positive control) at different concentrations over a suspension of human red blood cells (RBCs or erythrocytes), after 3 h of incubation at 37 °C. Differences were considered statistically significant with respect to the haemolyitic activity of 0.5% DMSO/water solution.

In the present study, the antileishmanial potential of various fractions (n-hexane (EWNX), ethyl acetate (EWEA), chloroform (EWCH) and aqueous (EWAQ)) of *E. wallichii* root extracts has been investigated against the promastigote and amastigote forms of *L. tropica* followed by investigation of their possible mechanism of action via reactive oxygen species [17] quantification, membrane permeability (via sytox green dye) and apoptotic assay (via AO/EB method) using fluorescent microscopy.

When compared with the positive control (tartar emetic and Glucantime), out of the four fractions, only EWEA and EWAQ were found to be highly active against both forms of *Leishmania* parasite (IC_{50}; 7.8 and 10.2 µg/mL against promastigotes while 9.9 and 13.3 µg/mL against amastigotes, respectively). The high antileishmanial activity of these fractions against *L. tropica* promastigotes and amastigotes can be correlated with the presence of higher quantity of tannins and saponins. Our previous findings have shown that fractions like EWEA and EWAQ contain higher amounts of tannins and saponins [11]. Wherease, tanins have been reported to disrupt the surface membrane in parasite like *Trypanosoma* and *Leishmania,* leading to the loss of cytoplasmic content and finally the death of the cell [25,26]. Saponins may also cause the lysis of the parasite by increasing permeability and thus result in the death of the parasite [17]. Our data showed that EWEA and EWAQ resulted in a time and dose dependent antileishmanial activity, which increased with increase in time of incubation and concentration of the extraccts as shown (Figs. 1 & 2 and Table 1).

Since fractions of *E. Wallichii* root extracts are known to carry higher quantity of tannins and saponins [11] and both these chemicals can cause membrane permeability [17,25,26]. We checked these fractions for the possible effect on membrane permeability of *L. tropica* using vital Sytox green, a fluorescent dye that penetrates the cell membrane only upon permeability and which can attaches/intercalates to the nucleic acid inside the cell resulting in the emission of the green fluorescence [27]. Our results showed that the two fractions i.e EWEA and EWAQ can potentially cause membrane permeability as confirmed by emission of the green fluorescence (Figs. 5 and 6 respectively). The presence of high quantity of saponins might be the key factor in the EWAQ, which can cause permeability of membrane through its high affinity towards the cholesterol present in cell membrane resulting in an insoluble complex. The lesion formed by saponins are micelle like insertion of saponins and cholesterol in membrane plane made the membrane permeable. While tannins in EWEA may be responsible for the disruption of surface membrane causing the loss of cytoplasmic content and eventually leading the death of parasite [25].

Notwithstanding the fractions were also found highly active against both the promastigote and amastigote forms of *Leishmania.* None of these extract was toxic to human blood erythrocyte at concentrations at which they were able to inhibit the growth of *Leishmania* parasites by half (IC_{50} range 10–188 µg/mL) as compared to the negative control (Fig. 4). Sanganuwan et al., (2015) has reported the ethyl acetate fraction of *Abrus precatorius* leaf to be least cytotoxic giving IC_{50} value over 100 µg/mL [28]. However, the slight increase in hemolytic activity of EWAQ and EWEA at concentration > 500 µg/mL may need further investigation as human erythrocytes are known to have more complex mechanism of defense compared with Leishmanial cell. The lower cytotoxicity values and high selectivity indice (SI) values of EWEA and EWAQ (Table 2) further increment their significace in the development of antileishmanial drug.

Induction of apoptosis can be characterized by membrane permeability and fragmentation of DNA. It is an established phenomenon that the induction of apoptosis is related to the activation of caspases [29]. When caspases initiate their activation, the cellular proteins start degradation, shrinkage of cell as well as condensation of chromatin occurs followed by splitting of DNA into fragments and membrane belbbing [29]. Flourescent mircropscpy analysis showed that EWEA and EWAQ were able to cause early apoptosis characterized by chromatin condensation [17], late apoptosis (LA) characterized by orange to red nuclei containing extremely condensed or fragmented chromatin as well as apoptotic bodies as indicated, compared and analyzed with the protocol of [18]. The permeability of membrane and the presence of apoptotic bodies in *L. tropica* cells treated with various fractions of *E. wallichii* root extracts, might indicate the possible mode of action as a function of apoptosis.

Reactive oxygen species production is inversely proportional to viability of *Leishmania* i.e.., the higher the ROS production, the lower will be the cell viability [15]. In this investigation, it was observed that small amount of non-persistent ROS were produced with early exposure of cells to different fractions of *E. wallichi*, however, they disappeared with the increase in time interval. Comperatively higher amount of ROS was observed for EWCH (a least active fraction against *Leishmania*),

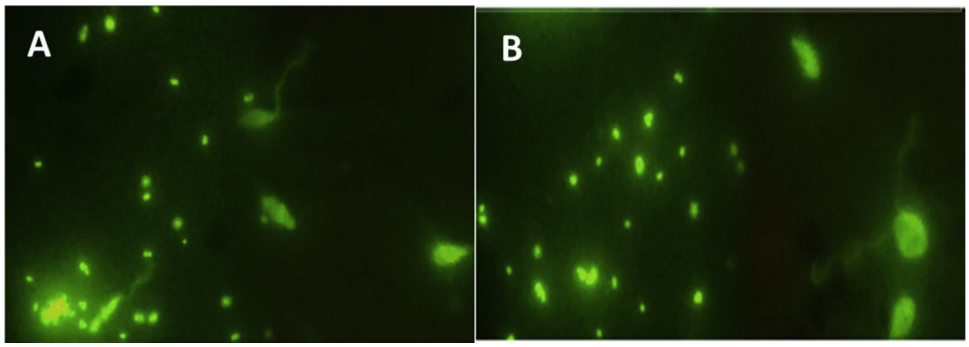

Fig. 5. Confirmation of membrane permeability caused by ethyl acetate fraction (EWEA) of *E. wallichii* root extract after visualizing the treated *L. tropica* cells stained with Sytox green after 24 h. A) 1000 µg/mL and B) 500 µg/mL.

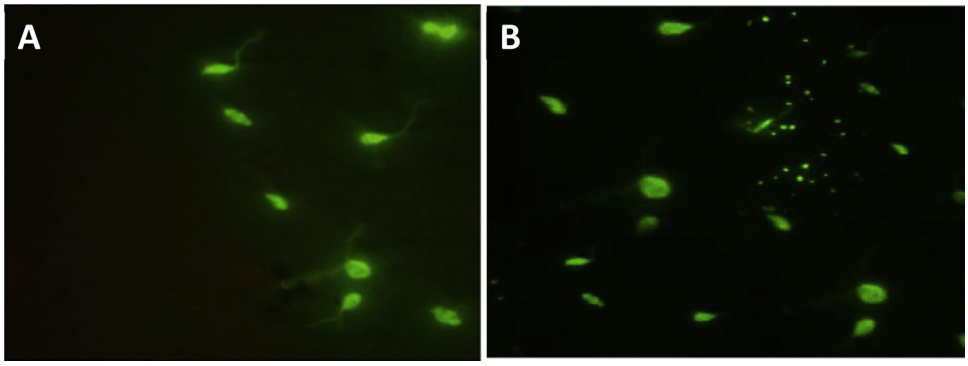

Fig. 6. Confirmation of membrane permeability caused by aqueous fraction (EWAQ) of *E. wallichii* root extract after visualizing the treated *L. tropica* cells stained with Sytox green after 24 h. A) 1000 µg/mL and B) 500 µg/mL.

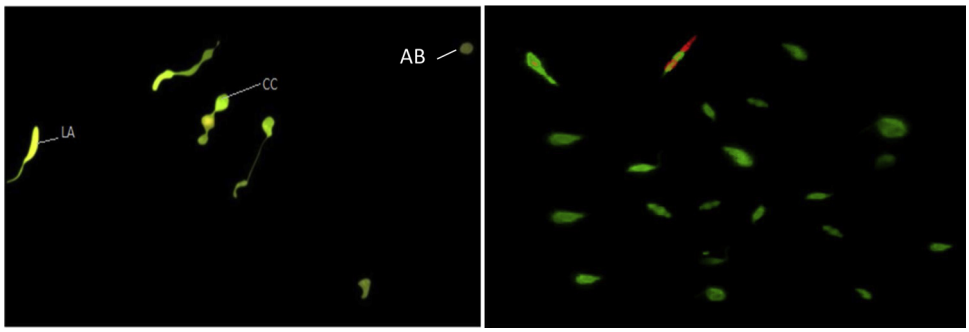

Fig. 7. Changes in morphology of *L. tropica* cells caused by its treatment with EWEA (1000 µg/mL) of *E. wallichii* root extract using AO/EB staining. The images were taken by fluorescence microscopy where LA-late apoptosis, CC- chromatin condensation and AB- apoptotic bodies.

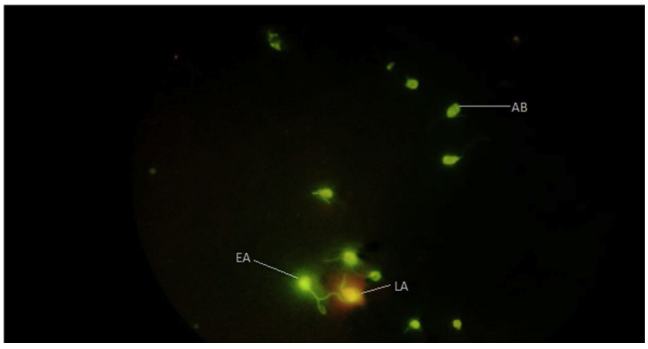

Fig. 8. Changes in morphology of *L. tropica* cells caused by its treatment with EWAQ (1000 µg/mL) of E. Wallichii root extract using AO/EB staining. The images were taken by fluorescence microscopy where LA-late apoptosis, CC-chromatin condensation and AB- apoptotic bodies.

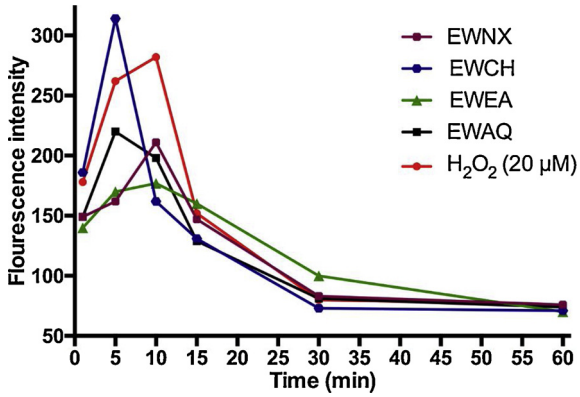

Fig. 9. Quantification of the reactive oxygen species generated by fractions of *E.wallichii* root extract at 1000 µg/mL after incubation with *L. tropica* promastigotes for 1–60 min at room temperature and in dark plate.

while the two active frations EWEA and EWAQ did not show any significant amount of ROS production. Reactive oxygen species [17] are capable of causing alteration in macromolecules, such as proteins, lipids, and nucleic acids, and can create a condition termed as oxidative stress and is concerned with a lot of physiological and pathological processes. These, oxygen derived free radicals are highly unstable, short-lived, and reactive, and seriously affect eukaryotic or prokaryotic cell integrity and viability thus leading to cell death [30].

5. Conclusion

In the current study, four foractions (n-hexane (EWNX), ethyl acetate (EWEA), chloroform (EWCH) and aqueous (EWAQ)) of *E. wallichii* root extracts were evaluated for their antileishmanial activity against promastigote and amastigote forms of *L. tropica* followed by the investigation of their possible mechanism of action. Ethyle acetate and

Table 2
Cytotoxic activities (CC_{50}) and selectivity index of different fraction of *E. wallichii* root extracts, and Triton –X100 at different concentrations over a suspension of human red blood cells (RBCs).

Fraction	CC_{50} [CI][a] (µg/mL)	(SI)[b]	(SI)[c]
EWNX	285.5 [240.7-338.5]	1.75	1.62
EWCH	1063 [993.5-1137.0]	7.55	5.64
EWEA	291.6 [264.2-321.9]	28.42	21.85
EWAQ	2693 [3255-6542]	219.30	137.39
Triton-X100	< 0.1%	–	–

CC_{50} = 50% haemolysis of human RBCs as compared to negative control;
[a] 95% confidence interval.
[b] Selectivity index: $CC_{50\ \text{in human red blood cells}}$ /$IC_{50\ \text{in promastigotes}}$.
[c] Selectivity index: $CC_{50\ \text{in human red blood cells}}$ /$IC_{50\ \text{in aromastigotes}}$.
- Not applicable.

aqueous frantions were found to be comepratively highly active against both the forms of *Leishamia*. It may be speculated from the current study that the EWEA and EWAQ contain biologically active phytochemicals that may kill the *Leishmania* cells. A small amount of ROS activation was also observed in leishmania when exposed to different concentration of *E. wallichi* root extract. Both the fractions (EWEA and EWAQ) were found non-toxic (non-haemolytic) towards the human blood erythrocytes (RBCs). Therfore, it is strongly reconmmended that these fractions of the *E. wallichii* root extracts could be further exploited for the isolation of bioactive compounds and evaluation of their efficacy in the *in vivo* model of the disease.

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