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Research Article

Antifungal and antioxidant activity of stem bark extracts of *Ficus religiosa* L.

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Abstract

Aspergillus niger is a major cause of black moulds, stem and root rots of several plant species. These diseases are mainly controlled by using agrichemicals. However, these chemicals have adverse effects on environment. To avoid the use of synthetic toxic chemicals, researchers are focusing on plant derived natural products which can inhibit the growth of these pathogens. The present study is based on evaluating different solvent fractions of stem bark of *Ficus religiosa* L. for antifungal activity against *A. niger* at 0.25, 0.5, 1 and 2 mg/mL concentrations using agar dilution method. Antioxidant potential of the extracts was also determined by DPPH radical scavenging assay. The plant showed significant antifungal activity with highest activity observed for the *n*-butanol fraction showing 66 to 77.5% inhibition of fungal growth at the tested concentrations. Least active was the ethyl acetate fraction with % inhibition ranging from 47.5% to 62.5% at the tested concentrations. The extracts also showed significant antioxidant activity with highest activity of 91.71% observed for dichloromethane fraction. Phytochemical analysis indicated the presence of glycosides, alkaloids, saponins, tannins, flavonoids, triterpenes, sterols and phenols in the extracts. The study concluded that the stem bark of *F. religiosa* can be a good source of antifungal compounds with antioxidant activity.

Keywords: *Ficus religiosa*; *Aspergillus niger*; Antifungal activity; Antioxidant activity; DPPH

Introduction

Fungi are found all over the world, attacking plants and causing diseases **[1]**. Fungi have been the cause of many famine and economic losses due to the diseases they cause and are reported in the history of many countries **[2, 3]**. The number of fatalities due to fungal diseases is often vast, because in addition to its effect on field and yield, losses to storage products are also considered **[4]**. Beside this, it may cause carcinogenicity, reproductive disorders, genotoxicity, nephrotoxicity, terratogenicity, hepatotoxicity, and immunosuppression **[5, 6]**.

Aspergillus spp., *Alternaria* spp., *Fusarium* spp., and *Penicillium* spp. are the most common postharvest and storage fungi of fruits **[7]**. In developing countries there are unfit storage facilities, inappropriate packaging and transportation which make the post-harvest product quite difficult to handle or market. Low temperature and high humidity are required to store and transport fresh products as low temperature lowers down the microbiological, biochemical and physiological activities which help to maintain the quality **[8]**. A large portion of agriculture produce in the world over

becomes unfit for consumption by man due to contamination of grains by mycotoxins produced by various fungal pathogens especially species of *Aspergillus*. Approximately 25% of the cereals all over the world are known to contain mycotoxins and there are almost 300 fungal metabolites that are known to be toxic to animals and human beings [9]. Approximately 400 distinct mycotoxins have been discovered. The most significant among them are aflatoxins and ochratoxin A [10]. Species of *Aspergillus* and *Penicillium* produce ochratoxin A. Ochratoxin A have numerous detrimental effects. It is neurotoxic, nephrotoxic, genotoxic, hepatotoxic, teratogenic and has immunosuppressive properties [11]. *Aspergillus niger* is the causal organism for black mould of garlic, onion and shallot; root stalk rot of Sansevieria; stem rot of Dracaena; and boll rot of cotton. The fungus causes spoilage of figs, vanilla, cashew kernels, dates, pods and dried prune and lives as a saprophyte in soil. Crown rot of groundnut is the most serious plant disease caused by *A. niger* [12]. As *Aspergillus* crown rot is a seed-borne disease so it drastically affects plant in the crop's life cycle and cause certain losses. They are recorded as frequent as two plants per meter of planted row [13]. *A. niger* also causes otomycosis, an ear infection in humans [14], pulmonary infections including pneumonia [15] and tracheobronchitis [16]. Agrichemicals are quite vital to fight against plant diseases and in maintaining high crop yields. However, excessive use of these chemicals has adverse effect on environment, including ecological imbalance of soils and making plants more susceptible to diseases and pests [17]. Increasing public awareness on environmental issues demands alternative disease management systems, which are dependent to a lesser extent on synthetic chemicals or based on naturally occurring compounds [18]. The plants are a rich source of phytochemicals that can be used as pesticides and are more ecofriendly than synthetic chemicals [19].

Approximately two-third of the world's population depends on ethnomedicines. Almost 7000 medicative/officinal compounds that are being utilized in drug formulation are plant based. This made natural-product chemists, botanists, ethno-pharmacologists and microbiologists to explore phytochemicals of medicinal importance [20]. Antifungal compounds are present in a number of plant species, which are used to restrain fungal pathogens [21]. In search for new standardized antifungals, green plants are an excellent choice, as secondary metabolites are cosmopolitan among green plants [22]. Genus *Ficus* (Moraceae) consists of about 40 genera and 1400 species of trees, shrubs, vines and herbs [23]. *Ficus religiosa* L. is one of the most important species of the genus widely used in traditional medicines. Stem bark of this plant is known to possess several biological activities and is used for the treatment of skin diseases, nervous disorders, diabetes, anxiety, urino-genital disorders, wounds and hemorrhoids [24] [25-27]. The present study was carried out to study the antifungal activity of the stem bark extracts of the plant against *A. niger*.

Materials and methods

Chemicals

All the solvents used for extraction were of analytical grade, purchased from Fischer scientific. Reagents used for phytochemical analyses, antioxidant activity and antifungal activity were purchased from Sigma Aldrich.

Equipment

In order to concentrate the extracts Rota vapor R-210 was used. UV-Visible spectra were obtained using MeOH with UV-Vis spectrophotometer (UV-6000).

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Collection and identification of plant material

Stem bark material of *F. religiosa* was collected from Lahore College for Women University, Jail Road, Lahore. For identification of plants, Flora of Pakistan and other available literature was consulted.

Extraction and fractionation

The stem bark was grounded to give fine powder. The powdered material was weighed and extracted in methanol at room temperature. The solvent was filtered and recovered in rotary evaporator at 35^0C to obtain crude methanol extract. The crude extract was dissolved in distilled water and subjected to liquid-liquid partitioning for initial separation of compounds based on their relative solubility in solvents of different polarities. For this purpose the crude extract suspended in distilled water was sequentially partitioned using *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. All the fractions were concentrated in rotary evaporator and were stored at a temperature of 4^0C till further use.

Table 1. Extraction yield of different solvent extracts of *F. religiosa*

Dry weight (g)	Crude methanol (g)	*n*-hexane (g)	Dichloro methane (g)	Ethyl acetate (g)	*n*-Butanol (g)	Aqueous (g)
120.57	8.4	1.1	4.56	0.42	0.8	0.1
% Yield	7 %	13 %	55%	5%	9%	1.1%

Phytochemical analysis

Qualitative phytochemical analysis of the extracts was carried out for the determination of preliminary phytoconstituents present in the plant extract using standard methods [28].

Tests for alkaloids

a) *Dragendorff's test*: For detection of alkaloids, 3-5 drops of Dragendorff's reagent (solution of potassium bismuth iodide) were added in extract solution prepared. After sometime, orange yellow precipitation were formed which confirmed the presence of alkaloids.

b) *Tannic acid test*: Extract of both plants was treated with tannic acid. Formation of buff color precipitates ensured the presence of alkaloids.

Tests for glycosides

For the detection of glycosides, 1 mL of KOH (10%) was added in 1mL of extract. Appearance of brick red precipitates indicated the presence of glycosides.

Tests for flavonoids

a) Alkaline Reagent Test: To the solutions of extract prepared few drops of 10% NaOH solution were added. Appearance of yellow color which disappeared on the addition of dil. acid indicated the presence of flavonoids.

b) In 3 mL of plant extract, 4 mL of 1% KOH was added. Appearance of dark yellow color was indicative of flavonoids.

c) Few drops of $AlCl_3$ were added to 2 mL of each plant sample. The solution turned yellow indicating flavonoids.

d) A few drops of conc. HCl were mixed with 2 mL of plant extract. Appearance of red color indicated the presence of flavonoids.

Tests for saponins

For detection of saponins 2 mL of the extract was mixed with 2 mL of distilled water and vigorously shaken. Formation of stable foam confirmed the presence of saponins.

Tests for xanthoproteins

In 1 mL of extract solution added few drops of conc. HNO_3 and NH_3 solution separately. Appearance of reddish orange precipitates detected the presence of xanthoproteins.

Tests for phenols

a) Ferric Chloride Test: To 2 mL of plant extract, 5% $FeCl_3$ were added. A deep blue coloration of the solution confirmed the presence of phenols.

b) Nitric acid test: Bark extract mixture was treated with dilute HNO_3. Appearance of reddish to yellow color confirmed the presence of phenols.

Tests for tannins

a) Ferric chloride test: Addition of 3 drops of 5% $FeCl_3$ to I mL of extract led to the production of a blue or greenish-black color which changed to olive green color on progressive addition of $FeCl_3$ indicating the presence of tannins.

b) Potassium dichromate test: 5mL of the extract was mixed with 1mL of potassium dichromate solution (10%). Appearance of yellow precipitates indicated the presence of tannins.

Tests for terpenoids

a) In 1 mL of bark extract added 5 drops of conc. H_2SO_4. Formation of red/blue green coloration indicated the presence of terpenoids.

b) To 5 mL of plant sample were added 2 mL of $CHCl_3$and 3 mL of conc. H_2SO_4. The presence of terpenoids was confirmed by formation of an interface with reddish brown coloration.

Test for sterols

In 5 mL extract solution, 2 mL of $CHCl_3$ was added. To the mixture formed added 3 mL of conc. H_2SO_4 carefully along the side of wall of test tube forming a layer. Formation of fused reddish brown coloration indicated the presence of terpenoids.

Tests for quinones

Conc. HCl was added in 1 mL of plant extract. Formation of yellow precipitates confirmed the presence of quinones.

Tests for anthraquinones

To each plant extract was added the solution of NaOH. Appearance of violet or pink color in base layer confirmed the presence of anthraquinones.

Determination of total phenolic content

For estimation of total phenols20 µl of each sample were mixed with 158 µl of de-ionized water and 100 µl of FC reagent and incubated for 10 min at room temperature. After incubation 300 µl of Na_2CO_3 solution (25% w/v) was added to the mixture and was again incubated at 40°C for 30 minutes and finally cooled to room temperature. Absorbance was measured at 765 nm against the methanol used as blank [29]. A calibration curve of the standard gallic acid was constructed under similar conditions (Figure 1) and TPC, measured as mg gallic acid equivalent (GAE)/g dry extract (dE), was calculated using the equation:

$y = 0.086x + 0.0065$ $R^2 = 0.842$

Where y is the absorbance and *x* is the concentration of gallic acid.

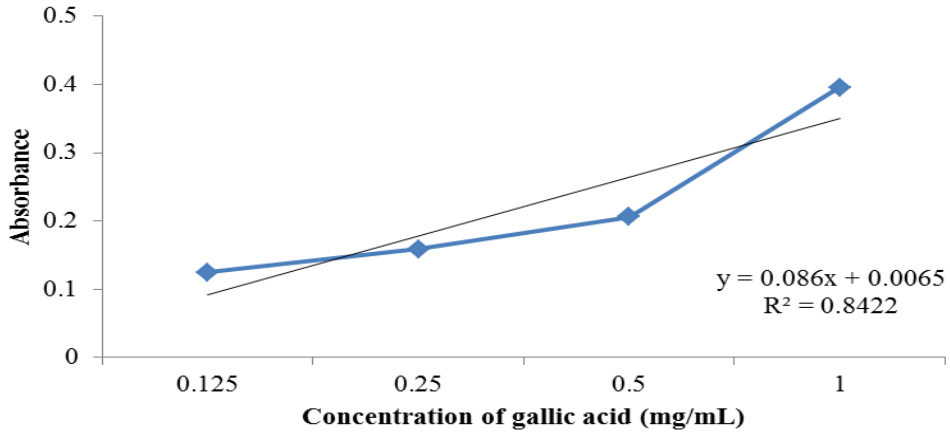

Figure 1. Calibration curve of gallic acid

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Determination of total flavonoid content

Flavonoid contents in each fraction were assessed by colorimetric method using $AlCl_3$ [30]. To 250 µl of the plant sample were added 400 µl de-ionized water and 90 µl of $NaNO_2$ solution (5%) and incubated for 6 min at room temperature. 180 µl of $AlCl_3$ (10%) was added to the above mixture and incubated again for 5 min at room temperature. Finally 600 µl of 1 M NaOH solution was added to the mixture and diluted to 3 ml with de-ionized water. Absorbance was measured with a spectrophotometer at 510 nm using methanol as blank. For calculation of flavonoid content, calibration curve of quercetin as standard was prepared under similar conditions (Figure 2). Total flavonoid content was expressed as mg quercetin equivalent (QE)/ g dry weight (dw).

$y = 0.9547x + 0.4935$, $R^2 = 0.968$

Where y = absorbance and

x = concentration of quercetin

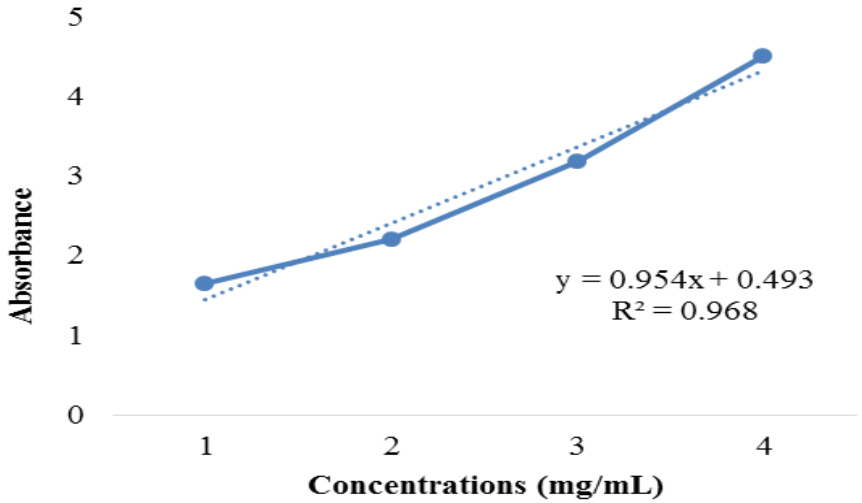

Figure 2. Calibration curve of quercetin

Determination of total tannin content

For determination of total tannins method Ram and Mehrotra was adopted [31]. To 0.1 ml of each plant extract, 0.5 ml of Folin Phenol reagent and 1 ml of 35% sodium carbonate solution was added. The final volume was made up to 10 ml with distilled water. The mixture was vigorously shaken and left to stand at room temperature for 30 min. Absorbance was measured at 725 nm. Blank contained water in place of plant sample. A calibration curve of gallic acid was prepared using different concentrations treated in the same manner as described above and total tannins were calculated by the following equation:

$y = 0.086x + 0.0065$, $R^2 = 0.842$

Where

y = absorbance

x = concentration of gallic acid

Results were expressed in terms of gallic acid mg/g of extract.

DPPH radical scavenging activity

To determine the antioxidant activity 5 mL of 0.05 mM of methanol extract of DPPH was mixed with 50 µl of plant samples (at 0.125, 0.25, 0.50, 1, 2, 4 mg mL^{-1}) and incubated for 30 minutes at room temperature. Absorbance was determined at 517 nm using methanol as blank. The control had all the reagents except plant sample. The change in color of reaction

mixture from violet to yellow indicated the occurrence of antioxidant activity. The % inhibition of DPPH radical was calculated using the following formula [32].

$$\% \text{ DPPH Scavenging activity} = \frac{[\text{Absorbance of control} - \text{Absorbance of extract}]}{(\text{Absorbance of control})} \times 100$$

Antifungal assay

Antifungal assay was performed using agar dilution method adopted by Javaid and Sammad [33].

Media preparation

Malt extract agar (MEA) medium was used for fungal growth. Medium was prepared by adding 2 g of malt and 2 g of agar in 100 ml of distilled water in 250 ml of conical flask and was sterilized by autoclaving at 15 lbs $inch^{-2}$ for 15-20 minutes at 121°C. After autoclaving the sterilized medium was cooled to room temperature. The sterilized medium was then placed in culture room until use.

Procurement and culturing of *A. niger*

The pure culture of *A. niger* strain taken from Fungal Biotechnology Laboratory, Department of Botany, Lahore College for Women University was placed on solidified 2% MEA medium. It turned into a colony within 10 days of incubation at 20°C.

Preparation of sample solutions

Stock solution (30 mg/mL) of each fraction was prepared by dissolving 300 mg of each fraction in 10 mL of distilled water. Working concentrations of each sample at 0.25, 0.5, 1 and 2 mg/mL of malt extract were prepared by using the following formula:

$C_1V_1=C_2V_2$

Where

C_1= concentration of stock solution

V_1= volume of stock solution

C_2= working concentration of tested sample

V_2= total volume required of test sample

Antifungal screening assay

Malt extract (ME) was prepared by adding 20 g ME in 1000 mL of distilled water and autoclaved at 121°C for 30 minutes in four conical flasks of 250 mL and cooled at room temperature. Teramycitin capsule at 50 mg/100 mL of the medium was added to avoid bacterial contamination. Four concentrations viz. 0.25, 0.5, 1 and 2 mg/60mL were prepared by adding 0.5, 1, 2 and 4 mL of stock solution in 59.5, 59, 58 and 56 mL prepared broth respectively, to make the total volume of the medium up to 60 mL. The 60 mL of each treatment was divided into three equal portions in 250 mL conical flasks to serve as replicates. Mycelial discs (5 mm) were prepared from 5-7 days old culture of *A. niger* using a pre-sterilized cork borer and were placed in each experimental flask. The flasks were incubated for 7 days at 25°C. After 7 days the fungal biomass from each flask was obtained by filtration of malt extract through pre-weighted Whatmann No.1 filter paper and dried up to stable weight in an electric oven and weighed. Fungal growth inhibition (%) was calculated by applying the formula:

$$\text{Growth inhibition (\%)} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

Medium containing malt extract, antibiotic and fungal disc without plant extract were used as positive control while DMSO replacing plant extract served as negative control. No antifungal activity was shown by negative control.

All the experiments were performed in triplicates and results were expressed as mean ± SE. The data was statistically analyzed by applying ANOVA followed by Duncan's Multiple Range Test at significance level of P = 0.05 using co-stat software.

Results and discussion

Phytochemical analysis

The qualitative analysis of crude methanol extract indicated the presence of glycosides,

alkaloids, flavonoids, phenolics, phytosterols, saponins, tannins, triterpenes, terpenoids, xanthoproteins, while anthraquinones, Coumarins and quinones were absent.

Quantitative phytochemical analysis

All the fractions were analyzed to quantify the total phenolic, total flavonoid and total tannin contents. The highest content of phenolics, flavonoids and tannins was determined for the ethyl acetate fraction (0.9 mg GAE/g dE, 0.47 mg QE/g dE and 2.54 mg GAE/g dE respectively) while least was in dichloromethane fraction (0.04 mg GAE/g dE, 0.03 mg QE/g dE and 0.23 mg GAE/g dE respectively) (Figure 3).These secondary metabolites have a major role in antifungal activity of plant extracts. Research has indicated that phenolic compounds synthesized in uninfected, healthy plant tissues, can act as antifungal compounds. These may include phenolic acids, simple phenols, flavanols and alkaloids [34]. Flavonoids are particularly strong agents against microbes, oxidative stress, allergies, cancer and inflammation [35].

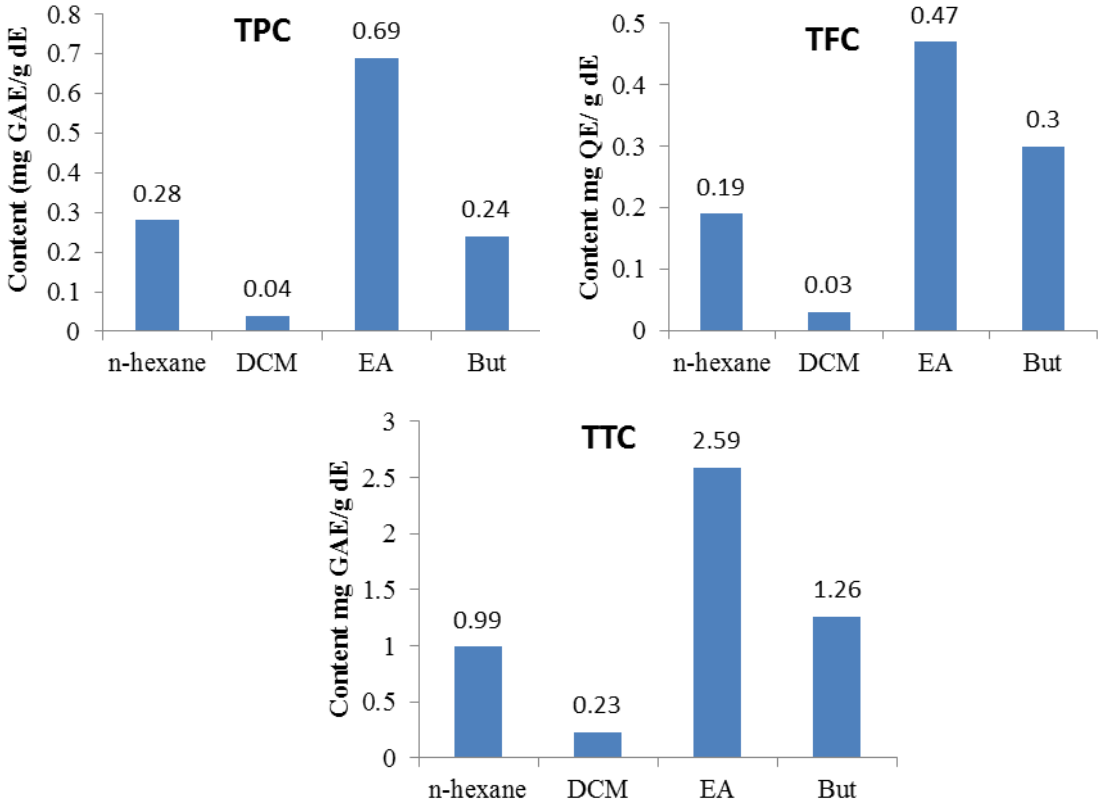

Figure 3. Total content of phenols, flavonoids and tannins in different fractions of *F. religiosa*

Antioxidant assay

The extracts showed significant antioxidant activity for all the fractions at all the tested concentrations. The inhibitory effect of the extracts ranged between 70.43-82.5% for *n*-butanol and ethyl acetate fraction respectively at the lowest tested concentration of 0.125 mg/mL. At highest concentration of 4 mg/mL maximum radical scavenging activity observed was 91.71% for dichloromethane fraction and minimum for *n*-butanol fraction with 86% inhibition (Figure 4). The results indicated that the compounds responsible for antioxidant

activity of stem bark range from non-polar in hexane and dichloromethane to medium polar in ethyl acetate and to highly polar in butanol fraction. This shows that the bark contains a wide range of antioxidants belonging to different classes of compounds. Antioxidant activity and quantitative analysis was correlated as ethyl acetate extract with highest phenols, flavonoids and tannin contents also had significant antioxidant activity. The results showed that all the extracts, specifically ethyl acetate extract, can be a rich source of natural antioxidants and can be used for treating diseases which result from oxidative stress. The phenolic, flavonoids and tannin contents in plant extract mostly act as antioxidants to prevent disease and promote the health. The observed antioxidant activity of the extracts may support the use of the plant in traditional medicines. Reports are available where the extracts of different *Ficus* species have shown antioxidant activity [36-38]. In all these studies the antioxidant activity was correlated to the presence of secondary metabolites including phenolics, flavonoids and anthocyanins.

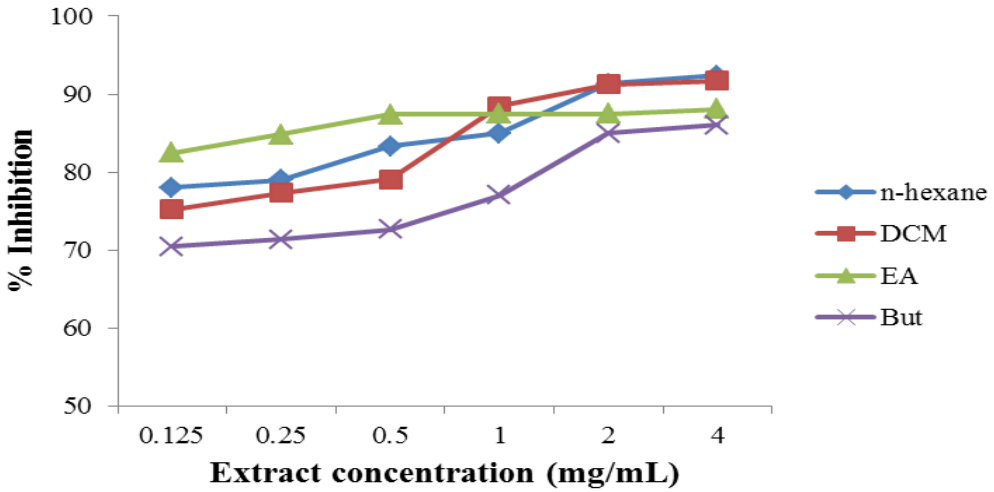

Figure 4. DPPH radical scavenging activity of *F. religiosa* stem bark extracts

Antifungal assay

In the present study different concentrations (0.25, 0.5, 1, 2 mg/mL) of respective fractions were applied *in vitro* against test fungus to check their antifungal potential. The results were presented in terms of fungal biomass and percentage of growth inhibition at different extract concentrations. A decrease in fungal biomass as compared to control was observed in the presence of all the plant extracts indicating growth inhibition potential of the plant material. The inhibitory effect was dose dependent. Highest antifungal activity in terms of decrease in fungal biomass was observed in *n*-butanol extract with % inhibition in fungal growth ranging from 66% to 77.5% at 0.125 and 2 mg/mL respectively while least effective was the ethyl acetate fraction with fungal biomass reduction of 47.5% and 62.5% at the same concentrations (Figure 5 and 6). Hemaiswarya et al. [39] reported the antifungal activity of leaf extracts of *F. religiosa* prepared in water, methanol and chloroform. Highest activity was observed for chloroform extracts. Rajiv and Sivaraj [40] also tested aqueous extracts of bark, leaves, stem and fruits of *F. religiosa* for antifungal activity. All the plant parts showed activity against *A. niger* with no significant difference in activity between different plant parts. Ramakrishnaiah and Hariprasad [41] screened the aqueous and methanol extracts of bark, leaves, stem and

fruits of *F. religiosa* for their antifungal activity against *A. niger* in terms of zones of inhibition. All the plant parts showed antifungal activity. However, the extract was used at a very high concentration (30, 40 and 100 mg/mL). In the present study the crude methanol extract was fractionated into different fractions using solvents of different polarities and the resultant fractions gave significant results reducing fungal growth at very low concentrations (0.125, 0.25, 0.5, 1 and 2 mg/mL) due to partitioning of targeted compounds in different solvents according to their polarity. The observed antifungal activity may be due to the presence of secondary metabolites including phenolics, flavonoids and tannins in the bark material which have previously been reported to possess antifungal activity [42-44]. The results clearly indicated that the bark material of the tested plant can be used as a source for plant based natural antifungal compounds active against *A. niger* which is a major issue in our agriculture sector affecting a large portion of agriculture produce due to poor storage conditions.

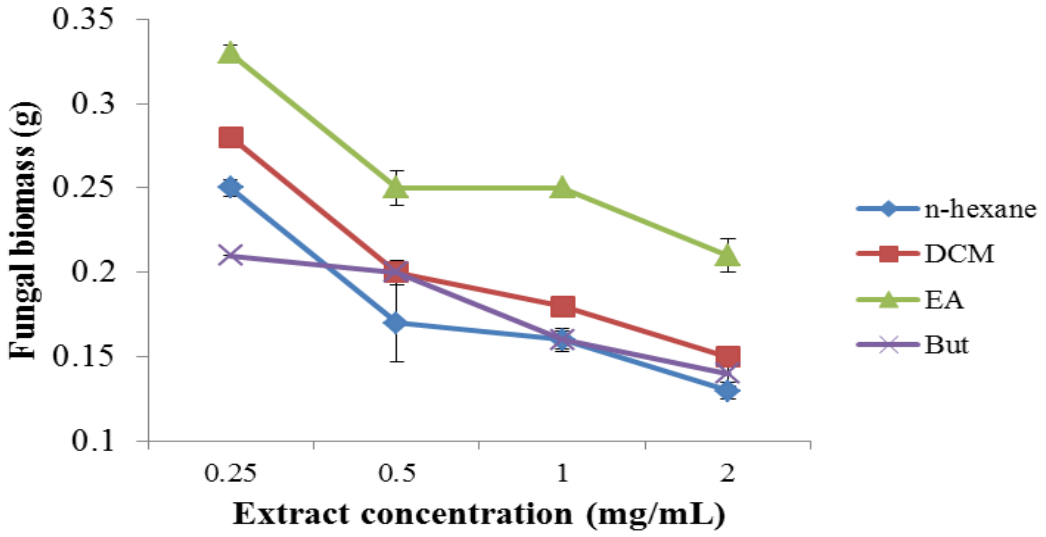

Figure 5. Decrease in fungal biomass of *A. niger* by *F. religiosa* bark extracts

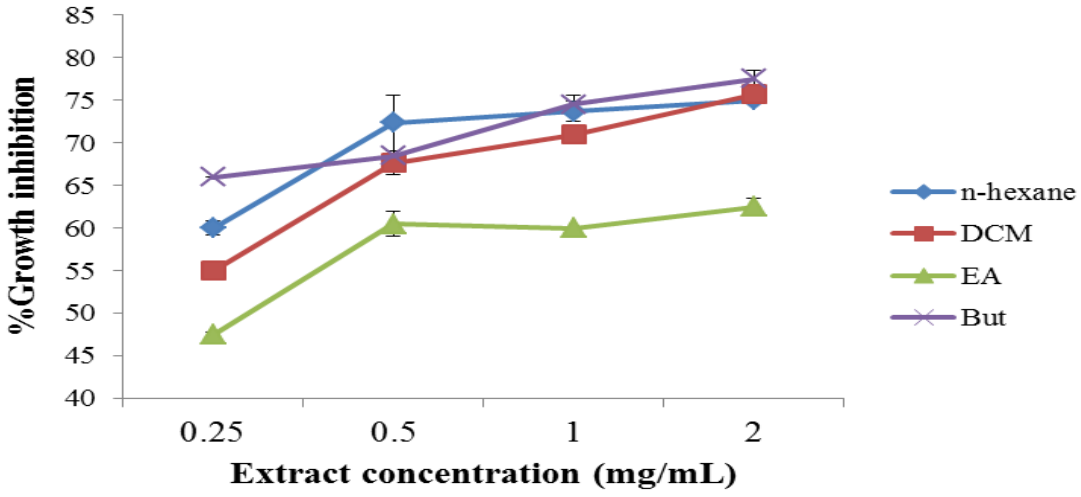

Figure 6. Inhibition of *A. niger* growth by *F. religiosa* bark extracts

Conclusion

The results concluded that the stem bark of *F. religiosa* is rich in phytochemicals with antifungal and antioxidant activity. The *n*-butanol fraction of the bark material had highest antifungal activity indicating that the fraction had maximum amount of phytochemicals with antifungal potential. The ethyl acetate fraction can be a good source of antioxidants.

Authors' contributions

Conceived and designed the experiments: Z Saddiqe & K Jabeen, Performed the experiments: A Shahid, Analyzed the data: A Shahid & Z Saddiqe, Contributed reagents/ materials/ analysis tools: Z Saddiqe & K Jabeen, Wrote the paper: Z Saddiqe.

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