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Native Yemeni *Plumbago auriculata* as a Promising Antioxidant and Antifungal Plant against Different *Fusarium* species

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Abstract
Plants and plant derived metabolites are believed as rich source of antifungal and antioxidant agents, with their fewer side effects, they are finding their way into pharmaceutical derivatives. The present study was undertaken to investigate the methanolic leaves and flowers extracts of *Plumbago auriculata* for their potential activity against phytopathogens and antioxidant efficiency. The extracts were evaluated against different pathogenic *Fusarium* species; *F. verticilloides, F. oxysporum* and *F. solani* using agar well diffusion method. *F. oxysporum* showed the higher susceptibility of inhibition with all extracts, followed by *F. solani* and *F. verticilloides* in case of leaves extracts, while *F. verticilloides* was the most sensitive after *F. oxysporum* in case of flowers extracts. It was clear that 75% concentration was the most effective among all concentrations tested. In DPPH (free radical scavenging assay) for antioxidant evaluation for the plant extract. The flowers extracts showed higher antioxidant activity than those of leaves with a dose-dependent manner. This study support the use of this herbal plant as antioxidant and antifungal agent to control *Fusarium* species especially *F. oxysporum* the causative agent of *Fusarium* wilt of tomato.
Keywords: Native, *Plumbago auriculata*, Antifungal, Antioxidant, Agar-well diffusion method.

INTRODUCTION

Fungi are ubiquitous in the environment and infection due to fungal pathogens has increased at an alarming rate in the world during the last two decades (Pfaller *et al.,* 2006; Rao *et al.,* 2009). Fungi are more dangerous to plants than bacteria for plant diseases, as those caused by fungi spread very rapidly. The damage is often very serious in nature which may even be more serious and widespread if prescribed control measures are not undertaken (Bibitha *et al.,* 2002).

Plant pathogenic fungi pose a major threat to crop plants, forest trees and plants of major economic importance (Rdorigual-Herrera *et al.,* 1999). In the past decades the use of fungicides to control crop diseases has contributed to increased production of food worldwide. Nevertheless, the massive use of synthetic fungicides in crop defense had severe environmental impact. Synthetic fungicides also pose serious health risks within our food chain and have been linked to increased occurrence of several types of cancer (Wilson *et al.,* 1997), which make the research on bioactive substances from plant sources has great scope and could lead to the development of antifungal agents that can combat fungal infections, and plants contain many components that are important sources of biologically active molecules (Newman *et al.,* 2003; Butler, 2004).

Plants have been utilized as important sources of medicinal drugs and health products since ancient time. Yemeni People especially in ruler areas use plants such as *P. auriculata* in their daily life as medicines. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants (Vinothapooshan and Sundar, 2010).

P. auriculata Lam (Family Plumbaginaceae), is a useful medicinal plant as its constituents are credited with potential therapeutic properties including anticancer, antifungal, anti-inflammatory, antibacterial, antifertility, antimalarial, antidiabetic, antiulcer, and antioxidant properties (Singh *et al.,* 2018).

The increasing number of fungal population with enhanced resistance to common fungicides urges the development for new formulations which are both effective and environmentally friendly. In this respect, plant extract may represent an ideal solution to the problem and can be easily tested *in vitro*. Heading from this idea, this research was designed to screen the ability of methanolic extracts of *P. auriculata* leaves and flowers for antifungal and antioxidant activity.

MATERIALS AND METHODS

Chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), was purchased from Sigma-Aldrich (St. Louis, MO). Ascorbic acid was purchased from Fisher Scientific (Loughborough, UK). Methanol and DMSO were purchased from Fisher Scientific (Fisher Scientific Co Ltd., Ottawa, ON).

Preparation of Plant Materials

Fresh *P. auriculata* Lam leaves and flowers were collected from the Faculty of Science's garden, Sana`a University, Yemen, identified and authenticated by a plant taxonomist at Department of Biology, Botany Division, Faculty of Science, Sana`a University; Yemen (Figure 1A,B). Leaves and flowers of the plant were washed thoroughly under running tap water and soaked in 2% solution of sodium hypochlorite for 20 min, rinsed thoroughly with sterilized distilled water and air dried at room temperature.

The scientific classification of *P. auriculata* is as follows (Fonden and Polter, 2015):

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Caryophyllales
Family: Plumbaginaceae
Genus: *Plumbago*
Species: *Plumbago auriculata* Lam.
Common names: Cape *Plumbago,* Cape Leadwort, Blue *Plumbago.*

(A) (B)

Fig. 1. Native Yemeni *Plumbago auriculata* A. *P. auriculata* flowers **B.** *P. auriculata* leaves.

Preparation of extracts

The dried plant materials were grinded to fine powder using electrical grinder (Waring Blender, Tokyo, Japan) at speed 6 for 2 min. 100g of the sample was soaked in methanol at 1:10 ratio for three days at room temperature, after that it was filtered through Whattman No. 1 filter paper. The supernatant was collected and allowed to evaporate until completely dry, then kept in sterilized

sealed bottles under refrigerated condition until subsequent analysis, and then 25%, 50% and 75% concentrations of the plant extract were used (Meignanalakshmi *et al.,* 2013).

Preparation of fungal spore suspension

Three *Fusarium* species were used in this investigation; namely *F. verticilloides, F. solani* and *F. oxysporum*. Different species were transferred to fresh slants in sterile conditions and were incubated at 28°C till sporulation. Suspensions of 7 days old conidia (1×10^5 conidia/ml) from each of *Fusarium* species using a hemocytometer were prepared (Rongai *et al.,* 2012**)**.

Determination of antifungal activity

Agar well assay

A drop of fungal spore suspension was placed in the centre of PDA plates (Hi Media, Mumbai), spread all over with a sterile glass spreader. Wells were bored with a sterile cup borer and different concentrations of (25%, 50% and 75%) of leaves and flowers extract samples were added (100ml) to each well. Plates were placed in the refrigerator for 10min and then transferred to the incubator held at 28°C and were incubated for 72 hours, and then they were observed for zone of inhibition.

Antifungal activity was calculated by measuring the diameter of the zone. The experiment was carried out in triplicate and mean of diameter of inhibition zone was calculated. 100% DMSO (Fisher Scientific Co Ltd., Ottawa, ON) used as a control (Ishnava *et al.,* 2011).

Determination of antioxidant activity

The antioxidant activity of leaves and flowers extracts, on the basis of scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, were determined by the method described by Blois (1958) with a slight modification by Gardeli *et al.* (2008). L-ascorbic acid was used as standard antioxidant and methanol was used as the control. An aliquot of 0.5ml of a methanolic solution of DPPH (50g DPPH/100 ml MeOH) was added into the different concentration (1.0, 0.5, 0.25 mg/ml) of each extract and ascorbic acid as long as control samples (both extract and ascorbic acid were dissolved in methanol). All samples were incubated in the dark at room temperature for 30min before absorbance values were read at 517nm (Amersham 2100 Pro, UV-vis spectrophotometer, UK). The decrease in absorbance was calculated as an IC_{50} and expressed as µg/ml, which is the concentration of sample required for 50% scavenging of DPPH radicals in the specified time period.

The radical scavenging effect was calculated as follows:

Radical scavenging effect (%)= Ac-As×100

Ac= absorbance of control

As= absorbance of test sample.

Where control is the absorbance of the DPPH radical + methanol.

Statistical analysis

The experimental results of the antifungal study are expressed as mean ± standard deviation (SD) of three replicates in each test**.** The data were subjected to two way analysis of variance (ANOVA) and followed by Scheffee multiple comparison tests to assess the statistical significance. The data were considered significant at $P<0.05$.

RESULTS

The antifungal activities of methanolic extracts of leaves and flowers of *P. auriculata* against tested *Fusarium* species were reported based on the observed clear zone of fungal inhibition surrounding the well on the plate. The range of inhibition zone varied with test organism based on different concentration of extracts.

In case of leaves extracts, *F. oxysporum* was the most susceptible to methanolic extracts with inhibition zone of 30±1.5mm at 25% followed by *F. solani* and *F. verticilloides* with inhibition zone of 23±2.0 and 15±2.0mm, respectively. A good zone of inhibition was recorded with *F. oxysporum* when treated with 50% of leaves extracts, followed by *F. solani* and *F. verticilloides.*

The concentration of 75% showed the strongest fungistatic effect against all *Fusarium* species tested starting with *F. oxysporum* which showed zone of inhibition of 77±1.5mm and ending with *F. verticilloides* 45±1.5mm. It was clear that the most sensitive fungus towards all concentrations of leaves methanolic extracts was *F. oxysporum*, while *F. verticilloides* and *F. solani* were most sensitive towards the conc. of 75%.

Similar observations were noticed in case of *P. auriculata* flowers extracts, in the sense of that the strongest fungistatic effect was obtained with the concentration of 75%, followed by 50% then 25%. Again *F. oxysporum* was the most susceptible species in all concentrations, followed by *F. verticilloides* and *F. solani.* It was worth mentioning that *F. verticilloides* was more susceptible for flowers extracts more than leaves extracts. An opposite situation was noticed in case of *F. solani.*

Results of analysis of variance on mycelial growth in different concentrations showed that the item was highly significant; Scheffee test also indicates significance in different concentrations of plant extract and radical growth (Table 1 and 2).

Table 1. Antifungal activity of methanolic leaves extracts of *P. auriculata* against tested *Fusarium* species (zone of inhibition mm)

***Fusarium* species**	**Concentration of methanolic extract of leaves**		
	25%	**50%**	**75%**
Fusarium solani	23±2.0[a]	33±1.5[a]	55 ±1.0[a]
Fusarium oxysporum	30±1.5[b]	52±2.0[b]	77±1.5[b]
Fusarium verticilloides	15±2.0[c]	24±1.5[c]	45±1.5[c]

Values are means of three replicates ± SD. Means followed by the different letter within column; differ significantly at 0.05.

Table 2. Antifungal activity of methanolic flowers extracts of *P. auriculata* against tested *Fusarium* species (zone of inhibition mm)

***Fusarium* species**	**Concentration of methanolic extract of flowers**		
	25%	**50%**	**75%**
Fusarium solani	13±1.5[a]	21±2.0[a]	35±0.6[a]
Fusarium oxysporum	29±1.5[b]	40±1.5[b]	45±1.5[b]
Fusarium verticilloides	15±1.5[a]	25±1.5[a]	38±1.0[a]

Values are means of three replicates ± SD. Means followed by the different letter within column; differ significantly at 0.05.

Antioxidant activity

In this study, the antioxidant capacity of methanolic extracts of *P. auriculata* leaves and flowers was assessed by the DPPH scavenging activity assay. IC_{50} of DPPH scavenging activities of each extract were compared to IC_{50} of ascorbic acid. Figure (2), showed the dose-response manner of DPPH radical scavenging activity of the methanolic extracts of leaves and flowers of *P. auriculata,* the scavenging activity of the pure antioxidant standard, ascorbic acid was 40% at lower concentration of 0.25mg/ml. The radical scavenging activity was following dose- dependent manner and it increased by concentration to 63 and 78% at 0.5 and 1.0mg/ml, respectively.

The overall picture revealed that the methanolic extracts of flowers had higher activity than those of leaves. As after 30 min of the reaction, methanolic extract of *P. auriculata* flowers at lower concentration 0.25mg/ml scavenged 29% of the total radicals in the reaction system.

It was obvious that the inhibitory effect of the tested extracts on DPPH radicals increased by concentration to 47 and 78% at 0.5 and 1.0 mg/ml in case of flowers extracts, respectively. Results of leaves extracts were parallel to its analogous flowers extracts, as they record 15% after 30min at lower concentration. Subsequently, the scavenging activity was increasing gradually by 26 and 66 % of the total radicals, respectively, at higher concentration 1.0 mg/ml.

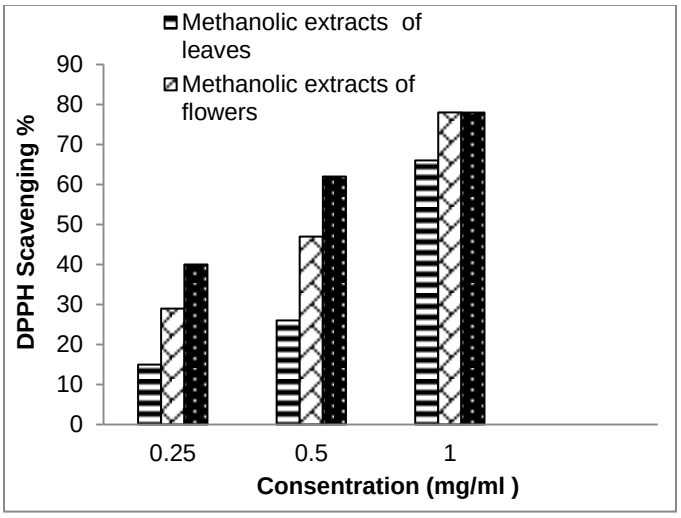

Fig. 2. DPPH scavenging activities of the methanolic extracts of the leaves and flowers of *P. auriculata.*

DISCUSSION

Plants naturally produce secondary metabolites, commonly referred to as phytochemicals or biologically active compounds which are essential for plant metabolism but play a great role in the plants' protection mechanism

(Ascensao *et al.*, 1997). In addition to plant protection, these bioactive compounds also serve as precursors for the development of natural, environmentally friendly and low toxicity pharmaceuticals, flavourants, fragrances, cosmetics and pesticides due to their therapeutic and aromatic properties (Thomas, 1991).

Results of the recent study indicated that methanolic extracts of leaves were the most effective against all *Fusarium* species tested in comparison to flowers. Their activity may due to that leaves contain α-amyrin, apigenin, luteolin, capensisone, α-amyrin acetate, isoshinanolene, β-sitosterol, diomuscinone, plumbagin and β-sitosterol-3β-glucoside (Rastogi and Mehrotra, 1995; Ariyanathan *et al*., 2011; Padhye *et al*., 2012; Saeidnia *et al.,* 2014; Khan and Hossain, 2015), from which plumbagin and isoshinanolene were of antifungal activity (Saji and Antony, 2015; Rawal and Adhikari, 2016). All parts of *P. auriculata* contain plumbagin with the highest amount of plumbagin accumulating in the leaves and stems of the plant in comparison to *P. zeylanica* and *P. rosea* (Jose *et al*., 2014). This shows that different species accumulate plumbagin in different parts of the plant.While flowers of the plant reported azalein, capensinidin, and capensinidin-3-rhamnoside (Harbome, 1962). The findings of the antifungal activity of plumbagin by Dzoyem *et al*. (2007) suggested that the naphthoquinone delayed germination of the fungus and was capable of inhibiting growth when administered at higher concentrations.

Antifungal activity was tested by spore germination assay on the following fungi: *Aspergillus fumigatus, Fusarium oxysporium, Aspergillus flavus, Curvularia lunata* and *Trichoderma* sp. Results of this study reported that the efficacy of the particle reflects on its size i.e. bigger paritcles were effective on larger spore such as *Curvularia lunata* and ineffective against smaller spore bearing fungi (Rajasekaran *et al*., 2015). The antifungal activity of the plant's leaves was recently mentioned by Kumar *et al.* (2018) against *F. oxysporum* and other fungal genera and species as it showed a good inhibitory activity in case of chloroform extract.

Antioxidant activity of the antioxidants is concerning with those compounds capable of protecting the organism system against the potential harmful effect of oxidative stress (Fernandez-Agullo *et al.,* 2013). Being of an effective antifungal activity; talking about *P. auriculata* leaves and flowers extracts; their antioxidant activity was of a great concern as limited if any information is available about this plant in Yemen. Radical scavenging activity of the extracts was compared using their respective IC_{50} values. A dose-dependent reducing activity was observed for the methanolic extract of the leaves and flowers of the plant and for L-ascorbic acid. Results obtained during this investigation confirmed the use of investigated plant in Yemeni traditional medicine, as the extracts showed a good antioxidant activity.

Among all chemical constituents mentioned before, plumbagin and β-sitosterol-3β-glucoside were of antioxidant potential. It seems that Plumbagin contributes to EGFR activation in ROS-related mechanisms (Padhye *et al*., 2012). It was also found to significantly reduce the catechol-induced DNA damage, and inhibit ascorbate and NADPH-dependent lipid peroxidation against mouse lymphoma cells (Demma *et al*., 2009).

On the other hand, *P. auriculata* was considered as the weakest plant on free radical scavenging assay among other plants used in the study of Shawarb *et al.,* (2017). This variance may explained by the idea that the antioxidant activity measured depends substantially on the test system used as mentioned by numerous studies (Janaszewska and Bartosz, 2002; Bauzaite *et al.,* 2003). Besides that chemical profile of a single plant may vary over a time, as it reacts to changing conditions (Cravotto, 2010).

An extensive review of African medicinal plants extracts with potent antioxidant activities by Atawodi (2005) indicated that the mechanism(s) of action extracts was by free radical scavenging. In addition, the synergistic effects of natural products also enhance their antioxidant activities (Bizimenyera, 2005).

CONCLUSION

Results of this investigation suggest that the bioactive constituent(s) of *P. auriculata* used in this investigation had both antioxidant and antifungal activities, and can serve as candidate for their exploitation as potent fungitoxicant for controlling *Fusarium* spp. especially *F. oxysporum* the causative agent of *Fusarium* wilt of tomato.

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CONFLICT OF INTEREST

All the authors have declared that no conflict of interest exists.

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