ANTIFUNGAL ACTIVITY IN EXTRACTS OF PLANTS FROM SOUTHWESTERN OKLAHOMA AGAINST *ASPERGILLUS FLAVUS*

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ABSTRACT

The use of medicinal plants has been an integral part of human civilization since antiquity. Naturally occurring pesticidal compounds are synthesized by the plant defense system, which includes antimicrobial proteins and lower molecular weight natural products. In this study, plants were collected from southwestern Oklahoma, and plant tissues were extracted and assayed for antifungal activity against *Aspergillus flavus*, a mycotoxin producing fungus. Out of the 84 plant tissue extracts tested, 40 extracts exhibited complete to very strong inhibition of fungal growth. Extracts were dialyzed in Tris buffer using 3,500 molecular weight cut-off dialysis membrane to remove low molecular weight compounds. After dialysis, the majority of the plant extracts lost antifungal activity against *A. flavus*. Four plant extracts, however, retained complete activity. The source plants of these four extracts were identified as belonging to Asparagaceae. Three of the extracts came from three different plants of the genus *Allium*. The fourth extract was from *Camassia scilloides*.

INTRODUCTION

The history of plant use for medicinal purpose is as old as human civilization. Six thousand year old excavated clay slabs from early Sumerian civilizations revealed recipes for drug preparation using over 250 different plants (Petrovska 2012). Additionally, an Egyptian scroll dated about 1500 BP mentioned more than 850 plant based medicines (Petrovska 2012). Hippocrates (460–377 BP) also believed in the power of plants to cure ailments and used 300 different plant species to heal his patients. Pedanious Dioscorides (50–70 AD) assembled *De Materia Medica* where he described comprehensive use, preparation, side effects, and cultivation of 600 plants (Sumner 2000). There are examples from every culture about healing abilities of plants. Modern research indicates that the majority of ethnobotanical claims are valid and correspond with our current knowledge of plant-derived compounds. Benefits of

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traditional plants have been explored via scientific research, which led to the discovery of many valuable drugs for the modern world. Examples include reserpine from *Rauwolfia serpentina* (Indian snake root), vincristine from *Catharanthus roseus* (Madagascar periwinkle), artimisinin from *Artemisia annua,* (sweet sagewort), capsaicin from *Capsicum annuum* (chili pepper), morphine from *Papaver somniferum* (poppy), atropine from *Atropa belladonna* (deadly night shade), silymarin from *Silybum marianum* (milk thistle), and ephedrine from *Ephedra sinica* (Chinese ephedra) (Farnsworth et al. 1985; Houghton 1995; Sumner 2000; Gupta, et al. 2005; Goutam 2015).

Plants provide a rich source for the discovery of new drugs. A number of bioactive compounds can be isolated from different parts of a single plant. *Azadirachta indica* (neem) is one such plant. Neem plant is known as the 'village dispensary' in India because of its wide spectrum of biological activities and no-cost availability due to the widespread growth of this plant in the region (Arora et al. 2008; Asif 2013). Over 135 bioactive compounds have been isolated from different parts of this plant. Some are well known to exhibit antiviral, antifungal, antibacterial, anti-insect, and antitumor activity. There are over 250,000 plant species in the world, but only 6% have been screened for biological activity. According to Farnsworth et al. (1985), there are 119 plant-derived drugs used today all over the world, and all of those came from less than 90 plant species. The possibility of finding novel compounds from plants that can be exploited for medicinal use is enormous.

Although the origin of many life-saving modern medicines came from natural sources, tremendous achievements in synthetic chemistry have made it possible for pharmaceutical companies to design and introduce drugs at a faster pace. To find medicinal compounds from a plant source, a large number of plants need to be screened. Once identified, the active compounds have to be purified and characterized. Many of these natural compounds are structurally complex. Therefore, to go from discovery to high throughput commercial production can be technically challenging and time consuming. Some of these issues have contributed to the decline of plant-based drug discovery (Gupta et al. 2005). However, over the last few decades, the world has watched the reemergence of infectious diseases once thought to be eradicated (Gupta et al. 2005; Lam 2007; Petrovska 2012). At the same time, incidences of pest and pathogen resistance against antimicrobial products have increased in alarming numbers. There is also an increase in the prevalence of multidrug resistant bacterial pathogens. In light of these facts, there is renewed interest in looking into nature's wealth for newer and better medicines.

Plants are a storehouse of naturally occurring pesticidal compounds that are molecularly diverse. Plants have developed an arsenal of defense mechanisms from protection against pests and pathogens. As a result, different and unique chemicals compounds are synthesized by plants. These diverse molecules in plants are under constant evolutionary selection. This makes the plant kingdom a continuously wealthy source for finding new antimicrobial compounds (Hossain 1999).

The compounds that are synthesized by plant defense systems, either to prevent pathogen attack or to destroy invading pathogens, include proteins and lower molecular weight natural products. Defense-related proteins produced by plants include hydroxyproline-rich glycoproteins; glycine-rich proteins; amylase inhibitors; proteinase inhibitors; toxic proteins such as lectins and thionins; hydrolases such as chitinases and β-1,3-glucanases; anti-microbial peptides such as defensins; and other cysteine-rich proteins (Hossain 1999). Lower molecular weight natural products include various

alkaloids, tannins, flavonoids, terpenes, etc. (Goutam 2015).

Southwestern Oklahoma has a rich history in the use of medicinal plants. Jordan et al. (2006) documented the use of over 100 species of vascular plants by the Plains Apache tribe. Thirty-nine of those species were used in rituals and for medicinal purposes. According to the study, out of the 105 documented species used by the tribe, 98 are native to southwestern Oklahoma and occur throughout the western U. S. and Great Plains (Jordan et al. 2006). Students from the Cameron University Biology Department collected plants from four different locations in southwestern Oklahoma. Aqueous crude and dialyzed extracts from collected plants were screened for antifungal activity against *Aspergillus flavus*, which was chosen because this fungus produces carcinogenic mycotoxins known as aflotoxins. This study was part of an assignment for a medicinal plants class. In this report we present the result of the study.

MATERIALS AND METHODS

Collection of Plant Materials

Forty-seven species of plants were collected from four different locations in southwestern Oklahoma (Table 1). These locations include Medicine Park, East Lawton, Stephens County, and Anadarko. Plants were collected based on ethnobotanical information (Jordan et al. 2006) as well as field observation. Therefore, not all of the collected plants have known medicinal use. The field observations of healthy plants growing in the midst of plants infested by pests and pathogens could indicate defense related compounds protecting these plants. Such field observations were a part of the collection process. On location, the collected materials were photographed, bagged, and kept in ice. Once transported to the laboratory, the plant materials were placed in -80^{o}C for long term storage.

Table1 List of plants screened for antifungal activity against *Aspergillus flavus*
PCN, Plant Collection Number. AF, Stephens County; MP, Medicine Park; CSL, East Lawton; AN, Anadarko. R, root; L, leaf; B, bulb; Fl, flower; Yl, young seedling. NI, not identified. Approximate latitudes and longitudes of the locations are: Lawton (N34.069424, W98.417781); Medicine Park (N 34.733270, W 98.483923); Anadarko (N35.069203, W96.265657); Stephens County (N34.36, W98.23).

Extract. #	PCN #	Scientific Name	Common Name	Family
1	AF#19 L	NI		
7	AF#19R			
2	AF#10R	*Yucca glauca* Nutt.	Soapweed	Agavaceae
4	AF#10L			
37	AF#10F			
3	AF#13L	*Artemisia* sp.		Asteraceae

5	AF#18L	NI	NI	NI
25	AF#18L			
6	AF#1L	*Ulmus* sp.	Elm seedling	Ulmaceae
8	AF#17L	*Callirhoe involucrata* (Torr. & A. Gray) A. Gray	Purple Poppy	Malvaceae
18	AF#17R			
9	AF#17Fl			
10	AF#15F	*Castilleja indivisa* Engelm	Indian Paint Brush	Orobanchaceae
15	AF#15R			
11	AF#9L	Rumex crispus L.	Curly Dock	Polygonaceae
29	AF#9R			
16	AF#	NI		
12	AF#5L	*Achillea* sp.	Yarrow	Asteraceae
14	AF#5R			
17	CSL#7L	*Nothoscordum bivalve* (L.) Britton	False garlic	Asparagaceae
38	CSL#7R			
16	AF#FR	NI		
19	AF#4L	*Daucus carota* L.	Wild carrot	Apiaceae
20	MPGA#1S	*Echinocereus reichenbachii* (Terscheck ex Walp.) J.N. Haage	Lace Echinocereus	Cactaceae
23	MPGA#1R			
21	AF#21S	*Opuntia* sp.	Prickly pear	Cactaceae
33	AF#21R			
24	AF #8 R	*Callirhoe involucrata* (Torr. & A. Gray) A. Gray	Purple Poppy seedling	Malvaceae
22	AF#14L	*Cirsium undulatum* (Nutt.) Spreng	Wavy-leaf Thistle	Asteraceae
27	AF#14R			
26	AN#1L	NI		
28	AF#6L	*Medicago lupulina* L.	Legume	Fabaceae
30	AF#11L	*Polygala senega* L.	Senega snake root	Polygalaceae
31	AF#2L	*Capsella bursa-pastoris* (L.) Medik.	Shepherd's Purse	Brassicaceae
39	AF#2Fl			
32	MPGP#1FL	NI		
35	MPGP#1L			

34	AF# 20L	NI		
36	CSL#10 F	NI		
40	AN#42	*Equiseteum* sp.	Rough horsetail	Equisetaceae
41	MP#23R	NI		
42	MP#24		Moss	
43	MP#9L	*Camassia scilloides* (Raf.) Cory	Wild hyacinth	Asparagaceae
76	MP#9F			
78	MP#9B			
44	MP#8L	NI		
46	MP#4L	*Glandularia bipinnatifida* (Nutt.) Nutt.	Verbena	Verbenaceae
50	MP#4F			
47	MP#5L	*Callirhoe leiocarpa* R.F. Martin	Tall poppy mallow	Malvaceae
60	MP#5F			
48	MP#1F	*Sapindus* sp.	Soap berry	Sapindaceae
49	MP#3F	*Tradescantia tharpii* E.S. Anderson &Woodson	Spiderwort	Commelinaceae
66	MP#3R			
45	MP#3L			
51	MP#6L	NI		
75	MP#6R			
52	MP#22Fl	*Allium canadense* L.	Wild onion	Asparagaceae
57	MP#22R			
70	MP#22B			
58	MP#19L	*Ambrosia* sp.	Ragweed	Asteraceae
85	MP#19R			
59	MP#26R	*Amsonia ciliata* Walter	Blue Star	Apocynaceae
61	MP#26Fl			
65	MP#26L			
62	MP#14L	NI		
79	MP#14R			
63	MP#15L	*Physaria gracilis* (Hook) O'Kane & Al-Shehbaz	Yellow-flowered bladderpod	Brassicaceae
80	MP#15R			
81	MP#15Fl			

64	CSL #B	*Allium drummondii* Regel	Drummond's onion	Asparagaceae
72	CSL#1L			
73	CSL#1Fl			
67	MP#11F	*Yucca glauca* Nutt.	Soapweed	Asparagaceae
68	CSL#3L	*Vicia sativa* L.	Common vetch	Fabaceae
69	MP#10 L	*Rosa* sp.	Wild rose	Rosaceae
71	MP#7R	*Allium canadense* L.	Wild onion	Asparagaceae
54	MP#7FL			
74	CSL#11L			
77	MP#13L	*Oenothera* sp.	Gaura	Onagraceae
82	MP#16L	NI		
83	MP#18L	*Erodium cicutarium* L.	Stork's bill	Geraniaceae
84	MP#18R			

Plant Tissue Extraction

Plant materials (seeds, fruits, leaves, roots, stems) were extracted in 10 mM Tris-HCl (pH 8.0) containing 0.2 g of insoluble PVP (polyvinylpolypyrrolidone; Sigma Chem. Co, Cat. # P6755) for each g of frozen plant tissue (Hossain 1999). Mostly, five volumes of buffer were used for each gram of fresh weight of tissue. The buffer volume was adjusted for mucilaginous and starchy tissue. All extractions were carried out at room temperature. Plant tissues were homogenized in liquid nitrogen using a mortar and pestle, and the homogenate was filtered through a double layer of Miracloth. The filtrate was centrifuged at 12,000 x g for 15 min in a Sorvall SS34 rotor. The supernatant fluid was collected, and the pellet containing debris and insoluble PVP was discarded. The clarified supernatant fluid, referred to as the crude extract was tested for antifungal activity.

Dialysis

To remove soluble, low-molecular-weight materials from the crude extract, 2 ml of each crude extract was dialyzed extensively against 10 mM Tris-HCl (pH 8.0) using a 3,500 molecular weight cut-off dialysis membrane (Spectra/Por). Dialysis was routinely carried out in 4 L beakers, and the dialysis buffer was changed at least three times over a 24–48 h period. Total volume of extracts dialyzed in each 4 L beaker was 20–30 ml. After dialysis, the crude extract (referred to as dialyzed extract) was tested for antifungal activity.

Source of Fungal Pathogen

The antifungal activity of all extracts was evaluated on the basis of activity against an *Aspergillus flavus* (ATCC # 22548) culture obtained from the American Type Culture Collection, Waldorf, MD. Working cultures of *A. flavus* (Fig. 1) were grown at room temperature on half-strength potato dextrose agar (PDA; Difco # 0013-17-6). Inoculated fungal plates were kept at room temperature for 10 days until the mycelial growth covered 75% of the plate. At that point, the plates were stored at 4ºC for future use.

Figure 1 *Aspergillus flavus* (ATCC 22548) on half strength Potato Dextrose Agar. Inoculated fungal plates were kept at room temperature for two to three weeks until the hyphal growth covered three fourths of the plate. For fungal assay, conidia were scraped from 2–3 weeks old plates.

Antifungal Bioassay

The assay used to detect antifungal activity in plant extracts was originally developed by Duvick et al. (Duvick et al. 1992). Conidia of *A. flavus* were collected by scraping the colony with a sterile loop and suspending the conidia in sterile water containing 0.01% Tween 20. Conidia from this stock solution were diluted with synthetic culture medium to a final concentration of ~290 conidia/90µl of growth medium. The latter contained 0.037 g NaCl, 0.0625 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaNO_3$, 2.5 g glucose, 0.25 g yeast extract, and 0.125 g casein enzyme hydrolysate in 1 L of 7.5 mM sodium phosphate buffer, pH 7.0. Ninety µl of the culture medium containing conidia were added to each well of a 96-well, U-bottom microtiter plate. Ten µl of crude extract or crude dialyzed extracts were added to each well. Four replicates (individual wells) were used for each sample. All of the assays were conducted in 96-well microtiter plates with four control wells in each plate. In these control wells, extraction buffer (10 mM Tris pH 8.0) was added to *A. flavus* conidia instead of plant extracts. The microtiter plate was covered with parafilm and incubated in the dark at 25ºC for 48 h. Conidial germination and fungal growth were observed after 48 h using a Nikon SMZ 1500 stereomicroscope equipped with digital CCD camera and NIS software. A rating scale of 0 to 4 was used to evaluate the inhibition of fungal growth (Fig. 2).

The ratings were based on the relative growth of fungi in comparison to the buffer control. A rating of zero indicated no inhibition of fungal growth, and a rating of four was given in the case of complete inhibition of fungal growth. Rating of 1–2 was based on approximately 50% or more hyphal growth compared to control. Rating of 3 was based on approximately 10–20% hyphal growth compared to control. Intermediate values (such as 1.5, 2.5, and 3.5) were assigned to distinguish between ratings when possible. Values from the four replicates were averaged.

Plant extract numbers were assigned by the students in the class, and the assay was performed by Dr. Tahzeeba Frisby. The plant collection numbers (PCN) matching the extracts were not given to Dr. Frisby, for an unbiased bioassay. Extracts were matched with their respective plant collection number after the bioassay data was collected.

Plant Identification

Plants collected for the study are common to southwestern Oklahoma. All plants were collected in the second week of April 2015. All of the plants were carefully identified using published field guides and keys (McCoy 1987; Freeman and Schofield 1991; Kindscher 1992; Ladd 1995; Loughmiller and Loughmiller 1996; Foster and Hobbs 2002; Barker 2006; Tyrl et al. 2008; Foley 2011). The Oklahoma Vascular Plants Database and the U. S. Wildflower Database of Wildflowers of Oklahoma were also consulted. Source for the authorities of the scientific names was the Integrated Taxonomic Information Service (www.itis.gov).

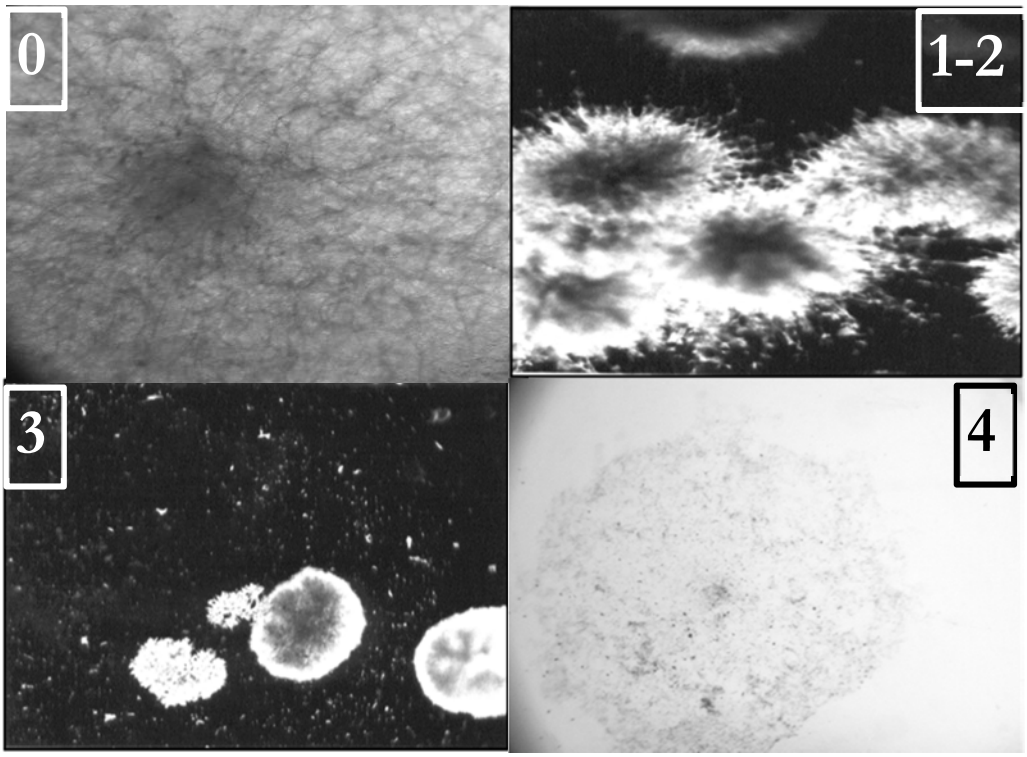


Figure 2 Antifungal rating based on the relative growth of fungi in the buffer control. Ratings: 0, no inhibition of fungal growth; 1, slight inhibition; 2, moderate inhibition; 3, strong inhibition; 4, no fungal growth.

RESULTS

Fungal Growth Inhibition by Crude Extracts

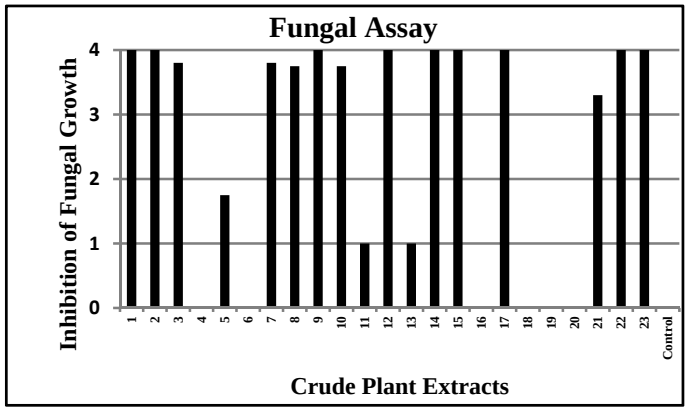
Eighty-four crude extracts obtained from 47 plant species (see Table 1) were screened for antifungal activity against *A. flavus*. Out of those, 29 exhibited complete inhibition of fungal growth (rating of 4; Fig. 3). An additional 18 extracts exhibited strong inhibition with a rating of 3.0 or above but less than 4. Nine more extracts exhibited a rating of 1 to 2.5. Twenty-seven extracts showed no inhibition (see Fig. 3). Growth was comparable to that observed in the control. Mycelial growth in the control buffer was extensive after 48 h incubation and covered the total surface of each well of the microtiter plate.

Fungal Growth Inhibition by Dialyzed Extracts

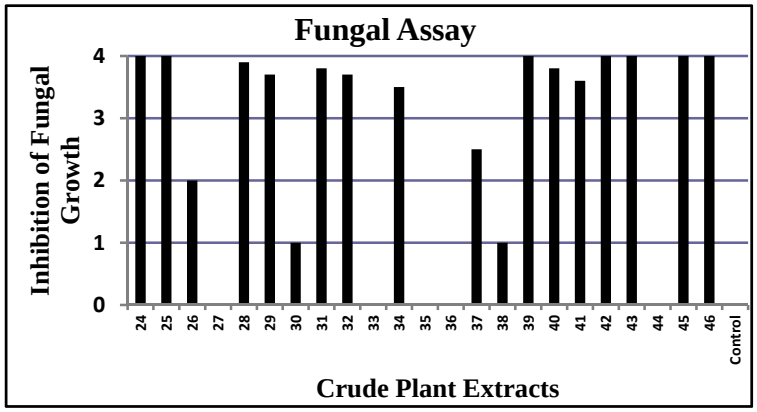
Forty-one crude extracts exhibiting very strong to complete inhibition of fungal growth (rating 3.5 to 4) were selected for further analysis. These extracts were exhaustively dialyzed (3,500 MWCO) in extraction buffer (10mM Tris pH 8.0) and assayed for antifungal activity against *A. flavus* as described above. Out of the 41 extracts, 29 exhibited a complete loss of antifungal activity after dialysis (Fig. 4). A loss of substantial activity was observed in extract 3, which was obtained from the leaves of an *Artemisia* species (Fig. 4A; Table 2). After dialysis, this extract showed slight inhibition of fungal growth after 48 hours. A partial loss of antifungal activity also was observed in dialyzed extracts from the root of *Yucca glauca* (extract 2) and from

the leaves of *Tradescantia tharpii* (extract 45), *Camassia scilloides* (extract 43), and *Oenothera* sp. (extract 77), but unlike extract 3, these retained moderate inhibition of *A. flavus* growth (see Fig. 4; see Table 2). However, dialyzed extracts 70 and 71 indicated strong activity with a rating of 3.4 and 3.1 respectively (Fig. 4B). Antifungal activity was also retained by dialyzed extracts 52, 54, and 73 and *Camassia* bulb extract 78 (see Fig. 4; Fig. 5, see Table 2). According to our bioassay results, these four dialyzed extracts completely inhibited conidial germination of *A. flavus* (see Fig. 5). Thus, among the 84 extracts screened for antifungal activity against *A. flavus,* these were the only four extracts that exhibited complete inhibition of fungal growth both before and after dialysis. Even after one week, no fungal growth was observed in these extracts.

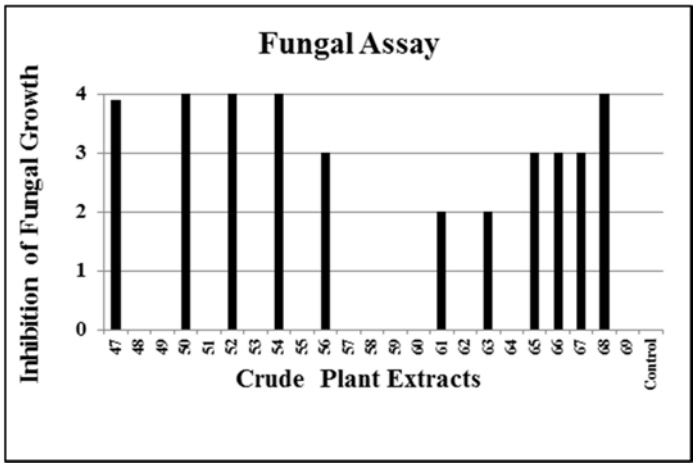
3A



3B



3C



3D

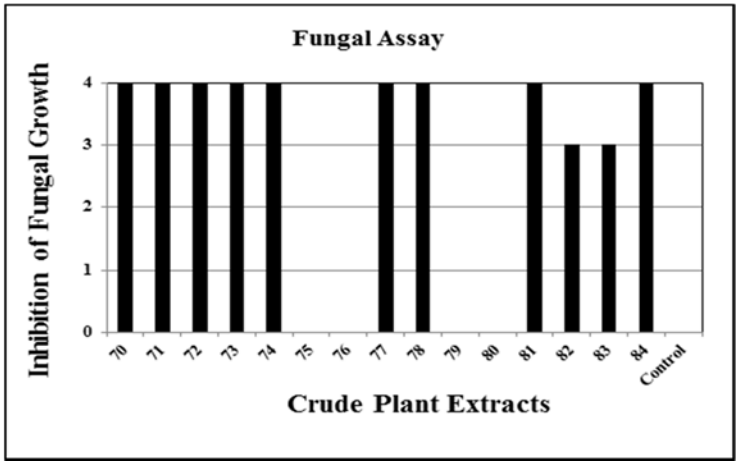
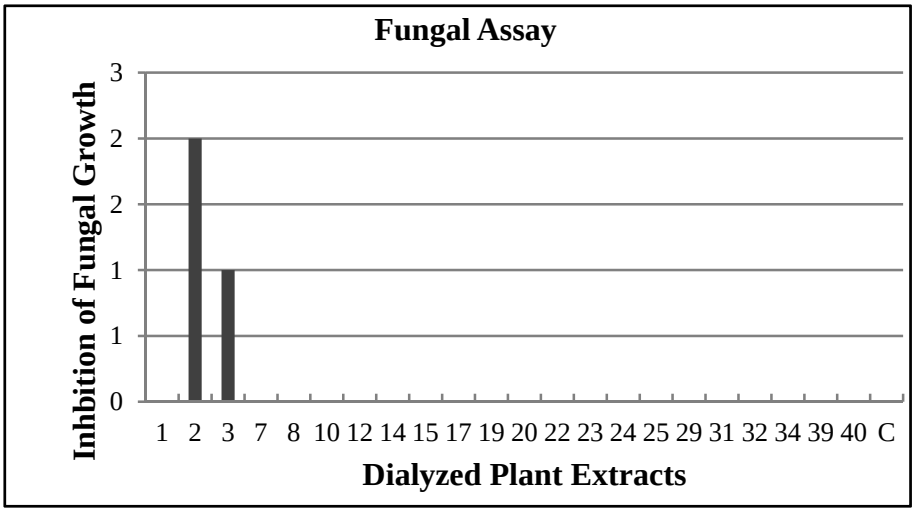


Figure 3 Evaluation of antifungal activity of crude extracts from Plant Collection Number (PCN) 1-84. (3A) Crude extracts from PCN 1-23. (3B) Crude extracts from PCN 24-46. (3C) Crude extracts from PCN 47-69. (3D) Crude extracts from PCN 71-84. Antifungal activity was measured using the standard assay with *Aspergillus flavus*. Rating of 0 = no inhibition of fungal growth and rating of 4 = complete inhibition of conidial germination and hyphal growth. PCN, plant collection number.

4A



4B

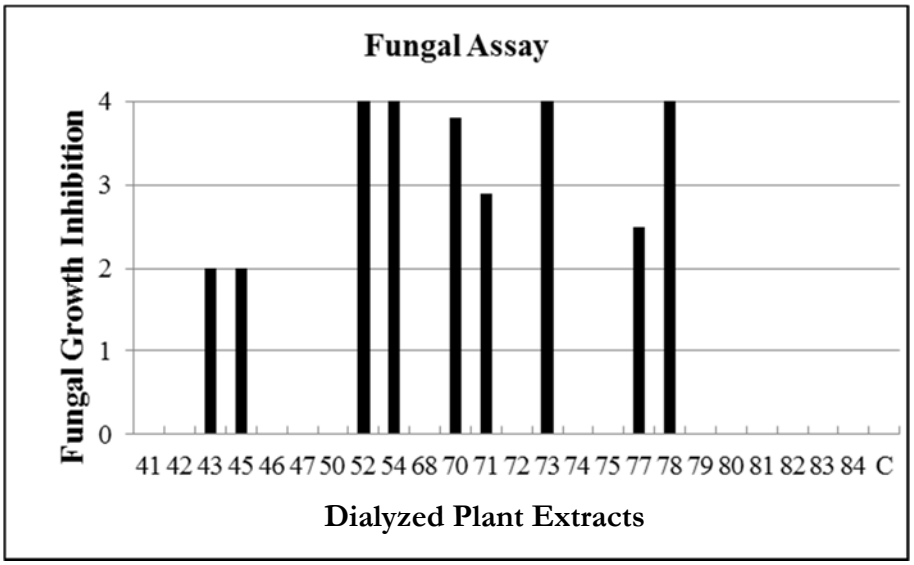
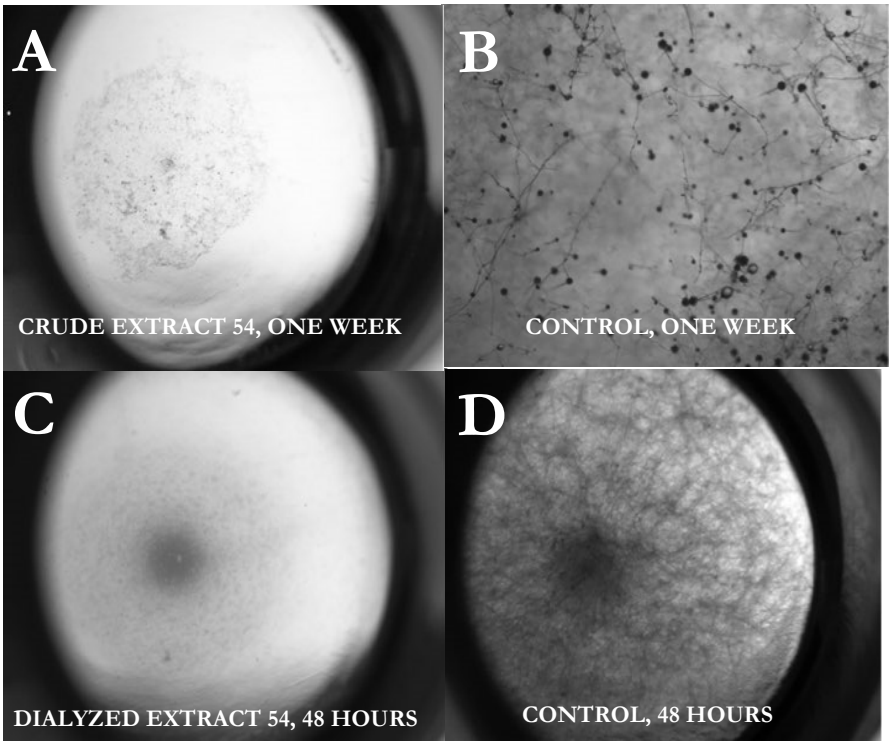
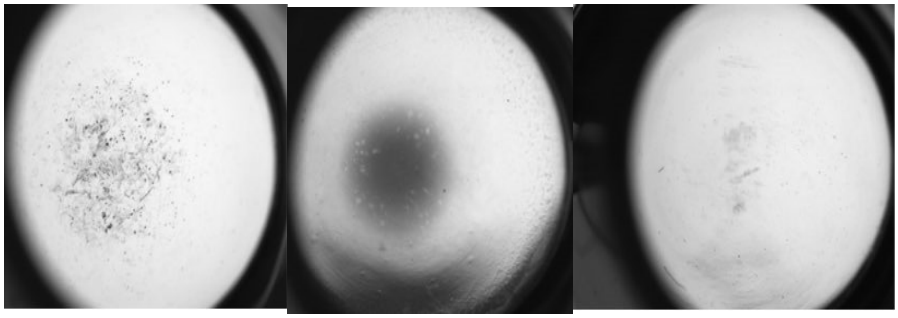


Figure 4 Evaluation of antifungal activity of dialyzed extracts from PCN 1-84 and Controls. (4A) Dialyzed extracts from PCN 1-40. (4B) Dialyzed extracts from PCN 41-84. Antifungal activity was measured using the standard assay with *Aspergillus flavus*. Rating of 0 = no inhibition of fungal growth and rating of 4 = complete inhibition of conidial germination and hyphal growth. PCN, plant collection number.



Panel 1 (A) Crude extract 54 retained complete inhibition of conidial germination and hyphal growth after one week. (B) *Aspergillus flavus* growth in Tris buffer control after one week. After one week of incubation conidia were visible in the control well. (C) Dialyzed extract 54 retained complete inhibition of conidial germination after 48 hours. (D) Control growth after 48 hours.



Panel 2 Antifungal activity of dialyzed extracts 52, 73 and 78. All three dialyzed extracts exhibited complete inhibition of conidial germination after 48 hours. Dark areas were due to the extract settling in the center of the well. Conidial germination and fungal growth were observed using a Nikon SMZ 1500 stereomicroscope equipped with digital CCD camera and NIS software.

Figure 5 Antifungal activity of crude and dialyzed extracts 54, 52, 73 and 78

Table 2 List of plants retaining antifungal activity after dialysis
PCN, Plant Collection Number. AF, Stephens County; MP, Medicine Park; CSL, East Lawton. R, root; L, leaf; B, bulb; Fl, flower.

Extract Number	PCN Number	Plant Tissue	Plant Name	Inhibition of Fungal Growth after Dialysis
2	AF #10R	Root	*Yucca glauca*	Moderate (rating 2)
3	AF #13L	Leaf	*Artemisia* sp.	Slight (rating 1)
43 78	MP#9L MP#9B	Leaf Bulb	*Camassia scilloides*	Moderate (rating 2) Complete (rating 4)*
45	MP#3L	Leaf	*Tradescantia tharpii*	Moderate (rating 2)
52 70	MP#22Fl MP22#B	Flower Bulb	*Allium canadense* (white flower)	Complete (rating 4)* Strong (rating 3.4)
54 71	MP#7Fl MP#7B	Flower Bulb	*Allium canadense* (light pink flower)	Complete (rating 4)* Strong (rating 3.1)
73	CSL#1B	Bulb	*Allium drummondii* (deep pink flower)	Complete (rating 4)*
77	MP#13L	Leaf	*Oenothera* sp.	Moderate (rating 2.5)

All six of the dialyzed extracts exhibiting strong to complete inhibition of *A. flavus* belong to two genera of Asparagaceae (see Table 2). Both the bulb and leaf of *Camassia scilloides* (PCN 9; Fig. 6C) possess antifungal activity against *A. flavus*, but the activity of the extract from the bulb of the plant is more potent. The other five extracts belong to the genus *Allium* (see Table 2). These plants were collected from East Lawton (CSL) and Medicine Park (MP). Extracts 52 and 70 were obtained from PCN MP#22 which had white flowers (Fig. 6A). Extracts 54 and 71 were from PCN MP#7 that had slightly pink flowers (Fig 6B). Extract 73 was obtained from CSL#1 which had a very distinct bulb and deep magenta-pink flowers (Fig. 7). According to the Oklahoma Vascular Plant Database, seven different *Allium* species are found in Comanche County, and *Allium* species exhibiting antifungal activity were tentatively identified based on the external morphology of the plants (see Table 2).

A. PCN MP#22 B. PCN MP#7 C. PCN MP#9

Figure 6 Plants exhibiting complete inhibition of fungal growth in crude and dialyzed extracts. (A and B), *Allium canadense*. (C) *Camassia scilloides*. Extracts from flowers of *A. canadense (ex #52 and 54)* and the bulb of *C. scilloides* (ex #78) exhibited complete inhibition of *A. flavus* growth. PCN, plant collection number.

A. PCN CSL#1 B. PCN CSL#1FL C. PCN CSL#1B

Figure 7 *Allium drumandii* collected from East Lawton. Both crude and dialyzed extracts (#73) from the flower of *A. drumandii* completely inhibited *Aspergillus flavus* growth in fungal bioassay. (A and B) Entire plant and flowers of *A. drumandii* respectively. (C) Characteristics fibrous structure around the bulbs of *A. drumandii*. PCN, plant collection number; Fl, flower; B, bulb.

DISCUSSION

In this study, most of the plant extracts that exhibited antifungal properties lost the activity after dialysis. Thus, it appears that most of the antifungal activity in crude extracts was due to soluble metabolites with molecular weights less than 3,500 Da. However, our results indicate that six extracts from two genera of Asparagaceae contained macromolecular compounds with molecular weights greater than 3,500 Da that were capable of strong to complete inhibition of fungal growth. To our knowledge, this is one of the first reports of antifungal activities from *Camassia scilloides*, *Allium canadense,* and *A. drummmondii* against *Aspergillus flavus*.

It is possible that the activity exhibited by some of the extracts may be due to the combinatorial effect of more than one type of compound. For example, crude extracts 2, 3, 43, 45, and 77, with ratings of 4, exhibited complete inhibition of conidial germination. After dialysis, however, these extracts lost approximately half or more of the inhibitory activity indicating a possible combined action of both low molecular weight compounds as well as molecules larger than 3,500 Da.

It is important to remember that the activities detected in the 84 aqueous extracts used in our study do not reflect the total antifungal activity present in the plant tissue or the potential to produce defense-related proteins and metabolites in response to fungal invasion. Furthermore, the loss of activity in many extracts may be due to protein denaturation and/or precipitation which may occur during storage and dialysis of the extracts. Also, many antifungal metabolites may not be soluble or may be only sparingly soluble in aqueous extracts. Therefore, organic solvent extraction would be required to isolate those compounds. Frequently, plant defenses are not expressed constitutively but are often produced in response to pathogen attack. These defense-related compounds include different types of proteins, which are induced upon infection by various pathogens, including fungi (Heisey and Gorgam 1992; Hu and Reddy 1997; Hu and Zhu 1997; Mohr et al. 1998; Cardoza et al. 2002). These defense-related proteins are comprised of enzymes responsible for the production of phytoalexins and other defensive metabolites, as well as pathogenesis-related proteins such as chitinase, glucanase and protease inhibitors. It is possible that extracts without antifungal activity are from plants that do not produce defense-related molecules constitutively and have not been induced. Even though antifungal compounds are not produced constitutively by these plants, they may very well possess the ability to activate defense genes in response to various elicitors of defensive compounds present in the tissue.

In this study, all 84 extracts were tested against *A. flavus* which is a filamentous ascomycete. Antifungal compounds present in the extracts may or may not have a broad range of antifungal activity. The activity may vary for different fungi due to different modes of action, or different fungi may be more or less sensitive to certain defense compounds. None of these extracts were tested against any representatives from oomycetes, such as *Phytophthora* sp. or *Pythium* sp. Many known antifungal proteins such as PR-1 and PR-5 specifically affect oomycetes, such as *Phytophthora infestans* (Woloshuk et al. 1991). Consequently, if these 84 extracts were tested for inhibitory activity against oomycetes, completely different results may have been obtained in this study.

The bioassays used in this study were rated after 48 hours of incubation, but the fungal growth in the microtiter plates was monitored and recorded for up to one week. Close observation of conidial germination and hyphal growth in the bioassay revealed that there may be different classes of mechanisms of antifungal activity

present in the extracts. In one class, conidial germination was completely inhibited after 48 h of incubation. Some examples from this category include crude extracts 1, 2, 14, 17, 25, 37, 43, 46, 50, 52, 54, 68, 73, and 78. In these extracts, conidia did not germinate even after 72 h of incubation. In the second class, conidial germination did occur, but the reduction in germination was coupled with an alteration in hyphal growth. For example, crude extract 56 strongly inhibited conidial germination and retarded hyphal growth, forming extremely branched and distorted hyphae (Fig. 8). Thus, our study indicates that extracts may exert their effect in at least two different ways. The first is to inhibit conidial germination and the second to distort hyphal growth.

The activities observed in our antifungal screens may not reflect all of the activities initially present in the extracts. The inhibitor may break down naturally or may be inactivated or detoxified through the action of endogenous activities in the extract (such as hydrolases) or reactive components in the extract (such as phenolic compounds or oxygen). The extraction conditions used in these studies were not designed to protect sensitive or unstable activities. Antioxidants, metal chelators, protease inhibitors, and/or reductants were not included in the buffer. The only protectant used was PVP, which was added to reduce the concentration of potentially reactive phenolics.

CONCLUSIONS

Our results showed that most of the extracts that exhibited antifungal activity before dialysis lost their activity after dialysis. This indicates that most of the antifungal activity was due to the presence of soluble metabolites with molecular weights less than 3,500 Da. Four extracts retained complete antifungal activity after dialysis. Three of the extracts were obtained from *Allium* and the fourth from *Camassia* sp. Our study also indicates that antifungal activity retained in these dialyzed extracts is due to macromolecular compounds with molecular weights greater than 3,500 Da.

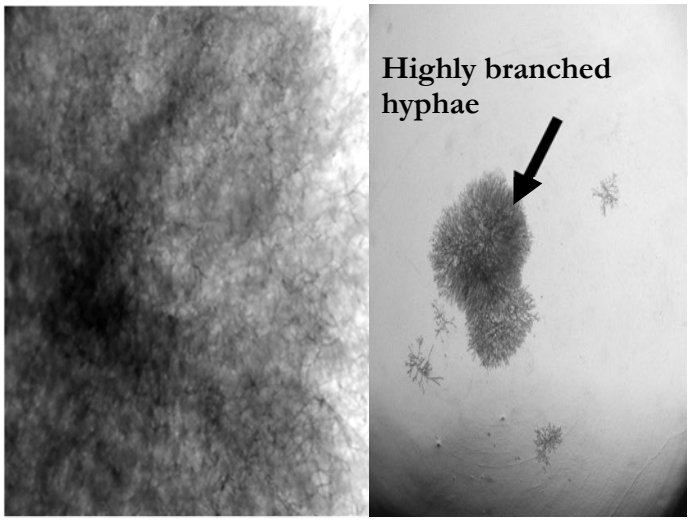


Figure 8 Antifungal activity of crude extract 56 after 48 hours. (A) Hyphal growth in control. (B) Crude extract 56 strongly inhibited conidial germination and retarded hyphal growth, forming extremely branched and distorted hyphae. Antifungal activity was measured using the standard assay with *Aspergillus flavus*. Rating of 0 = no inhibition of fungal growth and rating of 4 = complete inhibition of conidial germination and hyphal growth. Conidial germination and fungal growth were observed using a Nikon SMZ 1500 stereomicroscope equipped with digital CCD camera and NIS software.

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