

Evaluation of antioxidant, antimicrobial and cytotoxic potential in *Artemisia vulgaris* L.

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Abstract

Artemisia vulgaris L. (Mugwort or Afsantin) has been used to treat various diseases since ancient times by the inhabitants of Himalayan region-Pakistan. Methanolic fractions (HA1-HA9) obtained from the aerial parts of *A. vulgaris* were evaluated for their antioxidant, antimicrobial and brine shrimp cytotoxic activities. Fraction HA8 showed substantial phenolics content with value of $26.29 \pm 1.4 \mu\text{gEQ/mg}$ and DPPH scavenging ($82.84 \pm 3.01\%$). Conversely, total flavonoids content of $7.32 \pm 0.07 \mu\text{gEQ/mg}$ was determined in HA1 fraction. Fraction HA1 also showed significant cytotoxic effect with the value LD50 of $144.94 \mu\text{g/mL}$. Fractions HA7 and HA9 depicted maximum total antioxidant activity and ferric ion reduction (96.25 ± 3.29 and 176.91 ± 8 , respectively). All fractions showed encouraging results against bacterial strains *Bordetella bronchiseptica* and *Micrococcus luteus*, while HA2 fraction showed the highest percentage inhibition *Mucor* species with zone of inhibition of $13.25 \pm 0.35 \text{ mm}$. A total of 7 fractions showed significant antileishmanial activity with survival percentage ranging 0.00 to 19. To sum up, results of the current study indicated that the plant can be further explored for isolation of antileishmanial and antimicrobial compounds, which could be used for drug development.

Keywords: antioxidant, antimicrobial, antileishmanial, cytotoxicity, *Artemisia vulgaris*

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Introduction

More than 90% of therapeutic classes derived from natural prototypic products and two-thirds of the total population relies upon herbals

for basic pharmaceutical care [1]. Presence of bioactive compounds in plants is naturally gifted, since isolation of secondary metabolites from medicinal plants has led to breakthroughs in the form of new drug discovery [2-3]. Aspirin is a

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classic example of drug discovery, which was initially discovered as salicylic acid from willow bark and leaves. Likewise, taxol from the bark of yew trees has effectiveness against breast and ovarian cancers [2]. Systematic screening of microorganism, plants and animals is very fruitful in the discovery of novel antimicrobials [4]. Natural herbs possess a number of medicinal properties and are an excellent source of pharmaceutical and health care products [5]. It is well established that plant species have a cluster of antimicrobials. Therefore, screening of plant extracts was carried out to find new anti-infective agents [6-7]. The majority of the population in developing countries depends on natural resources, particularly medicinal plant species to treat infectious diseases [8-9]. Therefore, natural products have a leading role in the discovery of novel antibiotics [10]. For instance, most of the problems are associated with antibiotics, therefore significant research work has been focused on plant species which present remarkable antimicrobial effects [11-12].

Reactive oxygen species (ROS) have a major role in a number of pathological conditions. It is well accepted that in tissue injury a large amount of ROS is produced, which rapidly reacts with a number of biological macromolecules and causes oxidative alterations [13]. Free and oxidative radicals are the damaging entities because they affect proteins, membranes, lipids and nucleic acids and cause pulmonary diseases, inflammation, ischemia, cancer, diabetes and a number of degenerative disorders [14-24]. Plant based diets are suggested for patients suffering from cancer, cardiac dysfunctions, atherosclerosis, diabetes, hypertension and neurodegenerative disorders, because such diets are rich in secondary metabolites, which have anti-oxidative effects [21].

Phytochemicals are bioactive compounds, which are obtained from plants and contribute significantly to scavenge free radical species. Polyphenolic compounds with monocyclic aro-

matic ring and aldehydic, alcoholic or carboxylic group [22] are present in almost every plant [23]. These compounds are involved in the shaping of plants, affect reproduction, growth and resistance to parasites and environment [21]. In addition, phenolic compounds present in foods, medicinal plants and beverages have shown significant antioxidant properties and free radical scavenging [24-25]. Like phenolics, flavonoids are color pigments, involved in pollination by appealing to animals with charming colors and prevent plant species from UV damage. Flavonoids possess numerous pharmacological activities such as: antitumor, anti-inflammatory, analgesic, anti-HIV, anti-infective, antioxidant, anti-hepatotoxic, immune-stimulant, vasodilator and antiulcerogenic activities. Hesperidin and rutin are biologically active flavonoids and have the capacity to minimize capillary fragility. Likewise, quercetin is important for its antidiarrheal activity [22]. Screening of phytochemical characters is done by crude fractions of plant extracts [26], whereas antioxidant activity is determined by redox reactions [27-28] using various assays. Among these, 2, 2 diphenyl 1 picrylhydrazyl (DPPH) assay, which was introduced by Marsden Blois, working at Stanford University nearly 56 years ago [22] is very popular for determining the antioxidant properties of plant extracts. Likewise, Phosphomolybdenum complex assay is commonly used to determine total antioxidant capacity [29].

The number of resistant microbial strains is increasing every day due to the excessive and unfair use of drugs. Food spoilage by fungi causes considerable economic loss and constitutes a health risk for consumers due to mycotoxins [28]. Regardless of the survival of strong antifungal agents, multi resistant strains are incessantly emerging [30]. Mucorales (zygomycete fungi) are primary causes of food spoilage of soy cakes, used in traditional foods in Asia [31]. The higher occurrence of *Aspergillus niger* in tem-

perate and moist places is due to its capacity to survive over extensive pH range of 1.4-9.8 [32]. *Aspergillus fumigatus* produces countless conidia, which is released into the atmosphere and ultimately reaches alveoli in the lungs and causes serious health effects [33].

Brine shrimp '*Artemia salina*' is an invertebrate, which is marine in habitat and its larva (Nauplii) can be grown in saline solution. Potential toxicity of plant extracts and presence of anti-cancer agents can be tested in tubes containing a known number of brine shrimps. In this assay live brine shrimps are counted after 6 and 24h of the treatment and LD₅₀ value is calculated, which corresponds to 50% death [22]. Leishmaniasis disease is mainly caused by protozoa of the genus *Leishmania*. This disease appears at cutaneous and/or visceral levels and is common in hot and humid regions [34]. Even though antileishmanial drugs are accessible, first-line chemotherapy is still based on pentavalent antimonials, which are toxic and prone to drug resistance. Moreover, substituted drugs i.e. amphotericin B and pentamidine have also many side-effects. In this context, use of miltefosine as a first oral treatment of visceral leishmaniasis was a major step forward. However, new, safe and cost effective preparations are still desirable [35-36]. Pharmacological activity of crude extracts can be measured and standardized with the help of biological assays. Scientifically, economically and ethically *in-vitro* assays are better to perform prior to *in vivo*. Bioactive components are preceded for clinical trials and adverse effects studies following *in-vivo* assays [22]. Bioassays have a fundamental role in drug discovery and in finding new therapeutic entities for the treatment of diseases [37]. In this context the present study was mainly focused on *in vitro* bioassays as they are quick and reproducible [38].

Artemisia vulgaris (L.) belongs to Asteraceae family, commonly known as Mugwort and Afsantin. It is widespread in Himalayas region

of Pakistan and traditionally used as an antiseptic, diuretic, analgesic, antipyretic, diaphoretic, anthelmintic, hypoglycemic, antispasmodic, larvicide, expectorant and tonic. This species is also an effective remedy to treat colic, depression, asthma, rheumatism cancer, dyspepsia, hepatitis, epilepsy, cough, diarrhea, headache, hemorrhage and inflammation [39]. Additionally, anti-nociceptive [40] and hepato-protective [41] properties have been reported in this plant species. *A. vulgaris* is a well known medicinal plant species, which is traditionally used by the inhabitants of Himalayan region-Pakistan to treat various health disorders. However, its composition and properties have rarely been explored. Therefore, the present study was aimed to evaluate phenolics content, *in vitro* antioxidant, antimicrobial, antileishmanial and cytotoxic potential of *A. vulgaris*.

Materials and Methods

Sampling and extraction

Artemisia vulgaris was collected from Galyat Region of Abbottabad, Khyber Pakhtunkhwa, Pakistan and identified and voucher numbered 'PAC1007' by Dr. Muhammad Zafar, Plant Sciences Department, Quaid-i-Azam University, Islamabad-Pakistan. Afterwards plant specimen was submitted in the same herbarium. The aerial parts of plant were shade dried at room temperature and crushed into fine powder. The 2 kg powder was macerated in methanol for 10 days and then filtered. Finally, filtrate was evaporated under reduced pressure to obtain a thick semi-solid paste.

Fractionation

Fractionation was carried out by column chromatography. Column was packed with slurry of silica gel (Merck Cat No.1.07734.1000) prepared in chloroform and 120g dried extract was loaded on the top. The solvent was passed

through the column in increasing order of polarity as 3%, 6%, 10%, 15%, 20% and finally 25% of MeOH/CHCl₃. The behavior of different fractions was observed on TLC plates, developed in 5% MeOH/CHCl₃ and 9 fractions were obtained as HA1, HA2, HA3, HA4, HA5, HA6, HA7, HA8 and HA9.

Determination of total phenolics and flavonoids

Total phenolics content (TPC) in different fractions of *A. vulgaris* (HA1-HA9) fractions was determined using the method as described earlier [42]. In brief, 20 µl of each crude fraction was taken in a test tube, followed by the addition of 90 µl of the Folin-ciocalteu reagent. Samples were incubated for 5 min at room temperature, followed by the addition of 6% sodium carbonate (90 µL). The reaction mixture was again incubated for 1 hr at room temperature before taking absorbance at 715 nm using Gallic acid as standard. TPC was expressed as microgram Gallic acid equivalent per milligram (µg GAE/mg). Data were presented as ± of replicate analysis.

Total flavonoids content (TFC) was determined as explained before [43]. In short, 20 µL of each fraction was added in a separate well of 96 wells plate and 10 µL of potassium acetate was added in each well, followed by addition of 10 µL of 10% aluminum chloride. Afterwards, 160 µL of distilled water was supplemented to reconstitute volume up to 200 µL. The mixture was incubated for half an hour at room temperature. The absorbance of the reaction mixture was taken at 415 nm using quercetin as standard. TFC value was expressed as microgram quercetin equivalent per milligram (µg QE/mg).

Antioxidant assays

The antioxidant potential in different fractions of *A. vulgaris* was evaluated using different assays. DPPH free radical scavenging assay was performed using as reported previously

[44]. Briefly, 20 µL crude fraction was taken in 96 wells plate, followed by addition of 180 µL DPPH reagent. This mixture was incubated at 37°C for 1 hour. Ascorbic acid was used as a positive control, whereas methanol was negative control. Final reading was taken using micro plate reader at 517 nm. DPPH activity was intended by formula

$$\left(\frac{\text{Abs.}_{\text{sample}} - \text{Abs.}_{\text{control}}}{\text{Abs.}_{\text{control}}} \right) \times 100$$

Total antioxidant capacity was measured by phosphomolybdenum method [45]. Initially, 1.63 mL of 0.6 M sulfuric acid, 1.68 g NaH₂SO₄, 0.247 g of ammonium-molybdate was mixed, followed by addition of 50 mL distilled water. Afterwards, 1 mL of this reagent was taken in eppendorf tube and 0.1 mL of the sample was mixed, followed by incubation at 95°C for 90 min. After cooling, the absorbance of the reaction mixture was measured at 695 nm using a microplate reader. Total antioxidant capacity was measured as equivalent of ascorbic acid.

Ferric ion reducing power was estimated using protocol as describe previously [45]. The 0.2 M phosphate buffer, 1% potassium ferricyanide, 10% trichloroacetic acid and 0.1% ferric chloride were used as stock solution. Briefly, 200 µL of each fraction was taken in eppendorf tube and 500 µL of each solution: potassium ferricyanide and buffer were mixed. After incubation at 50°C for 20 min, 500 µL of trichloroacetic acid was mixed and centrifuged at 3000 rpm for 10 min. Then 100 µL of supernatant was poured in respective well of 96 well plate and 0.1% of ferric chloride was added along with 20 µL of distilled water in each well. The readings were taken at 630 nm on micro plate reader.

Antimicrobial assays

Disc diffusion method was used to determine antibacterial activity as reported previously by using one gram positive and two gram negative strains [46]. Antibacterial activity of HA1-HA9 fractions was checked against *Mi-*

Crococcus luteus (*M. luteus*) (ATCC 10240), *Bordetella bronchiseptica* (*B. bronchiseptica*) (ATCC 4617), *Salmonella typhimurium* (*S. typhimurium*) (ATCC 14028) bacterial strains using cefotaxime and DMSO as positive and negative controls, respectively. The test concentration of each fraction used was 20mg/mL. The inoculums of each bacterium were swabbed smoothly on the surface of the sterilized nutrient agar media present in each plate and 5µL of each fraction was loaded on autoclaved filter paper discs, placed on respective places in the petri plates, which were incubated at 37°C for about 24 hrs prior to measure the zone of inhibition. Each plate comprised two discs of positive and one disc of negative control.

The antifungal activity was evaluated using disc diffusion method as described previously [47] using Terbinafine and DMSO as positive and negative controls, respectively. Three fungal strains: *Mucor* species, *Aspergillus fumigatus* (*A. fumigatus*) and *Aspergillus niger* (*A. niger*) were used. Test concentration of each fraction was prepared as 20mg/mL. Assay was performed on solidified sterilized sabouraud dextrose agar (SDA) present in petriplate and different fungal solutions were swabbed on its surface. 5µL of each fraction were loaded on the surface of labeled filter paper disc, then incubated at 28 °C for 24 hrs before measuring the zone of inhibition.

Brine shrimp lethality assay

Brine shrimp lethality bioassay as explained earlier [43] was adopted to study cytotoxic effect in different concentrations: 1000, 100 and 10 µg/mL of each fraction and standard drug 250µL of sea water was added to each well of 96 well plate. Afterwards, 10 brine shrimps (*Artemia salina*) were transferred to each well and volume of the wells was raised up to 300µL to attain desired concentration. These plates were incubated for 24 hours at room temperature. Then shrimps

were taken from each well and survivors counted using magnifying glass. The lethal dose of crude fractions against brine shrimps was calculated using Finny software (1971).

Antileishmanial Activity

Antileishmanial activity was determined according to the protocol previously described [48]. *Leishmania tropica* KWH23 was kept warm at 24±1°C for 7 days in 199 medium having Fetal Bovine Serum (10%). Antileishmanial activity was performed in 96 well plates using different dilutions of stock solution (10,000ppm) of the extract. Amphotericin B and distilled water were used as positive and negative controls, respectively. The 96 well plates were incubated at 24°C for 72 hrs. Afterwards, test culture (15 µL) was transported to Neubauer counting chamber to calculate live promastigotes under microscope.

Results and discussion

Phytochemicals screening

Measured level of TPC and TFC in HA1-HA9 fractions are given in Table 1. TPC ranged from 9.24±0.001 to 26.29±1.4µgGAE/mg with decreasing order: HA8> HA9> HA1> HA3> HA7> HA5> HA6> HA4> HA2. The highest concentration of TPC was determined for HA8, whereas HA2 depicted the lowest value. These values were comparable to the reported levels of TPC in the methanolic extract of other medicinal plants [21-51]. The HA1 fraction exhibited the highest TFC (07.32±0.07 µgQE/mg), whereas the lowest value was calculated for HA3 (06.05±0.06 µgQE/mg). The decreasing order of TFC different fractions was: HA1> HA8> HA7> HA6> HA2> HA5> HA4> HA9> HA3.

Antioxidant activity in *A. vulgaris*

Comparison of DPPH free radical scavenging activity of HA1-HA9 fractions is given in

Table 1. Total phenolics and flavonoids contents in different fractions of *A. vulgaris*

Fractions	TPC ($\mu\text{g GAE/mg}$)	TPC ($\mu\text{g QE/mg}$)
HA-1	18.17 \pm 0.98	07.32 \pm 0.07
HA-2	09.24 \pm 0.01	06.61 \pm 0.06
HA-3	17.28 \pm 0.03	06.05 \pm 0.06
HA-4	09.37 \pm 0.03	06.47 \pm 0.05
HA-5	11.90 \pm 0.10	06.47 \pm 0.01
HA-6	11.36 \pm 0.10	06.75 \pm 0.02
HA-7	13.27 \pm 0.17	07.04 \pm 0.01
HA-8	26.29 \pm 1.40	07.00 \pm 0.02
HA-9	25.81 \pm 1.20	06.47 \pm 0.03

TPC. total phenolics content, TFC. total flavonoids contents, GAE. Gallic acid equivalent, QE. quercitin equivalent

Figure 1. In all fractions, the decreasing order of percentage DPPH scavenging activity was: HA8> HA9> HA1> HA3> HA5> HA7> HA6> HA2> HA4. HA8 fraction depicted the highest percentage DPPH scavenging capacity 82.84 \pm 3.010%, while HA4 showed the lowest scavenging (04.71 \pm 0.001%). These findings were comparable with previous reports [28-49]. Mighri and coworkers in 2010 reported that *A. herba-alba* showed weak antioxidant potential [52]. In another study *Artemisia abyssinica* extracts also showed similar antioxidant potential [53].

Results showing ferric ion reducing power are given in Figure 2, showing decreasing or-

der as: HA9> HA8> HA1> HA3> HA6> HA7> HA5> HA4> HA2. The highest ferric ion reducing power was determined for HA-9 fraction (176.9 \pm 8.45 mg/mL), followed by HA-8 and HA-1 (173.16 \pm 9.54 and 135.5 \pm 4.21 mg/mL, respectively), whereas the lowest value was noted for HA-2 (35.31 \pm 1.9mg/mL). These results were similar to previous studies [21-50].

Total antioxidant capacity of HA1-HA9 fractions of *A. vulgaris* as given in Figure 3 depicted decreasing order as: HA7> HA3> HA5> HA8> HA9> HA4> HA2> HA6> HA1. The HA7 fraction showed the highest total antioxidant capacity, followed by HA-3 and HA-5 (96.25 \pm 3.29,

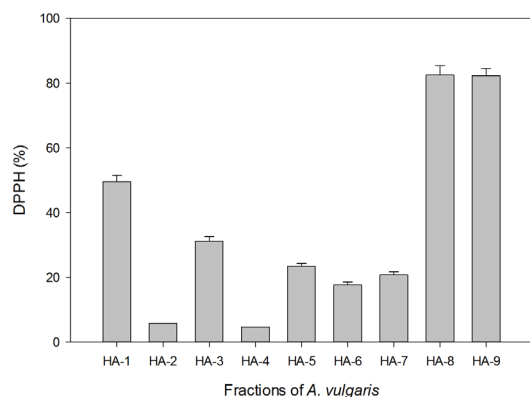


Fig. 1. Comparison of DPPH activity in HA1-HA9 fractions of *A. vulgaris*

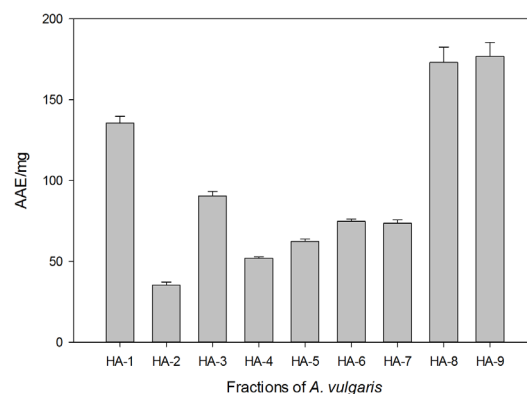


Fig. 2. Ferric ion reducing power in different fractions of *A. vulgaris*

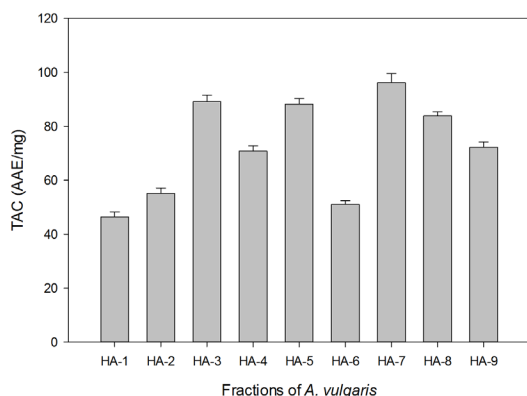


Fig. 3. Total antioxidant activity in different fractions of *A. vulgaris*

89.15±2.41 and 88.26±2.13AAE/mg, respectively), whereas the lowest value was calculated for HA-1 (49.36±1.91). Our results were comparable with previous reports [50].

Antimicrobial potential

Results showing antibacterial potential of HA1-HA9 fractions evaluated using disc diffusion methods against various pathogenic bacteria *S. typhimurium*, *B. bronchiseptica* and *M. luteus* are given in Table 2. Among bacterial strains *S. typhimurium* is a causative agent of salmonellosis in humans which is characterized by fever, acute intestinal inflammation, and diarrhea within 24 h after infection [54]. While *B.*

bronchiseptica is a respiratory pathogen rarely causing infections but recently investigated as a causative agent of bacteremia and pancreatic abscess [55]. *M. luteus* was previously considered as non-pathogenic but recently it is considered an opportunistic pathogen, especially in immunocompromised patients. It is also responsible for nosocomial infections [56]. Keeping in view the pathogenicity of these bacterial strains, they were used in the current study.

All fractions didnot show any activity against *S. typhimurium*. This finding contradicted previous results, in which significant activity was shown by plant extracts against *S. typhimurium* [57]. In addition all fractions exhibited similar antibacterial potential against *B. bronchiseptica* and *M. luteus*. However, these results were in agreement with previous work [43-58]. In 2015, Addo-Mensah and coworkers reported significant antibacterial activities of extracts *A. vulgaris* against *Staphylococcus aureus* and *Bacillus subtilis* [59]. In another study it is reported that *Artemisia dubia* showed antibacterial potential [60].

Antifungal activity of HA1-HA9 fractions was studied against fungal strains such as *Mucor* species, *A. niger* and *A. fumigatus* (Table 2). Two fractions HA1 and HA2 showed activity against *A.niger* and *Mucor spp.*, with values

Table 2 Antibacterial and antifungal activities (% inhibition) ofHA1-HA9fractions

Fractions	<i>Bacterial strains</i> (mm zone of inhibition)			<i>Fungal strains</i> (mm zone of inhibition)		
	<i>S. typhimurium</i>	<i>B. bronchiseptica</i>	<i>M. luteus</i>	<i>A. niger</i>	<i>Mucor spp</i>	<i>A. fumigatus</i>
HA1	---	6.000±00	6.000±00	11.00±0.11	08.50±0.71	07.50±0.71
HA2	---	6.000±00	6.000±00	---	13.25±0.35	08.00±10.41
HA3	---	6.000±00	6.000±00	---	9.625±0.88	06.50±0.71
HA4	---	6.000±00	6.000±00	---	10.75±3.18	02.50±3.54
HA5	---	6.000±00	6.000±00	---	09.75±0.35	02.50±3.54
HA6	---	6.000±00	6.000±00	---	06.50±0.71	03.00±4.24
HA7	---	6.000±00	6.000±00	---	07.50±2.12	---
HA8	---	6.000±00	6.000±00	---	06.25±0.35	---
HA9	---	6.000±00	6.000±00	---	07.00±1.41	07.00±1.41

of 11.00 ± 0.11 and 13.25 ± 0.35 mm respectively. Likewise, in other medicinal plant species significant activity has been reported against this fungal species [28]. All other fractions also showed activity against *Mucor* spp., such as HA4, HA5 and HA3 (10.75 ± 3.18 , 09.75 ± 0.35 and 9.625 ± 0.88 mm zone of inhibition, respectively). These results were in agreement with previous results [58].

Brine shrimp lethality assay

The cytotoxic effects of HA1-HA9 fractions determined by using brine shrimp lethality assay are given in Figure 4. As cytotoxicity is inversely proportional to LD_{50} , therefore HA1 showed significant cytotoxic effect because it has the lowest LD_{50} value. However, LD_{50} value was the highest for HA7 fraction, which depicts the lowest cytotoxic effect. The decreasing order of cytotoxicity was: HA1 > HA8 > HA4 & HA5 > HA3 > HA2 > HA6 > HA9 > HA7. These findings were in agreement with previous work [58].

Antileishmanial activity

Antileishmanial activity of HA1-HA9 fractions is given in Figure 5. In general, all fractions showed significant response except HA8 and HA9, as 0% survival of leishmania causing protozoa except fraction HA1 which showed

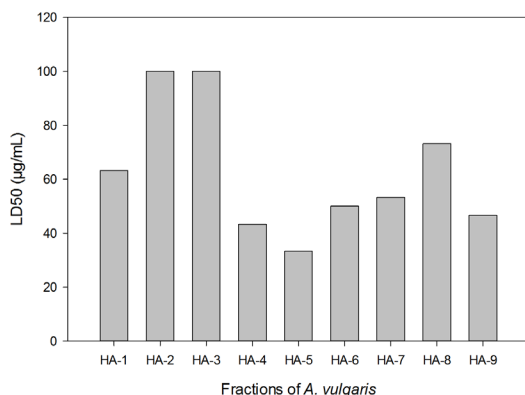


Fig. 4. Cytotoxic effects of *A. vulgaris* fractions

19% survival was calculated. These results were in agreement with various reports [59].

Conclusions

The present study revealed that aerial parts of *A. vulgaris* possess significant potential as antioxidant, antimicrobial and antileishmanial agent. Their antileishmanial activity is very promising, which may cope with the modern scenario of discovering new resources of drugs.

Conflicts of interest

We have no conflict of interest.

Authors' contribution

AH and MA conducted research work and prepared initial drafts of MS, SAK and NF helped in data analysis, AMA and AM supervised the project, were involved in writing and data interpretation.

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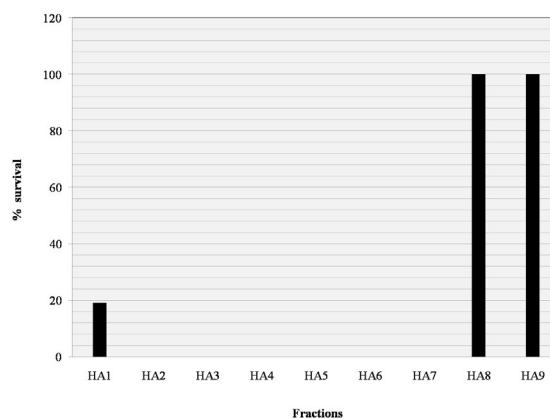


Fig. 5. Antileishmanial activity of HA1-HA9 fractions.

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