

In vitro Antioxidant, Antimicrobial and Antiprotozoal Activities of Ethanolic Extract and its Various Fractions from *Asphodelus fistulosus* Seeds

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ABSTRACT

Introduction: *Asphodelus fistulosus* (Asphodelaceae) has been traditionally used in the treatment of fungal and dermatomucosal infections. The present study aims to evaluate the antioxidant, antimicrobial and antiprotozoal activities of the ethanolic extract and its various fractions of seeds of the plant. **Materials:** *Asphodelus fistulosus* seeds were collected from Albaha region of Saudi Arabia. The chemicals used for the research work were purchased from Sigma Aldrich, Merck Germany and Loba India and used directly without further purification. **Methods:** The seeds were clean, dried and grinded to make it fine powder and extracted with 95% ethanol, ethanol and water were evaporated to get crude ethanolic extract. The crude ethanolic extract was fractionated into different fraction such as petroleum ether, dichloromethane and methanol. The extract and its various fractions were evaluated for antioxidant, antimicrobial and antiprotozoal activities. **Results:** The results of DPPH scavenging effect was found to be maximum for dichloromethane fraction followed by methanolic fraction and then ethanolic extract and petroleum ether fraction. The ethanolic crude extract, petroleum ether, dichloromethane and methanolic fraction were found to be active against the tested fungal strain *Candida albicans*. It was found that dichloromethane fraction was active against *Trypanosoma cruzi* and *L. infantum*. **Discussion:** The results of antioxidant study indicated that *A. fistulosus* contain polyphenols, proving them to be perfect sources of antioxidants. The plant showed significant antimicrobial and antiprotozoal activities. The metabolites, anthraquinones, flavonoids, terpenes and phenolic acids could be responsible for such activity of this plant. **Conclusion:** The results of antioxidant, antimicrobial and antiprotozoal activity of *Asphodelus fistulosus* supports its traditional use for the treatment of fungal and dermatomucosal infections.

Key words: *Asphodelus fistulosus*, Total phenolic content, Antiprotozoal, Antimicrobial, Antioxidant.

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INTRODUCTION

Nature has blessed mankind with medicinal plants to pursue a healthy life. Most of the drugs used in primitive medicine were obtained from plants and are principal

natural source of medicines. Plants serve as a reservoir of potentially useful biomolecules with unique properties which make them attractive candidates for the development of novel antimicrobial and antiprotozoal agents.^[1,2] Plant derived drugs have recently received more attention because of the increasing prevalence of antibiotic resistant bacteria and cost of the treatment.

Asphodelus fistulosus belongs to family Asphodelaceae. It is commonly known as hollow stemmed asphodel and onion weed.^[3,4] *A. fistulosus* is traditionally used for the

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treatment of dermatomucosal and fungal infections in various countries including Cyprus, Egypt, Libya, Palestine and Spain.^[5,6] The plant has been found to possess number of important biological activities like antileishmanial, antimalarial, cytotoxic, antimicrobial, anti-MRSA, anti-acne, lipoxygenase and tyrosinase inhibitory-activity.^[7-11] Chemical analysis of *A. fistulosus* reveals array of compounds including triterpenoids flavanoids, anthraquinones, carbohydrates and fatty acids.^[12-16] On the basis of the traditional uses of this plant, the present investigation was carried out to evaluate the antioxidant, antimicrobial and antiprotozoal activities of *Asphodelus fistulosus*.

MATERIALS AND METHODS

Plant material

The seed of *A. fistulosus* was procured from the Al Baha (Al Jadyah village) and were authenticated by taxonomist Dr Haider, Department of Chemistry, Al Baha University Kingdom of Saudi Arabia.

Extraction

The seeds of *A. fistulosus* were harvested and shade dried for 2-3 days and coarsely powdered. The ground part (2.5 kg) were extracted with 95% ethanol and the crude ethanolic extract (440 gm; 17 %w/w) were extracted with different solvents in increasing polarity of the solvent to get Petroleum ether fraction (374 gm), Dichloromethane fraction (930 gm) and methanol fraction (36 gm) in a Soxhlet apparatus. The extracts were concentrated under reduced pressure and kept at 4°C until further use. The extract and its successive fractions were evaluated for DPPH-assay, antimicrobial (antibacterial, antifungal) and antiprotozoal activity.

Chemicals

DPPH (2,2-diphenyl-1-picrylhydrazyl), Ascorbic acid (Vitamin C), NBT (nitro blue tetrazolium), riboflavin, PMS (phenazine methosulphate), Folin-Ciocalteu reagent, sodium carbonate, amoxicillin, fluconazole, benznidazole, suramin, chloroquine, miltefosine, SDA (Sabouraud dextrose agar)

Antioxidant activity

DPPH radical scavenging activity

The antioxidant activity were evaluated by measuring the reduction of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The samples (10-500 µg/mL) were dissolved in methanol and then added to a methanol solution of DPPH (60 mM).^[17] After 30 min, the UV absorbance of the resulting solutions was recorded at

λ 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. Vitamin C was used as the positive control. The free radical scavenging activity was calculated as a percentage inhibition of the DPPH radical by the sample or positive control.

$$\% \text{ scavenging} = [(A_0 - A_1) / (A_0)] \times 100$$

A₀ = control absorbance; A₁ = sample absorbance

Superoxide anion scavenging assay

The assay was done by riboflavin light NBT system.^[17] Briefly, 1 ml of sample was taken at different concentration (25 to 500 µg/ml) and mixed with 0.5 ml of phosphate buffer (50 mM, pH 7.6), 0.3 ml riboflavin (50 mM), 0.25 ml PMS (20 mM) and 0.1 ml NBT (0.5 mM). Reaction was started by illuminating the reaction mixture using a fluorescent lamp. After 20 min of incubation, the absorbance was measured at 560 nm. Ascorbic acid was used as standard. The scavenging ability was determined by following formula:

$$\% \text{ scavenging} = (1 - A_1 / A_0) \times 100$$

A₀ = control absorbance; A₁ = sample absorbance

Estimation of Total Phenolic content

Method to determine total polyphenolics content was adapted from Alves *et al.*^[17] The assay consisted leaching of 100 mg of the extract with 250 ml of methanol/water (60:40, v/v, 0.3% HCl) and filtering it through a 0.45 µm Millipore filter. 100 µl of filtrate so obtained was mixed with 100 µl of 50 % Folin-Ciocalteu reagent and 2 ml of 2.5% sodium carbonate. After incubating at room temperature for 2 hours, the absorbance was measured at 750 nm. Quantitation was based on standard curve of gallic acid (0~0.5 mg/ml) dissolved in methanol/water (60:40, v/v; 0.3 % HCl).

Antimicrobial activity

Disc Diffusion Method

All the antimicrobial studies were performed at Albaha Regional Research Laboratory, Albaha, Kingdom of Saudi Arabia. The crude extracts and the different fractions were dissolved in dimethylformamide (DMF) to prepare chemicals of stock solution of 5 mg/mL and simple susceptibility screening test was carried out using reported disc diffusion method.^[18] Each microbial strain was suspended in Mueller Hinton (MH) (Difco, Detroit, MI) broth and diluted approximately to 10⁶ colony forming unit (cfu)/mL. They were "flood-inoculated" onto the surface of MH agar and Sabouraud Dextrose Agar (SDA) and then dried. For *Candida albicans*, SDA were used. For *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, Macconcy agar was used and for *Escherichia coli*

and *Staphylococcus aureus* Muller Hinton agar were used. Six-millimeter diameter disc were prepared and 500 µg and 250 µg of the extracts were loaded. Antimicrobial activity was evaluated by measuring the zone of inhibition against the tested organism. Amoxicillin (20, 10 µg) and Fluconazole (10 µg) were used as standard drugs. Dimethylformamide was used as solvent (negative controls).

Antiprotozoal activity

Standard Drugs

For the different tests, appropriate reference drugs were used as positive control: chloroquine for *P. falciparum*, miltefosine for *L. infantum*, benznidazole for *T. cruzi* and suramin for *T. brucei*. The integrated panel of microbial screens and standard screening methodologies were adopted as previously described.^[19] All assays were performed in triplicate at Albaha regional research laboratory, Albaha, Kingdom of Saudi Arabia. Plant extracts were tested at concentrations (10-100 µg/mL) to establish a full dose-titration and determination of the IC₅₀ (inhibitory concentration 50%).

Antiplasmodial Activity

Chloroquine-resistant K1-strain was cultured in human erythrocytes O⁺ at 37°C under a low oxygen atmosphere (3% O₂, 4% CO₂ and 93% N₂) in RPMI-1640, supplemented with 10% human serum. Infected human red blood cells (200 µL, 1% parasitaemia, 2% hematocrit) will be added to each well and incubated for 72 h. After incubation, test plates were frozen at 20°C. Parasite multiplication was measured using the Malstat assay, a colorimetric method based on the reduction of 3-acetylpyridine adenine dinucleotide (APAD) by parasite-specific lactate-dehydrogenase (pLDH).^[20]

Antileishmanial Activity

L. infantum MHOM/MA(BE)/67 amastigotes was collected from the spleen of an infected donor hamster and will be used to infect primary peritoneal mouse macrophages. For *in-vitro* antileishmanial activity, 3 × 10⁴ macrophages were seeded in each well of a 96-well plate. After 2 days outgrowth, 5 × 10⁵ amastigotes/well, was added and incubated for 2 h at 37°C. Pre-diluted plant extracts was subsequently added and the plates was further incubated for 5 days at 37°C and 5% CO₂. Parasite burdens (mean number of amastigotes/macrophage) will be microscopically assessed after Giemsa staining on 500 cells and expressed as a percentage of the blank controls without plant extract. The results are expressed as IC₅₀ (inhibitory concentration 50%).

Antitrypanosomal Activity

Trypanosoma brucei Squib-427 strain (suramin-sensitive) was cultured at 37°C and 5% CO₂ in Hirumi-9 medium,^[21] supplemented with 10% fetal calf serum (FCS). About 1.5 × 10⁴ trypomastigotes/well will be added to each well and parasite growth will be assessed after 72 h at 37°C by adding resazurin.^[22] For Chagas disease, *T. cruzi* Tulahuen CL2 (benznidazole-sensitive) strain will be maintained on MRC-5 cells in minimal essential medium (MEM) supplemented with 20 mM L-glutamine, 16.5 mM sodium hydrogen carbonate and 5% FCS. In the assay, 4 × 10³ MRC-5 cells and 4 × 10⁴ parasites will be added to each well and after incubation at 37°C for 7 days, parasite growth will be assessed by adding the β-galactosidase substrate chlorophenol red β-D-galactopyranoside.^[23] The results are expressed as IC₅₀ (inhibitory concentration 50%).

RESULTS

Antioxidant Activity

The results of DPPH assay and superoxide scavenging assay are shown in Figure 1 and 2. From Figure 1, it can be seen that the DPPH scavenging effect was found to be maximum for dichloromethane fraction followed by methanolic fraction and then ethanolic extract. Petroleum ether fraction was least active. From the superoxide scavenging results as shown in Figure 2, the methanolic fraction scavenge more superoxide radical than the dichloromethane fraction followed by ethanolic extract and then Petroleum ether fraction. Although the DPPH and superoxide scavenging of the extracts and different fractions are less than the standard Vitamin C. these results are supported from the results of total phenol contents of the extract and its fractions. Total phenolic content of the extract and different fractions was solvent dependent and expressed as mg of gallic acid equivalent. The results are summarized in Figure 3. The methanolic fraction showed highest phenolic content (124.1±3.2) whereas dichloromethane and ethanolic extract showed phenolic content of 92.7±2.8 and 88.5±1.3, respectively.

Antimicrobial activity

In the present study, the disc diffusion observations reveal that ethanolic crude extract, petroleum ether, dichloromethane and methanolic fraction were active against the tested fungal strain *Candida albicans*, whereas the ethanolic crude extract and various fraction showed moderate activity against the tested gram positive and gram negative strains. From the results as shown in

Table 1: *In vitro* Antiprotozoal activity of ethanolic crude extract and its various fractions of *Asphodelus fistulosus*.

Extract & Fractions	Antitrypanosomal activity ^a		Antileishmanial activity ^a	Antiplasmodial activity ^b
	<i>T. cruzi</i>	<i>T. brucei</i>	<i>L. infantum</i>	<i>P. falciparum</i>
Ethanolic extract	63.5	43.8	50.4	51.2±4.2 (8.9±1.3)
Petroleum ether fraction	58.3	25.2	44.2	68.2±1.8 (5.7±0.3)
DCM fraction	20.8	58.9	39.8	45.9±3.2
Methanolic fraction	>100	79.6	>100	11.4±2.4
Benznidazole	2.0	---	---	---
Suramin	---	3.0	---	---
Miltefosine	---	---	2.0	---
Chloroquine	---	---	---	(0.7)

^aThe antileishmanial and antitrypanosomal activity of the extract and its various fractions are expressed as IC₅₀ values in µg/ml. Highly Active= IC₅₀ <10 µg/ml; Active= IC₅₀ : 10-50 µg/ml; Moderate active= IC₅₀ : 50-100 µg/ml; Inactive= IC₅₀ >100 µg/ml; *T. cruzi*: *Trypanosoma cruzi*; *T. brucei*: *Trypanosoma brucei*; *L. infantum*: *Leishmania infantum*; *P. falciparum*: *Plasmodium falciparum*
^bAntiplasmodial activity is expressed as percentage of inhibition at 10.0 µg/ml. Values in parenthesis correspond to IC₅₀ value.

Table 2: Antimicrobial activity of ethanolic crude extract and various fractions of *Asphodelus fistulosus*.

Extracts	Antibacterial Activity ^a 500 (250) µg/disc								Antifungal Activity ^a
	Gram Positive Bacteria				Gram Negative Bacteria				Yeast
	S.e	S.a	E.f	S.p	E.c	K.p	P.m	P.a	C.a
Ethanolic extract	12 (---)	---	14 (10)	12(---)	16(10)	14(10)	10(---)	10(---)	18 (12)
Petroleum ether fraction	10 (---)	---	16 (12)	18(12)	16(12)	16(12)	12 (---)	---	16 (10)
DCM fraction	14 (10)	12 (---)	18 (13)	10(---)	12(8)	14(10)	14 (8)	10 (---)	14 (8)
Methanolic fraction	12 (8)	10 (---)	10 (---)	12(8)	12(8)	15(10)	-----	---	12 (---)
Amoxicillin	18 (12)	>26	16 (12)	18(13)	26(16)	24(14)	24(16)	20(10)	NT
Fluconazole	NT	NT	NT	NT	NT	NT	NT	NT	16

^aActivity is expressed as zone of inhibition at two concentrations, 500 µg/disc and 250 µg/disc
 For antibacterial activity, amoxicillin (20 and 10 µg/disc) is used as a standard drug; for antifungal activity, fluconazole (10 µg/disc) is used as a standard drug.
 S.e: *Staphylococcus Epidermidis* (ATCC 12228); S.a: *Staphylococcus aureus* (ATCC 25923); E.f: *Enterococcus faecalis* (ATCC 29212); S.p: *Streptococcus pneumoniae* (ATCC 49619); E.c: *Escherichia coli* (ATCC25922); K.p: *Klebsiella pneumoniae* (ATCC 700603); P.m: *Proteus mirabilis* (ATCC 13376); P.a: *Pseudomonas aeruginosa* (ATCC 27853); C.a: *Candida albicans* (ATCC10231); NT: Not Tested; ---: No zone of inhibition

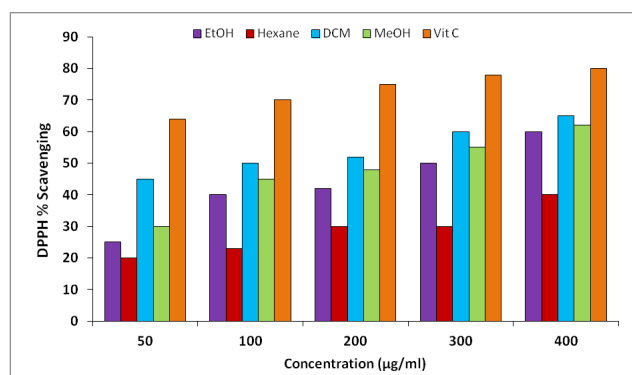


Figure 1: DPPH radical scavenging activity of ethanolic crude extract and its various fractions of *Asphodelus fistulosus*. Each value represents a mean ±SD (n=3). EtOH: Ethanolic crude extract; Pet. ether: Petroleum ether; DCM: dichloromethane fraction; MeOH: methanolic fraction; Vit C: Vitamin C (standard).

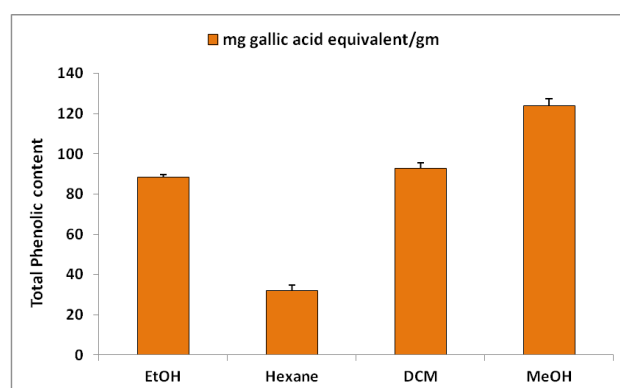


Figure 3: Total phenolic content (expresses as mg gallic acid equivalent/gm) of ethanolic crude extract and its various fractions of *Asphodelus fistulosus*. EtOH: Ethanolic crude extract; Pet. ether: Petroleum ether; DCM: dichloromethane fraction; MeOH: methanolic fraction.

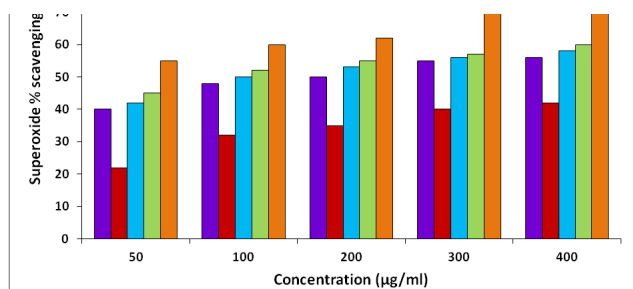


Figure 2: Superoxide radical scavenging activity of ethanolic crude extract and its various fractions of *Asphodelus fistulosus*. Each value represents a mean $\pm$ SD (n=3). EtOH: Ethanolic crude extract; Pet. ether: Petroleum ether; DCM: dichloromethane fraction; MeOH: methanolic fraction; Vit C: Vitamin C (standard).

Table 2, it was observed that gram negative bacterial strains were more sensitive towards the various extracts compared to tested gram positive bacterial strains. The petroleum ether and dichloromethane fraction showed more activity compared to crude ethanolic extract and methanolic fraction. The ethanolic crude extract were more active against *E. coli* and *C. albicans*, showing zone of inhibition of 16 (500 µg/disc) and 10 mm (250 µg/disc) for *E. coli* and zone of inhibition of 18 (500 µg/disc) and 12 (250 µg/disc) for *C. albicans*. Similarly, the petroleum ether fraction was active against two bacterial strains, *E. faecalis* and *S. pneumoniae*. showing zone of inhibition of 16, 12 mm for *E. faecalis* and 18, 12 mm for *S. pneumoniae* at 500 µg/disc and 250 µg/disc respectively which were comparable to the standard drug amoxicillin at concentration of 20 and 10 µg/disc. The ethanolic crude extract and petroleum ether fraction showed comparable zone of inhibition against *C. albicans* at 500 µg/disc with the standard drug fluconazole at concentration of 10 µg/disc.

Antiprotozoal activity

The antiprotozoal activity (IC_{50}) for the extract and different fraction are shown in Table 1. From the table, it was observed that dichloromethane fraction was active against *Trypanosoma cruzi* (IC_{50} =20.8µg/ml) whereas ethanolic extract (IC_{50} =63.5 µg/ml) and Petroleum ether fraction (IC_{50} =58.3µg/ml) were moderately active against *Trypanosoma cruzi*. Further, it can be seen that the ethanolic extract (IC_{50} =43.8 µg/ml) and Petroleum ether fraction (IC_{50} =25.2µg/ml) was found to be active against *Trypanosoma brucei* whereas two fractions, dichloromethane and methanolic were moderately active. The antileishmanial activity (Table 1) indicated that the ethanolic extract was active against *L. infantum* (IC_{50} = 50.4µg/ml). Among its fractions, Petroleum ether (IC_{50} = 44.2

µg/ml) and dichloromethane fractions (IC_{50} = 39.8 µg/ml) were active whereas methanolic fraction was found to be inactive (IC_{50} >100 µg/ml). On the other hand, ethanol extract and Petroleum ether fraction exhibited good activity against *Plasmodium falciparum* (IC_{50} <10µg/ml). The dichloromethane and methanolic fractions were inactive against *P. falciparum*.

DISCUSSION

The production of oxygen and free radicals in the body, probably involves the development of many diseases such as inflammation, cancer, rheumatoid arthritis, Parkinson's and Alzheimer's diseases.^[24] The natural antioxidants cause less toxic side effect and can provide protection against oxidative degradation therefore they are useful in the treatment of these diseases. It has been observed that the extracts and fractions of the plant possess antioxidant property. The high antioxidant properties of dichloromethane fraction and methanolic fraction may be attributed due to the presence of polyphenols. The antioxidant results reveal that the polyphenols are present in the extract and its fractions. The results of the antioxidant study indicate that *A. fistulosus* contain polyphenols, proving them to be perfect sources of antioxidants. *A. fistulosus* is traditionally used for the treatment of dermatomucosal and fungal infections in various countries including Cyprus, Egypt, Libya, Palestine and Spain.^[5,6] *Asphodelus* spp extracts have antiseptic and anti-inflammatory properties, antifungal and antimour activities.^[25] Anthraquinones, flavonoids, terpenes and phenolic acids are the most important metabolites.^[12-16] These metabolites could be responsible for antiprotozoal activity of this plant.

CONCLUSION

The present study shows the antioxidant, antimicrobial and antiprotozoal activities of *Asphodelus fistulosus*. The tested plant showed significant radical scavenging activity suggesting that the plant can be used as a source of antioxidants. The antimicrobial antiprotozoal activities showed significant results against tested pathogens. The present study supported the traditional use of this plant as antifungal and antiparasitic agents. Finally we conclude that *Asphodelus fistulosus* may be a potent medicinal plant for the treatment of microbial and protozoal infections. The work is in progress to isolate and to characterize the active compounds present in the ethanolic extract and the various fractions of *Asphodelus fistulosus*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

NBT: Nitro Blue Tetrazolium; **PMS:** Phenazine Methosulphate; **SDA:** Sabouraud Dextrose Agar; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **UV:** Ultra Violet; **APAD:** 3-acetylpyridine adenine dinucleotide; **pLD:** parasite-specific lactate-dehydrogenase; **FCS:** Fetal Calf Serum ; **DCM:** Dichloromethane; **IC:** Inhibition Concentration; **DMF:** Dimethylformamide; **MH:** Mueller Hinton; **MEM:** Minimal Essential Medium; **EtOH:** Ethanol Extract.

SUMMARY

The present study shows that *Asphodelus fistulosus* extract and its various fractions exhibited significant antioxidant, antimicrobial and antiprotozoal activities. Among different fractions, DPPH scavenging effect was found to be maximum for dichloromethane fraction followed by methanolic fraction and then ethanolic extract whereas the methanolic fraction scavenge more superoxide radical than the dichloromethane fraction followed by ethanolic extract and then petroleum ether fraction. The antimicrobial activity was significantly exhibited by the petroleum ether and dichloromethane fraction against tested microbial strains. Dichloromethane fraction was active against *Trypanosoma cruzi* and *L. infantum* whereas and petroleum ether fraction exhibited good activity against *Plasmodium falciparum*.

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