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In vitro antileishmanial and antioxidant potential, cytotoxicity evaluation and phytochemical analysis of extracts from selected medicinally important plants

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ABSTRACT

The antileishmanial drugs presently available have stern limitations regarding efficiency, safety and cost that make it crucial to hunt for novel therapeutic approaches from natural products. In current study crude methanolic extracts (CMEs) of four medicinally important plants were evaluated for their phytochemical constituents, antioxidant potentials, cytotoxicity and antileishmanial activities. *Digera muricata* (L) and *Cannabis sativa* (Linn) showed highest total phenolic and flavonoid content (273.4 μg GAE/mg DW and 206.8 μg QA/mg DW, respectively), amongst others. Total antioxidant capacity determined *in vitro* by phosphomolybdenum assay exhibited that *Saccharum spontaneum* (L) and *Mangifera indica* (L) were the strongest antioxidants (~108 and ~100 μg AAE/g of DW, respectively) plants amongst other tested. The *S. spontaneum* showed remarkable scavenging activity (EC_{50} 44.9 μg/mL), while *M. indica* showed to possess the lowest scavenging activity (EC_{50} 105.7 μg/mL) on 1,1-diphenyl-2-picrylhydrazyl. *S. spontaneum* and *C. sativa* were the least toxic (CC_{50}, 113.0 and 109.4 μg/mL respectively), while *M. indica* remained the most toxic (CC_{50} 42.46 μg/mL). Moreover, the *in vitro* evaluation of antileishmanial potential of plants CMEs, demonstrated a dose-dependent growth inhibition of *L. major* promastigotes and axenic amastigotes. *M. indica* and *D. muricate* extracts exhibited to possess antileishmanial potential at minute concentrations (IC_{50} 5.2 and 18.9 μg/mL, respectively) comparable to that of tartar emetic (IC_{50} 4.7μg/mL) against *L. major* promastigotes. Each CME was significantly active ($p < 0.0001$) against *L. major* axenic amastigotes when compared to Glucantime®. This study encourages the isolation of bioactive compounds from these plants for antileishmanial drug development.

1. Introduction

Beside their role as antioxidants, the uses of numerous plants and their extracts, and bioactive compounds extracted from various natural sources have been reported for the treatment of many disorders including infectious diseases such as malaria, trypanosomiasis and leishmaniasis (Dvorkin-Camiel and Whelan, 2008; Ullah et al., 2016). Leishmaniasis, a protozoan parasitic disease caused by the parasites of genus *Leishmania* and one of the most neglected tropical diseases (NTDs), has a huge impact on public health, with an estimated 350 million people at threat and mortality rate of 70,000 per annum around the globe (Blum et al., 2004; Torres-Guerrero et al., 2017).

Rodents, dogs and other wild animals are the reservoirs of the parasite *Leishmania*, which are transmitted to the human or animal hosts by the bite of infected female sandfly of the genus *Phlebotomus* and *Lutzomyia* (Mishra et al., 2007). Clinical manifestation of

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Abbreviations

CME	crude methanolic extract
DPPH	1,1-diphenyl-2-picrylhydrazyl
QE	quercetin equivalent
GAE	gallic acid equivalent
AAE	ascorbic acid equivalent
ROS	reactive oxygen species
TPC	total phenolic content
TFC	total flavonoid content
FRP	ferric reducing power
TAC	total antioxidant capacity
DW	dry weight of extract
TA	Potassium antimonyl(III) tartrate
PBS	phosphate buffered saline
EC_{50}	the extract concentration required to decrease DPPH free radical by 50% as compared to non-treated control
IC_{50}	the extract concentration required to inhibit *Leishmania* growth by 50% as compared to non-treated control
CC_{50}	the extract concentration required to stimulate hemoglobin release by erythrocytes by 50% as compared to non-treated control

leishmaniasis varies from cutaneous and mucocutaneous forms (affecting skin and mucosa, respectively) to visceral form (affecting liver, spleen and/or bone marrow) (Desjeux, 2004). Cutaneous leishmaniasis is the most common form, caused by *Leishmania* (*L.*) *amazonensis* and *L. tropica* in the New World and L. *donovani* and *L. major* in the Old World (Alrajhi et al., 2002; Islam et al., 2014). Leishmaniasis is endemic in the tropical and neotropical regions such as Iran, Saudi Arabia, Syria, Afghanistan and Pakistan, and in some South American countries, especially Brazil (Kassi et al., 2008).

The first-line drugs for the cure of leishmaniasis are pentavalent antimonials (Glucantime® and Pentostam®), which are effective in all forms of the disease. But they are, unfortunately, associated with severe side effects and the emergence of resistance in some parts of India. Other alternatives such as miltefosine and liposomal amphotericin B, despite their high efficacy rate, are also severely toxic and expensive (Frézard et al., 2009; Islam et al., 2014). Insufficient treatment options concerned toxicities and the emergence of resistance to the clinically available drugs in endemic areas strongly encourages the search for new, efficient, cost-effective and safe antileishmanial therapeutics. Natural products are valuable source of novel medicinal agents for many diseases including leishmaniasis (Bekhit et al., 2018; Fan et al., 2018; Ullah et al., 2016). Plants and their extracts have largely been investigated for their antileishmanial potential. Chemical constituents isolated from *Leguminosae* members were shown to possess antileishmanial activity against *L. braziliensis*, *L. amazonensis* and *L. panamensis* (Araujo et al., 1998; Kedzierski et al., 2009). Some of diterpenes and triterpenes extracted from *Lamiaceae* were reported to have considerable *in vitro* antileishmanial activity against *L. major* and *L. donovani* (Tan et al., 2002).

Moreover, plants and their secondary metabolites are well known for their antioxidant properties. Antioxidants are micronutrients possessing the potential to either scavenge ROS directly or prevent their generation (Bursal and Gülçin, 2011). Therefore, extraction of antioxidant components from natural resources such as plants are getting significant attention since the last few decades due to the apprehension that synthetic antioxidants are harmful to human health (Kumar and Jain, 2014). Medicinal plants and herbs are playing important roles as powerful antioxidants due to the occurrence of polyphenolic compounds (secondary metabolites) in abundance (Kumar and Jain, 2014). Phenolic compounds decrease the risk of serious health concerns due to their resistance to the oxidative damage by reactive oxygen species (ROS) (Meot-Duros and Magne, 2009; Tourtoglou et al., 2014). *Cannabis sativa* (L) has traditionally been used against pain, skin cancer, inflammation and as antimicrobial agent (Dogrul et al., 2003; Yesilyurt et al., 2003). *Digera muricata* (L) is an important medicinal herb known for their antibacterial, laxative, antifungal and anthelmintic activities (Muhammad Rashid Khan and Ahmed, 2009; M. R. Khan, Rizvi, Khan, Khan and Shaheen, 2009). *Saccharum spontaneum* (L), locally known as Kasa in Indian subcontinent, and *M. indica* are also reported for their huge range of pharmacological properties (Ghani, 1998; Ioset, 2008; Shah et al., 2010; Kaith et al., 2010; Goyal, 2013). In the current study crude methanolic extracts (CMEs) from the leaves of four traditionally important medicinal plants namely; *Digera muricata* (L) (Amaranthaceae), *Cannabis sativa,* Linn (Cannabaceae), *Saccharum spontaneum* (L) (Poaceae) *and Mangifera indica* (L) (Anacardiaceae) were assessed for phytochemical constituents, antioxidant activities, cytotoxicity and antileishmanial potential against promastigote and axenic amastigote forms of *L. major* (an etiological agent of cutaneous leishmaniasis in Pakistan).

2. Materials and methods

2.1. Reagents and chemicals

Reagent grade chemicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium antimonyl tartrate, ascorbic acid, gallic acid, quercetin, phenol reagent, Folin-Ciocalteu's reagent, calcium chloride dihydrate, aluminum chloride hexahydrate ($AlCl_3.6H_2O$), potassium chloride, disodium hydrogen phosphate, sodium bicarbonate, potassium dihydrogen phosphate, methanol and Triton-X 100 were purchased from Sigma-Aldrich (St. Louis, Mo. USA). Commercial Glucantime® (300 mg/mL; Sanofi-Aventis) was kindly provided by Adnan Traders® for research purposes only. All other solvents and chemicals were of at least analytical grade and obtained from Sigma-Aldrich. Double-distilled deionized water was used throughout the experiments.

2.2. Collection of plant material

Four different medicinal plant *spp.* were collected during the months of November and December from the tropical region of Mardan division, KPK, Pakistan, and were botanically identified as *Cannabis sativa* Linn. (Cannabaceae) (Accession number 103), *Digera muricata* (L) (Amaranthaceae) (Accession number 207), *Mangifera indica* (L) (Anacardiaceae) (Accession number 293) and *Saccharum. spontaneum* (L) (Poaceae) (Accession number 97). A voucher samples of these plants are saved in the Herbarium at Department of Botany, Abdul Wali Khan University Mardan (AWKUM), Pakistan for futuristic purpose.

2.3. Preparation of methanolic extracts of selected plants' leaves

Shade-dried leaves of selected experimental plants were carefully washed with deionized water and manually crushed into fine powder through electric grinder at room temperature. The air-dried leaf powder (100 g) of each plant was immersed into methanol (200 mL) and incubated at room temperature for 72 h. Subsequently, the methanol was evaporated from the residue (extracts) and the extraction was repeated for an additional 96 h. Each crude methanolic extract (CME) was filtered through a Whatman No.1 filter paper, the filtrate was concentrated to a constant weight at 40 °C under reduced pressure in a Vacucell (MMM Group, Planegg, Germany). CMEs were stored at 4 °C until further use.

The yields of CMEs were 9.34% (*S. spontaneum*), 11.58% (*C. Sativa*), 8.69% (*D. muricata*) and 6.48% (*M. indica*).

2.4. Stock solutions of CMEs

Stock solutions of CME of each plant was prepared by dissolving 20 mg of CME in 1 mL of dimethylsulfoxide (DMSO) and diluted with appropriate solvent or culture media when required. Samples were filtered with syringe filter (pore size of 0.5 μm) (Sigma-Aldrich). 0.5% Triton X-100 (stock solution 5 μg/mL) prepared in phosphate-buffered saline (PBS) was used as a positive control. Stock solutions of Glucantime® (300 μg/mL) and potassium antimonyl(III) tartar emetic (TA) (80 μg/mL) were prepared in deionized water.

2.5. Phytochemical analysis

The preliminary phytochemical investigations were carried out for relative quantification of the different chemical groups present in the CME of each plant leaves.

2.5.1. Determination of total phenolic content (TPC)

The total phenolic content of the extracts was assessed by the Folin–Ciocalteu method (Kaur and Kapoor, 2002) with slight modifications (Ullah et al., 2015). From the stock solutions of CMEs (20 mg of extract/mL DMSO), 200 μL of each solution was mixed with 3.0 mL of Folin–Ciocalteu reagent (diluted 10-fold with deionized water previously). The mixture was kept at room temperature for 5 min followed by the addition of 3.0 mL of 6% (w/v) sodium carbonate solution. The resulting reaction mixture was allowed to stand at 25 ± 1 °C for 90 min in dark and the absorbance was measured at 725 nm using spectrophotometer (Agilent-DAD, 8453, Agilent Tech., Germany). The standard calibration curves were constructed from various gallic acid (GA) concentrations (0–50 μg/mL) and deionized water was used as reagent blank. The total phenolic content was calculated from the standard calibration curves and the results obtained in three separate experiments were expressed as μg of gallic acid equivalent (GAE) per mg of dry weight (DW) of CME of each plant (μg GAE/mg DW).

2.5.2. Determination of total flavonoid content (TFC)

The total flavonoid content in CME of each plant was examined by aluminum chloride colorimetric method (Chang et al., 2002) with slight modifications (Jafri, Saleem, Ihsan ul, Ullah, & Mirza, 2017). In brief, from stock solutions of CMEs, (20 mg of extract/mL DMSO), 300 μL of each sample solution was separately mixed with 3.4 mL methanol (30%), 0.15 mL of 10% aluminum chloride hexahydrate ($AlCl_3.6H_2O$; 0.3 M), 0.1 mL potassium acetate (CH_3COOK; 1 M) and 2.8 mL of deionized water. After incubation for 30 min at room temperature, the reaction mixture absorbance was recorded at 506 nm against reagent blank on spectrophotometer. Calibration curves were constructed at various quercetin (Q) concentrations (0–100 μg/mL). The total flavonoid content was calculated from the standard calibration curves and the results obtained in three separate experiments were expressed as μg of quercetin equivalent (QE) per mg of dry weight (DW) of CME of each plant (μg QE/mg DW).

2.6. Determination of antioxidant activities

2.6.1. Total antioxidant capacity (TAC) by phosphomolybdenum assay

The total antioxidant capacity of extract of each plant was spectrophotometrically determined by phosphomolybdenum assay using the method as described previously (Jafri et al., 2014; Prieto, Pineda & Aguilar, 1999). This assay is routinely used to evaluate the total antioxidant capacity (TAC) of plant extracts, which is based on the molybdenum(VI) reduction to molybdenum(V) by the plant extracts (antioxidant compound) and the formation of a green phosphomolybdenum(V) complex under acidic conditions, with a maximal absorption at 695 nm (Nagendra Prasad et al., 2009; Prieto, Pineda & Aguilar, 1999). Briefly, an aliquot of 0.1 mL with final concentration of 100 μg/mL of plant extract in methanol was mixed with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95 °C for 90 min. Followed by incubation, the samples were allowed to cool at room temperature and absorbance of the mixture was recorded at 695 nm against reagent blank (0.1 mL methanol without plant extract) on UV–visible spectrophotometer. A typical reagent blank solution consists of appropriate volume of the same reagent solution, solvent and was incubated under same conditions as used for the samples. For samples of unknown composition, antioxidant capacity can be expressed as equivalents of ascorbic acid (Prieto et al., 1999). The total antioxidant capacity was calculated from the ascorbic acid calibration curves (0–500 μg/mL) and the results obtained in three separate experiments were expressed as μg of ascorbic acid equivalent (AAE) per mg of dry weight (DW) of CME of each plant (μg AAE/mg DW).

2.6.2. Ferric reducing power (FRP) assay

Ferric reducing or antioxidant power of the extracts was calculated according to the method previously described (Oyaizu, 1986; Zhao et al., 2008). In brief, 500 μL of extract from the stock solution of each CME was mixed with 500 μL of phosphate buffer (pH 6.6; 0.2 M) and 500 μL of 1% potassium ferricyanide [$K_3Fe(CN)_6$], and incubated at 50 °C for 20 min followed by the addition of 500 μL of trichloroacetic acid (10%). The tubes were then centrifuged at 10,000 rpm for 10 min. The upper layer of the mixture was mixed to an equivalent volume of distilled water and 100 μl of 0.1% (w/v) ferric chloride ($FeCl_3$). The ferric reducing power activity is based on the reduction of Fe(III) to Fe (II) in the presence of CMEs/standard (Fejes et al., 2000). The presence of Fe(II) can be examined by computing the development of Perl's Prussian blue at 700 nm. The greater the absorbance, the greater will be the reducing power. The results were expressed as Ascorbic acid equivalent (AAE μg/mg dry weight (DW) of sample). DMSO (200 μl) was used as blank.

2.6.3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

The free radical scavenging activity of plant extracts was measured *in vitro* against the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the method described previously (Brand-Williams et al., 1995). Fresh radical DPPH solution was prepared in methanol (6×10^{-5} M) daily before the absorbance measurements. The DPPH solution (3 mL) was mixed with 100 μL of plant extracts at various final concentrations (50, 100, 200, 400 μg/mL) against reagent blank. The mixture was shaken vigorously and kept at room temperature for 1 h in the dark. The ability of the corresponding extracts to donate hydrogen atoms or electrons was measured, from the bleaching of purple-colored methanol solution of DPPH to light yellow-colored solution, at 517 nm on a UV visible light spectrophotometer. The experiment was carried out in triplicate. Ascorbic acid was used as a positive control.

The percent (%) inhibition of DPPH radical was calculated by the following formula (I):

$$\text{Percent (\%) DPPH scavenging effect} = [A_0 - A_1/A_0] \times 100 \quad \text{(I)}$$

where, A_1 and A_0 = the absorbance recorded at 517 nm of the reagent blank and the sample, respectively.

The antiradical activity was expressed as EC_{50} (μg/mL), the extract dose required to cause a 50% decrease of the absorbance at 517 nm. A lesser EC_{50} value corresponds to a greater antioxidant activity.

3. Biological activities

3.1. Cytotoxicity assays (Ex vivo red blood cell hemolysis assay)

Venous blood samples from healthy human subjects with no current history of infectious disease were collected by a trained health professional after obtaining 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 red blood cells were washed 3 times with PBS to perform cytotoxicity assay as performed by (Alrajhi et al., 2002; Nadhman et al., 2015). CME of each plant dissolved in DMSO was mixed with red blood cells suspended in PBS, at a final concentration of 909.1, 90.1, 45.5, 9.1 and 4.5 µg of CME per mL. The final concentration of DMSO was < 0.5%. The resultant mixture was incubated at 37 °C for 3 h and centrifuged at 1000 rpm for 5 min. The supernatants were collected, and the released hemoglobin was monitored at 576 nm using spectrophotometer. PBS or Triton X-100 (0.5%) were used as negative or positive control, respectively. The hemolytic percentage in the presence of various concentrations of CMEs was calculated by using the following formula;

Hemolysis (%) = [(O.D. 576 nm in the sample solution – O.D 576 nm in PBS)/(O.D 576 nm in 0.5% Triton X-100 – O.D 576 nm in PBS) x100.

3.2. Antipromastigote activity

3.2.1. Parasite culture

Leishmania major isolates kindly donated by Department of Zoology, University of Peshawar, Pakistan, were grown in RPMI-1640 culture medium (Gibco®) with 10% heat-inactivated fetal calf serum (HIFCS; Gibco®) in tissue culture flasks (25 cm^2) (TPP® Sigma-Aldrich) in the presence of 100 µg/mL of each penicillin and streptomycin solution at 23 °C. After 4 days of incubation, the parasite culture was monitored using inverted microscope (Olympus®) and passaged for further growth.

3.2.2. Promastigote proliferation measurements by MTT [3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

The promastigote forms of *L. major* were seeded in 96-well microtiter plates (2.5×10^6 per well) in RPMI-1640 and 10% HIFCS and incubated for 72 h in the presence of 0.1% DMSO (negative control), TA (1–50 µg/mL; positive control) or CME of each plant at various final concentrations (909.1, 90.1, 45.5, 9.1 or 4.5 µg/mL) at 25 ± 1 °C. . After the incubation period, a 100 µL of 5 mg/mL MTT (3-(4,5- dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) solution (Sigma Chemical Co., St. Louis, Mo.), was added to each well of 96-well plates and incubated for 4 h at 25 ± 1 °C (Mosmann, 1983). The enzymatic reaction was stopped by the addition of 100 µL of 50% isopropanol and 10% sodium dodecyl sulfate (SDS). 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 was shown to produced by the action of mitochondrial dehydrogenases of metabolically active cells to compare with the number of viable cells. All plants extract and controls were assayed in triplicate.

3.3. Antiamastigote activity

3.3.1. Culture of L. Major amastigotes

For conversion of promastigotes form of *L. major* to axenic amastigotes, promastigotes were centrifuged and re-suspended in medium-199 (Sigma-Aldrich) containing Hank's balanced salts, 20% HIFCS, 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 incubated for 7 day at 31 °C in 5% humidified CO_2.

3.3.2. Antiamastigote assay

100 µL of *L. major* axenic amastigotes (final count 1×10^6 parasites) were added to each well of the 96 wells microtiter plates and incubated with 0.1% DMSO (negative control), commercial Glucantime® (1, 50 and 100 µg/mL; positive control) or in the presence of each plant extract (CMEs) at different concentrations (909.1, 90.1, 45.5, 9.1 and 4.5 µg/mL) at 24 °C for 72 h. After the incubation period, each well was pipetted with 100 µL of MTT solution and incubated for 4 h at 24 °C 100 µL of solubilization solution (0.04 M HCl in absolute isopropanol:acidified isopropanol) was added to solubilize the dye and readings were taken at 570 nm on microtiter plate reader (Bio Tek ELx-800).

3.4. Statistical analysis

All the experiments were performed in triplicates and the data analyzed were presented as mean ± standard deviations (SD). The IC_{50}, CC_{50}, and EC_{50} values were calculated using nonlinear regression curve fit (GraphPad Prism Software, Version 6 Inc., La Jolla, CA). The satisfactory level of significance was 95% ($p < 0.05$). The association coefficients between total phenolics and the methods of antioxidant activity were verified using Microsoft Excel (2010).

4. Results and discussion

4.1. Phytochemical analysis (total phenolic and flavonoid contents)

The importance of plants in medicine is well-established and their constituents have been recognized as natural antioxidants, potential therapeutic agents targeting cancer, pathological angiogenesis and cardiovascular diseases (Dawidowicz et al., 2006; Dell'Agli, Buscialà and Bosisio, 2004). The medicinal and biological benefits of plants are attributed to their polyphenolics and flavonoids constituents. Total phenolic content (TPC) and total flavonoid content (TFC) have been reported to have a direct correlation with antioxidant activity. These compounds are known as powerful chain-breaking antioxidants (Cakir et al., 2003; Ksouri et al., 2008). In this context, we investigated the crude methanolic extracts of the four selected medicinally important plants for their total phenolic and flavonoid contents. The total phenolic contents (TPC) and total flavonoid contents (TFC) present in each extract were determined from linear regression equation of calibration curve from different concentration of gallic acid or quercetin, respectively, and the results were expressed as gallic acid equivalent (GAE) in µg/mg or quercetin equivalent (QE) in µg/mg of plant extracts, respectively.

Our results demonstrated that the CMEs from the selected plants showed large variations in TPC levels (Table 1). The highest TPC level was determined for *D. muricata* (273.4 ± 5.2 µg GAE/mg DW), followed by *C. sativa* (240.8 ± 4.3 µg GAE/mg DW) and *M. indica* (138.9 ± 3.9 µg GAE/mg DW). *S. spontaneum* was found to possess comparatively minor TPC (12.9 ± 6.8 µg GAE/mg DW). Moreover, differences were observed for TFC in the extracts from different plants. The TFC level of *C. sativa* (206.8 ± 2.5 µg QE/mg DW) remained the highest and that of *D. muricata* (55.6 ± 9.9 µg QE/mg DW) the lowest. *S. spontaneum* and *M. indica* exhibited to contain TFC in a similar quantity (138.9 ± 3.9 and 137.3 ± 9.3 µg QE/mg DW, respectively).

These results showed that the selected plants contained distinctive but significant amount of phenolic and flavonoid contents. Phenolic compounds are well known for their antioxidant activity, that can be ascribed to their ability to scavenge free radicals, donate protons or

Table 1
Total phenolics content (TPC) and total flavonoid content (TFC) in crude methanolic extracts (CMEs) of the four selected medicinally important plants including *S. spotaneum, C. sativa, D. muricata* and *M. inidica*.

Plants CMEs	TPC (µg GAE/mg DW)	TFC (µg QA/mg DW)
S. spontaneum	12.9 ± 6.8	138.9 ± 3.9
C. sativa	240.8 ± 4.3	206.8 ± 2.5
D. muricata	273.4 ± 5.2	55.6 ± 9.9
M. indica	138.9 ± 3.9	137.3 ± 9.3

Each value in the table is presented as a mean ± SD (n = 3); CMEs = crude methanolic extracts; DW = dry weight; GAE = gallic acid equivalent; QE = quercetin equivalent.

Table 2
Total antioxidant capacity (TAC) and ferric reducing power (FRP) in crude methanolic extracts of the four selected medicinally important plants, *S. spontaneum, C. sativa, D. muricata* and *M. inidica*.

Plants CMEs	TAC (μg AAE/mg DW)	FRP (μg AAE/mg DW)
S. spontaneum	81.7 ± 2.8	61.2 ± 7.0
C. sativa	96.0 ± 4.2	104.6 ± 3.1
D. muricata	108.2 ± 4.6	268.7 ± 1.1
M. indica	100.2 ± 5.1	167.7 ± 8.9

Each value in the table is presented as a mean ± SD (n = 3); CMEs = crude methanolic extracts; DW = dry weight; AAE = ascorbic acid.

Table 3
Percent DPPH free radical scavenging activity by the crude methanolic extracts (CMEs) of selected plants. EC_{50}[(a)] concentration of CME of *S. spontaneum, C. sativa, D. muricata*and *M. indica* plants.

Plants CMEs concentrations (μg/mL)	DPPH radical scavenging activity (%) (mean ± SD (n = 3))			
	S. spontaneum	*C. sativa*	*D. muricata*	*M. indica*
400	87.2 ± 1.2	87.9 ± 2.3	95.2 ± 1.9	60.7 ± 1.8
200	71.3 ± 1.1	72.1 ± 2.8	82.3 ± 1.3	54.7 ± 3.6
100	57.5 ± 2.1	60.2 ± 3.2	60.3 ± 2.1	49.7 ± 3.3
50	51.3 ± 1.3	48.0 ± 2.1	47.9 ± 2.2	39.2 ± 2.1
[a]EC_{50} [[b]CI] [a]μg/mL)	55.3 [44.5–68.9]	55.4 [47.4–64.8]	57.3 [51.1–64.2]	128.7 [107.5–154.1]

[a] EC_{50}: dos[a] at which DPPH free radicals are scavenged by 50% as compared to the non-treated reagent control.

[b] CI: 95% c[b]nfidence interval; Results were expressed as the mean ± SD (n = 3).

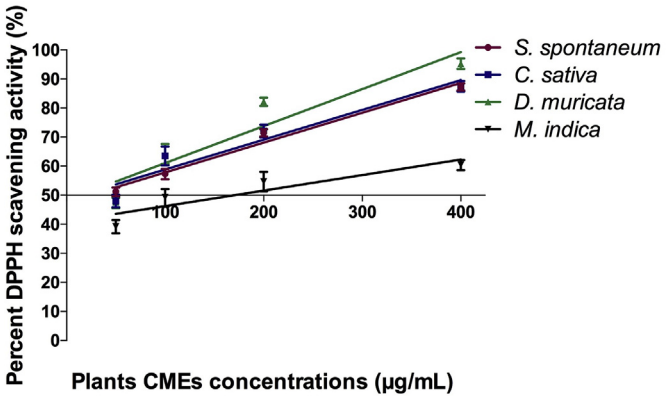

Fig. 1. Graphical representation of the percent DPPH free radical scavenging activity against various concentration of CMEs (μg/mL) of *S. spontaneum* ($Y = 0.1026^{*}X + 47.57$; $R^2 = 0.9729$), *C. sativa* ($Y = 0.1025^{*}X + 48.60$; $R^2 = 0.9010$), *D. muricata* ($Y = 0.1273^{*}X + 48.33$; $R^2 = 0.8795$) *and M. indica* ($Y = 0.05342^{*}X + 40.90$; $R^2 = 0.7716$), quercetin ($Y = 0.0503^{*}X - 0.0129$; $R^2 = 0.9981$), gallic acid ($Y = 0.0781^{*}X - 0.3247$; $R^2 = 0.9995$).

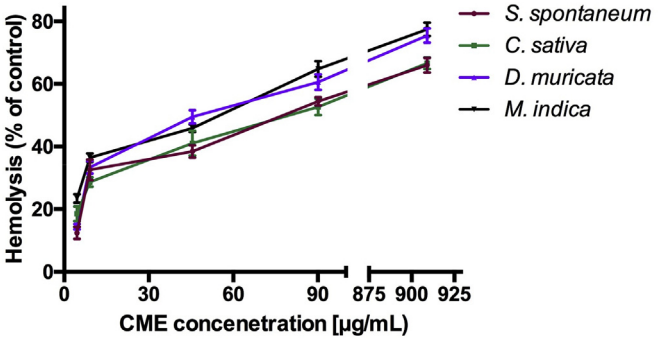

Fig. 2. Hemolytic activity of CMEs of the four selected medicinally important plants, expressed as CC_{50}, at a final concentration of 909.1, 90.1, 45.5, 9.1 and 4.5 μg/mL. Data (non-linear regression) analysis performed on GraphPad Prism (version 6.0) software.

electron or chelate metal cations (Afanas' ev, Dcrozhko, Brodskii, Kostyuk, & Potapovitch, 1989; Amarowicz et al., 2004). The antioxidant activity of phenolic acids depends on the numbers and positions of hydroxyl group in relation to the carboxyl group and increases with increasing degree of hydroxylation (Robards et al., 1999). In addition, the mechanism of action of flavonoids has also been correlated with the scavenging of free radicals or chelation of metal ions (Kessler et al., 2003). This ability of scavenging free radicals confers upon them the strong antioxidant and antitumor potential (Sahreen et al., 2010; Shon et al., 2004). Keeping into consideration the medicinal and biological importance of phenolics and flavonoids compounds, *D. muricata* and *C. sativa* due to their high TPC and FPC can be further investigated as a natural source for the extraction of antioxidants and anticancer compounds.

4.2. Antioxidant activities of plants CMEs

4.2.1. Total antioxidant capacity (TAC)

Plants are the rich sources of antioxidants that reduce the oxidative stress in cells and are, therefore, useful in the treatment of several human diseases, including cancer, cardiovascular diseases and inflammatory diseases (Krishnaiah et al., 2011). As presented in Table 2, the total antioxidant capacity (TAC) of CME of each plant was measured based on the principle of Mo(VI) reduction to Mo(V) in the presence of antioxidant compounds. The Mo(VI) reduction to Mo(V) results in the formation of a green colored complex of phosphomolybdenum(V) that can be observed spectrophotometrically at 695 nm. This is a quantitative method of measuring TAC and employs cost-effective reagents (Prieto et al., 1999). TAC of all CMEs was measured and expressed as ascorbic acid equivalent (AAE μg/mg of dry weight (DW) of extract). The antioxidant activity of CME of *D. Muricata* and *M. Indica* (108.2 ± 4.6 and 100.1 ± 5.1 μg AAE/g of DW of extract) was 1.12 fold and 1.32 fold higher than that of *C. sativa* and *S. spontaneum* extracts. These results showed that CMEs from all the plants contains compounds that present elevated antioxidant capacity and this can be correlated with the presence of high amount of phenolics and

Table 4
Percent hemolytic activity and cytotoxic activity (CC_{50}) values of the crude methanol extracts of selected plants in red blood cells at various concentrations.

Plants CMEs	Test concentrations (μg/mL)					**CC_{50} [μg/mL] [[a]CI]**
	909.1	90.1	45.5	9.1	4.5	
	Percent hemolytic activities of plants extracts (mean ± SD)					
S. spontaneum	65.9 ± 2.3	54.30 ± 1.8	38.5 ± 2.1	32.9 ± 3.7	12.4 ± 2.1	113.0 [70.6–180.7]
C. sativa	66.8 ± 2.0	52.4 ± 3.3	41.1 ± 4.1	29.0 ± 1.4	18.5 ± 1.3	109.4 [82.5–145.0]
D. muricata	75.3 ± 1.7	60.6 ± 2.1	49.7 ± 1.2	33.6 ± 4.6	14.5 ± 1.0	53.10 [39.1–72.0]
M. indica	77.7 ± 3.7	65.4 ± 3.9	44.6 ± 1.6	36.4 ± 1.6	23.7 ± 4.4	42.46 [32.2–55.9]

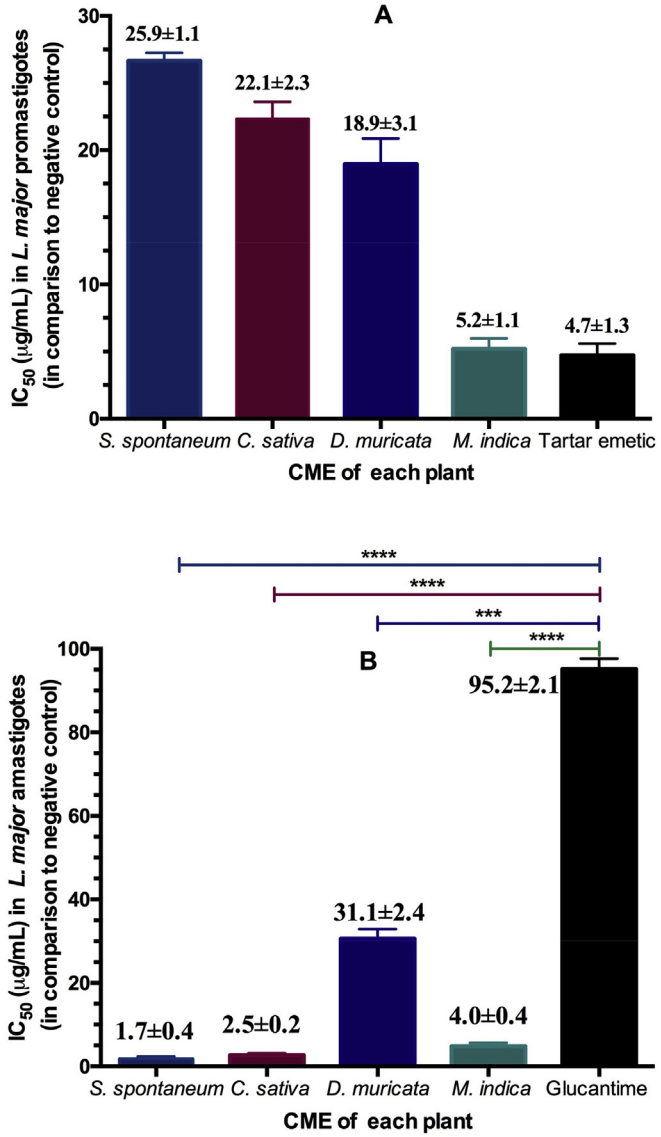

Fig. 3. Growth inhibitory concentrations (IC_{50}) of crude methanolic extracts of selected medicinal plants on promastigote form (**A**) and amastigote form of *L. major* (**B**). Data is presented as mean ± SD (n = 3). ***$p < 0.001$; ****$p < 0.0001$, Test t.

flavonoids. This correlation was also found for *Lanneacoro mandelica* extracts as reported recently (Kumar and Jain, 2015).

4.2.2. Ferric reducing power (FRP)

Reducing power assay is often used to evaluate the ability of antioxidants to donate electron (Oyaizu, 1986). A number of reports exhibited a positive correlation between total antioxidant activity and reducing power of plant extracts (Halliwell and Gutteridge, 1990). Our results also showed a parallel correlation between TAC and FRP (Table 2), where the plant extract from *D. muricata* (108.2 ± 4.6 µg AAE/mg DW) and *M. Indica* (100.2 ± 5.1 µg AAE/mg DW) with highest TAC also showed the highest FRP values (268.7 ± 1.1 µg AAE/mg DW and 167.7 ± 8.9 µg AAE/mg DW, respectively). This might be due to the fact that antioxidants are proton or electron donors that reduce ferric ion to ferrous ion by donating electron (Shon et al., 2004). Furthermore, our results indicated that the higher the total phenolic contents in a plant extract, the more it will have the reducing power ability as previously demonstrated (Jafri et al., 2014).

4.2.3. DPPH free radical scavenging activity

Medicinal plants and herbs play important roles as powerful antioxidants due to the occurrence of polyphenolic compounds (secondary metabolites) in abundance. Phenolic compounds decrease the risk of serious health concerns due to their resistance to the oxidative damage by reactive oxygen species (ROS). Previous studies have reported antioxidant activity along with antileishmanial activity in which good antileishmanial compounds were good antioxidant too (Ali et al., 2016; Jain et al., 2013; Meot-Duros and Magne, 2009; Shah et al., 2014; Tourtoglou et al., 2014). The DPPH is a constant radical with a highest absorption at 517 nm that can readily undertake scavenging by antioxidant (Y. Lu and Foo, 2001). It has been broadly used to assess the capability of compounds as hydrogen donors or free-radical scavengers and the antioxidant activity of plant extracts (Da Porto, Calligaris, Celotti, & Nicoli, 2000; Soare et al., 1997). During the reaction, DPPH accept electron or hydrogen radical from antioxidant and change its characteristic purple color to yellow, which can be measured at 517 nm on spectrophotometer (Čanadanović-Brunet et al., 2014). As shown in Table 3, the free radical scavenging potentials (%) of CME of each plant at different test concentrations were found in the following order: *S. spontaneum* > *C. sativa* > *D. muricata* > *M. indica*. Percent DPPH free radical scavenging activity of CMEs was greater at highest CMEs concentrations and vice versa. Interestingly, *S. spontaneum* extracts showed stronger DPPH radical scavenging activity than *D. Muricata* and *C. sativa*, which were comparatively rich in phenolics. However, at the highest test concentration (400 µg/mL), *D. muricata* showed highest antioxidant activity by scavenging 95.2 ± 1.9% DPPH free radicals, which indicated that *D. muricata* may contain some compounds that are more effective at higher doses. The scavenging effect of CMEs on the DPPH radical were also expressed as EC_{50} values (the CME concentration at which 50% of the DPPH free radicals are inhibited) were found in the following order: *S. spontaneum* > *C. sativa* > *D. muricata* > *M. indica*. EC_{50} values were calculated by non-linear regression of the data using GraphPad 6.0 software.

Percent DPPH scavenging activity graph was constructed via linear regression data using GraphPad Prism 6.0 software. Equations and the resulting graphs are presented in Fig. 1 for *S. spontaneum, C. sativa, D. muricata* and *M. indica* percent DPPH scavenging activity.

4.3. Biological activities

4.3.1. Cytotoxicity assay (hemolytic assay)

Determination of cellular toxicity of a drug towards human erythrocytes is the basic step of drug development against any particular disease. Hemolytic assays for all the CMEs were carried out due to the fact that compounds possessing potent biological activity may not be appropriate in pharmacological preparations if they have hemolytic effect. This assay is usually used to check the biocompatibility of potential drug against erythrocytes in which the interaction of molecules with the cells (RBCs) results in the formation of pores in the membrane that ultimately leads to the release of hemoglobin (Katsu et al., 1986; Lieber et al., 1984). In the current study, the *in vitro* hemolytic activity of various concentrations of the CMEs of the selected plants on human erythocytes was evaluated. The total hemolysis (%) was obtained using 0.5% Triton X-100 as positive control and phosphate saline buffer as negative control. Table 4 represents the percent hemolytic activity and cytotoxic activity (CC_{50}) of each CME. As can be seen from Table 4 and Fig. 2, *M. indica* and *D. muricata* extracts were comparatively more cytotoxic towards human erythrocytes with CC_{50} of 42.46 µg/mL and 53.10 µg/mL, respectively, than those of *C. sativa* (109.4 µg/mL) and *S. spontaneum* (113.0 µg/mL). However, cytotoxicity presented by these CMEs were found comparable to those reported for other medicinal plants (Vinjamuri et al., 2015; Zohra and Fawzia, 2014). These results showed that toxicity is dose dependent, which decreases with decrease in extract concentrations.

CC_{50}: EC_{50}: CME concentration that is toxic to eryhtrocytes by 50%

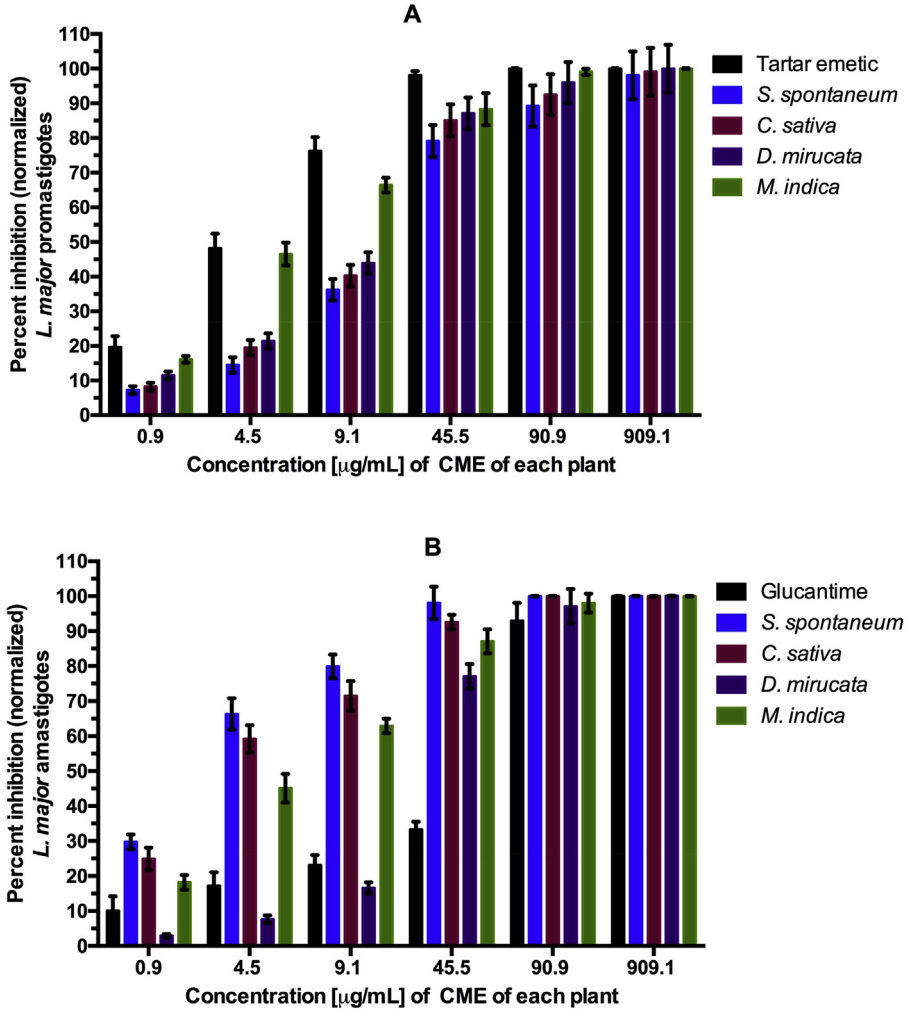

Fig. 4. Dose-dependent activity graph of the crude methanolic extracts (CMEs) of selected medicinal plants on promastigote form of *L. major* (**A**) and amastigote form of *L. major* (**B**). Data is taken as mean ± SD (n = 3).

Table 5
Selectivity indices (SI) of CMEs of *S. spontaneum, C. sativa, C. sativa* and *M. indica*plants against promastigote and amastigote forms of *L. major*.

Plants CMEs	[a]SI (CC_{50}/I^{a}_{50})	[b]SI (CC_{50}/I^{b}_{50})
S. spontaneum	4.3	66.4
C. sativa	4.9	43.7
D. muricata	2.8	1.7
M. indica	8.1	10.6

[a] SI: calcu[a]ated as the ratio between CC_{50} in human erythrocytes and IC_{50} in *L. major* promastigotes.
[b] SI: calcu[b]ated as the ratio between CC_{50} in human erythrocytes and IC_{50} in *L. major* amastigotes.

as compared to the non-treated reagent control; Results were expressed as mean ± SD (n = 3); [[a]CI] = 95% confidence interval.

4.3.2. In vitro antileishmanial activity in L. Major promastigotes and amastigotes

The life cycle of *Leishmania* alternates between two main morphological forms: (i) the motile promastigote form in the midgut of sand fly vector and (ii) the intracellular amastigotes in the mammalian host (Killick-Kendrick, 1990). Potassium antimonyl(III) tartrate(TA) was primarily used for the treatment of leishmaniasis but due to their adverse cytotoxic effect such as cardiac intoxication, the treatment was switched to less toxic form Sb(V). Sb(V) based drugs such meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam®) were better tolerated but have repeatedly reported to have threatening effects on human (S.-T. E. LU & LIU, 1963; Ribeiro et al., 1999). It is thus desirable to seek for new antileishmanial drugs with maximum antileishmanial activity and minimum or no cytotoxic effects. In this regard, bioactive compounds are considered to be the most suitable candidates due to the fact that they are comparatively less harmless than synthetic ones (Gachet et al., 2010; Sharif et al., 2007)

In this work, CMEs of all the selected plants were evaluated for their antileishmanial potential both in promastigote and amastigote forms of *L. major* and the growth inhibitory concentration (IC_{50}) in the presence of test extracts were determined in comparison to the standard TA drug (positive control) or commercial Glucantime® drug (positive control), respectively (Fig. 3A and B). The results showed that the CMEs of all the selected plants were active against the promastigote forms of *L. major*, inhibiting their growth by 50% at micromolar concentrations (IC_{50} values 25.9 ± 1.1 µg/mL (*S. spontaneum*), 22.1 ± 2.3 µg/mL (*C. sativa*), 18.9 ± 3.1 µg/mL (*D. muricata*) *and* 5.2 ± 1.4 µg/mL (*M. indica*)), when compared to that of saline buffer (non-treated negative control) (Fig. 3A). Although, when compared to that of the positive control, TA (IC_{50} 4.7 ± 1.3 µg/mL), the antipromastigote activity of the CMEs from all the plants was not significantly greater but considerable.

Intriguingly, all the extracts were found to be comparatively more active in amastigote forms of *L. major* than in promastigote forms, at micromolar concentrations (IC_{50} values 1.7 ± 0.4 µg/mL (*S.*

spontaneum), 2.5 ± 0.2 μg/mL (*C. sativa*), 4.0 ± 0.4 μg/mL (*M. indica*) and 31.1 ± 2.4 μg/mL (*D. muricata*)). These studies also exhibited that all the plant extracts were significantly more active than the clinically available antileishmanial standard drug. Glucantime® ($p < 0.05$) in both promastigote and amastigote forms of *L. major* (Fig. 3B). Based on the activity of CMEs from all the plant in both promastigote and amastigote forms of *L. major*, it is worth notifying that their activity was not stage-specific and a correlation between the phytochemical constituents and antileishmanial activity was found.

Moreover, as sketched in Fig. 4A and B, the activities of CMEs of all plants were in a dose-dependent manner, where the growth inhibition rate of promastigote or amastigote forms of *L. major* increases with the increase in CME concentration.

Surprisingly, considering the selectivity index, calculated as the ratio between CC_{50} in human erythrocytes and IC_{50} in *L. major* parasites (promastigotes or amastigotes form) (*See* Table 5), CMEs from all the plants were found to be selective (broadly or narrowly). Among the others, *M. indica* remained the more selective in promastigotes form (SI = 8.1). On the other hand, *S. spontaneum* was the most active and selective in amastigotes form of *L. major*. It is noteworthy that CMEs from all plants were not only highly selective but also considerably active in both forms of *L. major*. In brief, our findings were in accordance previous study (Sharma et al., 2011), *Plumeria bicolor* extracts against the amastigote and promastigote form of *L. donovani* reported to be highly potent. Our results suggested that extracts from these plants can be further used for the isolation and structural characterization of valuable bioactive compounds and investigated in *in vivo* experimental model of the disease.

5. Conclusion

Antioxidant properties of plants or their products are of great interest due to their less harmful nature than synthetic compounds. Medicinal plants and herbs are possess compound that acts as antioxidants mainly due to the presence of polyphenolics and flavonoids. The present study showed that CMEs from the selected plants, including *S. spontaneum, C. sativa, D. Muricata* and *M. indica* possessed considerable amount of polyphenolic and flavonoid contents and were exhibited to have antioxidant as well as free radical scavenging potential in the phenolic compounds, which may be attributed to presence of hydroxyl groups. The current study also suggests further exploitation of these plants as natural sources of antioxidants or other bioactive compounds. Furthermore, the antileishmanial potential of CMEs from all plants exhibited considerable activity at minute concentrations *in vitro* both in promastigotes and axenic amastigote forms of *L. major*. Interestingly, when assayed for their cytotoxic evaluation against human erythrocytes, all the CMEs showed toxicity at concentrations only several to many folds higher than their concentration at which they were active in *L. major* promastigotes and amastigotes. Our results further suggest that CMEs form the plants selected in this study, could be further investigated for the isolation and purification of the active antileishmanial constituents. These plants were reported for the first time to have promising antileishmanial potential and phytochemical constituents.

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Authors' contribution

N.U. designed and supervised the study. S.M. performed the biological experiments and antioxidant potential analysis. I.U., M.Q. and A.W. performed the phytochemical analysis. A.I. performed the biological experiments, analyzed the results and wrote the manuscript. N.U., M.Q. and M.K.A. revised the manuscript.

Conflicts of interest

The authors declare that there is no conflict of interests.

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