AN APPROACH TO ENHANCE SECONDARY METABOLITE PRODUCTION OF ENDOSYMBIOTIC FUNGI THROUGH THE INCORPORATION OF RESIN INTO CULTURE MEDIA

By

Lauren Kelly West

A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors degree

With Honors in

Molecular and Cellular Biology

THE UNIVERSITY OF ARIZONA

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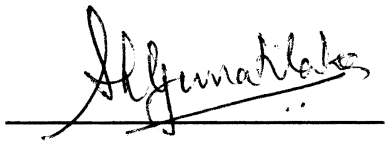

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Abstract

Many fungi produce biologically active secondary metabolites when stressed. Some of these metabolites have proven to have agricultural and medicinal uses. Methods to improve the yield of these fungi would allow large-scale production of biologically active metabolites with potential for drug discovery and development. This project was designed to test if yields can be improved through the incorporation of a resin. Six endosymbiotic fungal strains were studied: *Paraphalasphaeria quadriseptata, Verticillium chlamydosporium, Ulocladium sp.,Gretetia reticulosperma, Phaeosphaeria/ Phaeosphaeriopsis* and *Geopyxis* sp. nov**.** These strains were grown under four different media conditions: Potato dextrose broth (PDB), PDB with 0.25 mM $CuSO_{4,}$ PDB with resin, PDB with resin and 0.25mM $CuSO_{4.}$ The relative yields of the extracts produced by these fungi were compared and the extracts were investigated for compounds of interest by Thin-layer Chromatography.

1. General Introduction

When stressed, certain fungi have been known to produce interesting and biologically active metabolites; these secondary metabolites have shown medicinal and therapeutic potential. Many metabolites are often produced in low yields as they may cause harm to the fungus due to their potent antifungal activity. Increasing yields of these metabolites may allow low-yielding fungal strains to improve production of biologically active small-molecule natural products.

Secondary metabolites of both fungi and plants are topics of interest and research in natural product chemistry. Familiar fungal metabolites include penicillin and cephalosporin, which are marketed for their antibiotic uses. As well as ergot alkaloids which have been used to treat headaches and control bleeding (Keller et al., 2005). Secondary metabolites are not essential to the common metabolic pathways of the fungi and are often only produced when these fungi are stressed. Many of these compounds have been shown to inhibit bacterial and fungal growth as well as the growth of parasites, viruses, and cancer (Keller et al., 2005). For this reason, the search for new secondary metabolites as well as new applications for previously discovered secondary metabolites may assist in the development of novel pharmaceuticals.

Although adsorbent resins are commonly used when culturing soil streptomyces, it is not typically applied to increase secondary metabolite yields in fungi (Singh et al., 2009). This project proposes that the yield of these metabolites can be enhanced through the use of a resin (e.g. HP-20). Our hypothesis is that removing the toxic metabolites with resin as they are produced will make the media non-toxic to the fungus allowing for unhindered growth of fungus and improved yield of secondary metabolites.

Five fungal strains were selected for this study based on their known secondary metabolite production: *Paraphalasphaeria quadriseptata, Verticillium chlamydosporium,*

Ulocladium sp.,Gretetia reticulosperma, and *Phaeosphaeria/ Phaeosphaeriopsis.* In addition, a control was also tested: *Geopyxis* sp. nov. Five strains were cultured under four different media conditions: Potato dextrose broth (PDB), PDB with 0.25 mM $CuSO_4$, PDB with resin (HP-20), PDB with resin and 0.25mM $CuSO_4$. PDB is commonly used when culturing fungi and bacteria. $CuSO_4$ has been shown to improve the yield of secondary metabolites probably by providing additional stress to the fungus (Paranagama et al., 2007). The fungi were allowed to consume all of the glucose present in the media which is known to trigger the production of secondary metabolites as a result of stress. The yields of the total fungal extracts were obtained through extraction of each of the species cultured in the four different media specified above. Thin-layer chromatography (TLC) analysis was performed for each of the extracts and compared with previously isolated compounds. High pressure liquid chromatography (HPLC) was also performed on selected extracts.

The six strains were selected because they represent a range of different fungi which produced bioactive compounds in low yields. *Paraphalasphaeria quadriseptata* is a rhizosphere fungus isolated from the Christmas cactus common in the Sonoran desert (Wijeratne et al., 2006). The compound of interest found in *Paraphalasphaeria quadriseptata* is monocillin I. Monocillin I along with radicicol and zearalenone are fungal polyketides which are compounds of interest for developing novel treatments for cancer and neurodegenerative diseases (Wang et al., 2008)

Verticillium chlamydosporium is also a rhizosphere fungus which has shown to have nematicidial activity (Kerry et al., 2006). The ability of this fungus to prevent parasitic attack emphasizes the symbiotic relationship between *V. chlamydosporium* and the plant that it populates. The compound of interest for *V. chlamydosporium* is radicicol which is an important metabolite due to its potent anti-cancer properties. It functions through an inhibitory mechanism

of the chaperone Hsp90 whose repression causes cancer pathways to be down regulated (Wang et al., 2008).

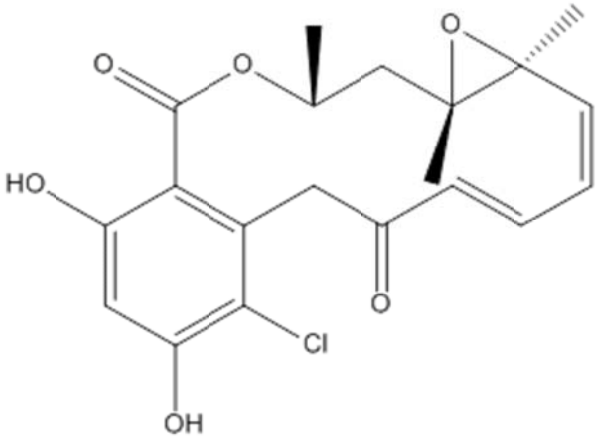
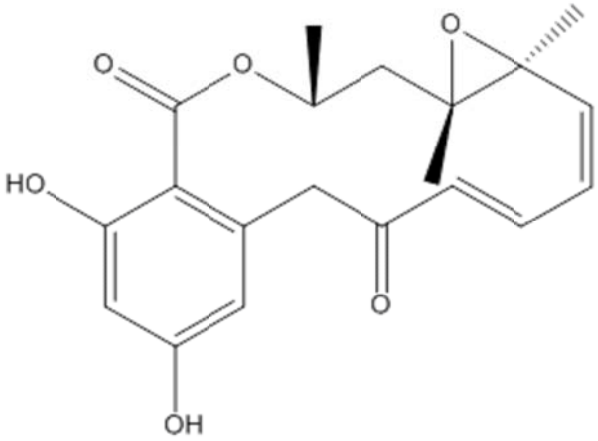
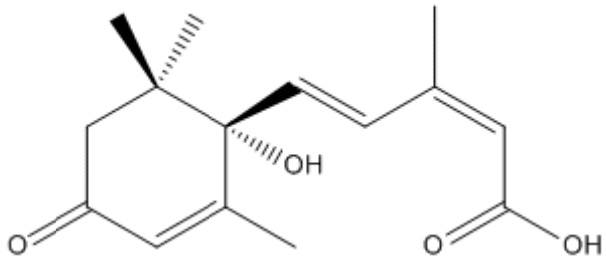
Greletia reticulosperma is a fungus found in the lichen Usnea whose compound of interest is sphaeropsidin A. This compound exhibits plant pathogenic qualities (Schrader et al., 2010). *Ulocladium sp.* contains a variety of fungi which are known to spoil food and infect plants. Many *Ulocladium* fungi are classified as saprobes (Anderson et al., 2008). The fungi which belong to *Ulocladium* secrete enzymes capable of degrading cellulose, amylase, xylan, and other complex polysaccharides which facilitate their saprotrophic activity (Pedersen et al., 2009). The compound of interest in *Ulocladium sp.* is abscisic acid, which is an important hormone involved in plant signaling, growth, and stress adaptation (Giraudat, 1995).

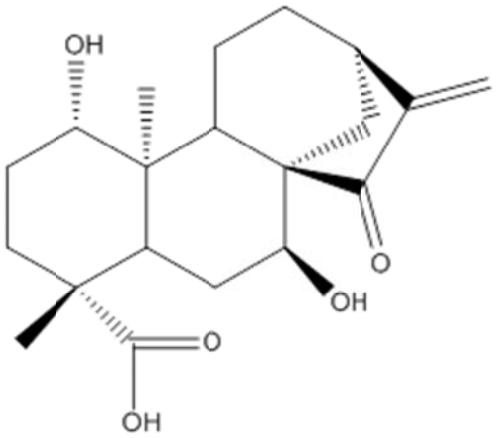
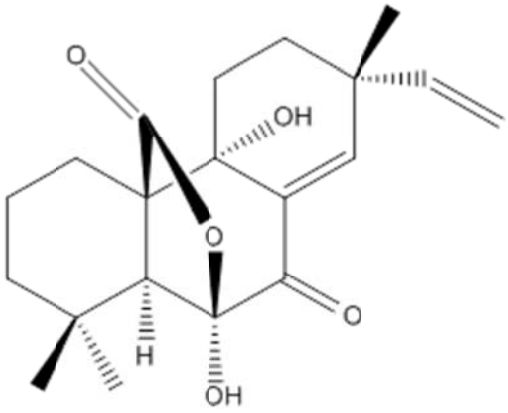
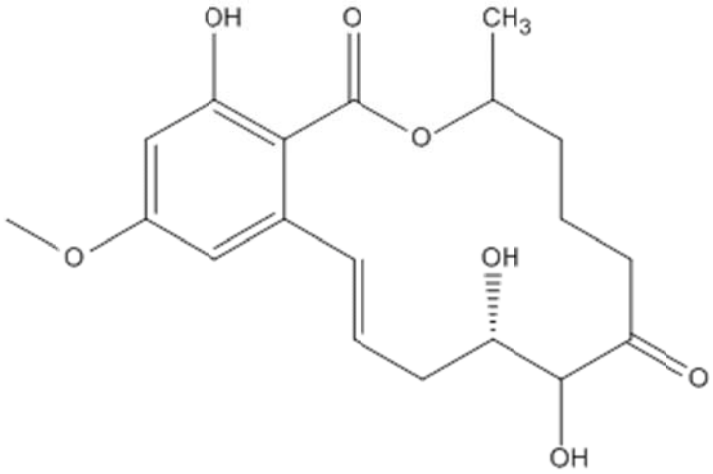
Many of the fungi which are classified in the genus *Phaeosphaeria* are often parasites which feed on grasses, cereals, and other plants (Shoemaker et al., 1988) The subgenus *Phaeosphaeriopsis* was created to specify fungal strains within *Phaeosphaeria* which produce specifically 4–5-septate ascospores, which are the reproductive cells produced in ascomycete fungi (Câmara et al., 2003). The compound of interest in *Phaeosphaeria/ Phaeosphaeriopsis* is a zearalenone-related compound with a macrolide ring (Ellestad et al., 1978). Macrolides, similar to the metabolite generated by *Phaeosphaeria/ Phaeosphaeriopsis*, are known to have antibiotic properties (Back, 1977). In addition, this compound is classified as a natural polyketide which, as discussed above, are a class of molecules of extreme interest due to their potential biological applications (Navickas and Maier, 2010).

Geopyxis sp. nov. represents a genus of fungi encompassed in the family Pyronemataceae which is a large and diverse family of fungi (Perry et al., 2007) The compound of interest is structurally similar to ent-kaurane diterpenoids which exhibit cytotoxic activity and selectivity towards human cancer cells (Lee et al., 1996). The structures of all the

compounds mentioned are shown in figure 1. This study reports the relative yields of the extracts derived from these six fungal strains grown in four different culture media as well as the investigation of extracts for the production of secondary metabolites of interest.

Figure 1. Structures of compounds of interest in fungal strains investigated

Fungal strain	Compound of Interest
Verticilliam chlamydosporium (ATCC 16289)	 Radicicol
Paraphaeosphaeria quadriseptata (Opl-1-F20)	 Monocillin I
Ulocladium sp (CS-69-141)	 Abscisic Acid

Geopyxis sp. nov. (AZ 0066)	 *Geopyxis* Compound
Gretetia reticulosperma (AZ 0721)	 Sphaeropsidin A
Phaeosphaeria/ Phaeosphaeriopsis (DC 3330)	 *Phaeosphaeria* compound

2. Results

2.1 Yields of Fungal Extracts

Table 1.

Fungal Species	Culture Conditions [a]	Extract from Filtrate Yield (mg)	Extract from Mycelia Yield (mg)	Total Yield of Extract (mg)
Verticilliam chlamydosporium	PDB	9.22	116.23	125.45
	PDB + resin	2.64	83.49	86.13
	PDB + $CuSO_4$	5.85	150.76	156.61
	PDB+ resin + $CuSO_4$	4.26	104.38	108.64
Paraphaeosphaeria quadriseptata	PDB	4.51	61.05	65.56
	PDB + resin	0.88	36.43	37.31
	PDB + $CuSO_4$	0.801	56.62	57.421
	PDB+ resin + $CuSO_4$	0.42	30.47	30.89
Ulocladium sp	PDB	34.67	57.87	92.54
	PDB + resin	13.98	26.29	40.27
	PDB + $CuSO_4$	13.52	53.91	67.43
	PDB+ resin + $CuSO_4$	4.87	79.93	84.80
Geopyxis Sp. Nov.	PDB	61.48	52.61	114.09
	PDB + resin	23.51	231.27	236.78
	PDB + $CuSO_4$	41.80	32.29	55.8
	PDB+ resin + $CuSO_4$	24.62	212.96	237.58
Gretetia reticulosperma [b]	PDB	3.99	11.45	15.44
	PDB + resin	0.59	21.98	22.57
	PDB + $CuSO_4$	No Growth	No Growth	No Growth
	PDB+ resin + $CuSO_4$	No Growth	No Growth	No Growth
Phaeosphaeria/ Phaeosphaeriopsis	PDB	3.62	76.55	80.17
	PDB + resin	0.810	107.1	107.97
	PDB + $CuSO_4$	7.85	75.86	83.71
	PDB+ resin + $CuSO_4$	1.28	69.38	70.66
Paraphaeosphaeria quadriseptata [c]	PDB	4.23	65.95	70.27
	PDB + resin	0.120	107.56	107.68
	PDB + $CuSO_4$	11.35	97.78	109.13
	PDB+ resin + $CuSO_4$	1.21	121.18	122.39

a. 100mL of liquid culture

b. *Gretetia reticulosperma* was inoculated on 6/3/10 and showed no growth with $CuSO_4$. Therefore the results for this sample were only obtained from the strain growth in PDB and PDB with resin.

c. Strain re-inoculated due to initial low yield

2.2 TLC Investigation

Table 2. Rf values for TLC spots in the extract of *Ulocladium* sp.

Eluent: 8% MeOH/DCM Adsorbant: Silica Gel 60 RP-18 F_{254} S

	PDB	**PDB +$CuSO_4$**	**PDB +$CuSO_4$ + resin**	**PDB + resin**
Filtrate **Extract**	Compound of interest with Rf value of 0.52 not present in sample. Compounds found with Rf values of 0.85 and 0.79.	Compound of interest at 0.41 not present in sample. Compounds found with Rf values of 0.65 and 0.83.	Compound of interest with Rf value of 0.40 was found to be present along with other trace compounds.	Compound of interest with Rf value of 0.5. Compound of interest present in extract with Rf value of 0.48 along with other trace compounds.
Mycelia **Extract**	Compound of interest with Rf value of 0.47 was found to be present along with other trace compounds.	Compound of interest with Rf value of 0.45 was present in sample along with other trace compounds	Compound of interest with Rf value of 0.45 was present in sample with Rf value of 0.44.	Compound of interest with Rf value of 0.42 was found to be present in sample

Table 3. Rf values for TLC spots in the extract of *Verticillium chlamydosporium*

Eluent: 10% MeOH/DCM Adsorbant: Silica Gel 60 RP-18 F_{254} S

	PDB	**PDB +$CuSO_4$**	**PDB + resin**	**PDB + $CuSO_4$ + resin**
Filtrate Extract	Compound of interest with Rf value of 0.7 was found to be present in the sample. Additional compound with a Rf value of 0.84 present.	Compound of interest with Rf value of 0.63 was found to be present in the sample.	Compound of interest at 0.7 not present in sample. Additional compounds found with Rf values of 0.87 and 0.09 present in sample.	Compound of interest with Rf value of 0.67 was not present in sample. Additional compound found in sample with a Rf value of 0.09.
Mycelia Extract	Compound of interest with a Rf value of 0.7 not present in sample. Compound present in sample with a Rf value of 0.09.	Compound of interest with a Rf value of 0.7 not present in sample. Compound present in sample with a Rf value of 0.09.	Compound of interest with a Rf value of 0.7 not present in sample. Compound present in sample with a Rf value of 0.09.	Compound of interest with a Rf value of 0.7 not present in sample. Compound present in sample with a Rf value of 0.09.

Table 4. Rf values for TLC spots in the extract of *Greletia reticulosperma*

Eluent: 8% MeOH/DCM Adsorbant: Silica Gel 60 RP-18 F_{254} S

	PDB	**PDB + resin**
Filtrate Extract	Compound of interest with a Rf value of 0.51 present in sample. Weak presence of compound of interest with a Rf value of 0.47 in sample along with many other trace compounds.	Compound of interest with a Rf value of 0.52. Presence of compound of interest in sample at 0.53. Additional major compounds with a Rf value of 0.84 present along with other trace compounds.
Mycelia Extract (MeOH)	Compound of interest with a Rf value of 0.5. Weak presence of compound of interest present in sample with a Rf value of 0.48 with other major compounds having Rf values of 0.92 and 0.56.	Compound of interest with an Rf value of 0.51. Presence of compound of interest in sample with a Rf value of 0.53 along with other trace compounds.
Mycelia Extract (EtOAc)	Compound of interest with a Rf value of 0.48 presence in sample along with other major compounds having Rf values of 0.81, 0.4, and 0.25.	Compound of interest with a Rf value of 0.50. Presence of compound of interest in sample with a Rf value of 0.49 with another major compound with a Rf value of 0.84.

Table 5. Rf values for TLC spots in the extract of *Paraphaeosphaeria quadriseptata*

Eluent: 8% MeOH/DCM Adsorbant: Silica Gel 60 RP-18 F_{254} S

	PDB	**PDB + resin**	**PDB+$CuSO_4$**	**PDB +$CuSO_4$ + resin**
Filtrate Extract	Compound of interest with a Rf value of 0.67 present in sample. Additional compounds found to have Rf values of 0.85, 0.55, and 0.15.	Compound of interest with a Rf value of 0.63 present in sample. Additional compounds in sample found to have Rf values of 0.51 and 0.83 along with other trace compounds.	Compound of interest with a Rf value of 0.63 present in sample. Additional compounds in sample found to have Rf values at 0.45, 0.57, and 0.82.	Compound of interest with a Rf value of 0.53 present in sample. Additional compounds with Rf values at 0.78 and 0.22 present.
Mycelia Extract (MeOH)	Compound of interest with a Rf value of 0.41 present in sample along with other trace compounds.	Compound of interest with a Rf value of 0.53 present in sample. Additional compounds found with Rf values of 0.8 and 0.11	Compound of interest with a Rf value of 0.5. Compound of interest present in sample with a Rf value 0.48. Additional major compound in sample with a Rf value of 0.66 along with other trace compounds.	Compound of interest with a Rf value of 0.59 present in sample along with other trace compounds.
Mycelia Extract (EtOAc)	Compound of interest with a Rf value of 0.52. Compound of interest present in sample with a Rf value of 0.50 with additional major compound present with a Rf value of 0.81.	Compound of interest with a Rf value of 0.46 present in sample. Additional major compounds present with Rf value of 0.8, 0.38, and 0.18.	Compound of interest at 0.48 present in sample. Additional major compounds present with a Rf value of 0.80 along with other trace compounds.	Compound of interest at 0.41 present in sample. Additional major compounds with Rf values of 0.35 and 0.67 present along with other trace compounds .

Table 6. Rf values for TLC spots in the extract of *Phaeosphaeria/ Phaeosphaeriopsis*

Eluent: 8% MeOH/DCM Adsorbant: Silica Gel 60 RP-18 F_{254} S

	PDB	**PDB+ resin**	**PDB +$CuSO_4$**	**PDB +$CuSO_4$ + resin**
Filtrate Extract	Compound of interest with a Rf value of 0.41. Compound of interest present in sample with a Rf value of 0.39. Additional compound with Rf value of 0.84 present with traces of minor compounds.	Compound of interest with a Rf value of 0.39 not present in sample. Additional compounds with Rf values of 0.24 and 0.92 present in sample.	Compound of interest with a Rf value of 0.38 present in sample. Additional compounds with Rf values of 0.22 and 0.14 present in sample.	Compound of interest with a Rf value of 0.42 not present in sample. Additional compound present with a Rf value of 0.24 with traces of minor compounds
Mycelia Extract (MeOH)	Compound of interest with a Rf value of 0.39.Compound of interest present in sample with a Rf value of 0.41. Additional compounds with Rf values of 0.45 and 0.61 present with traces of minor compounds.	Compound of interest with a Rf value of 0.38. Compound of interest present in sample with a Rf value of 0.35. Additional compounds with Rf values of 0.48 and 0.83 present with traces of minor compounds	Compound of interest with a Rf value of 0.38 present in sample. Additional compounds with Rf value of 0.53 and 0.83 present with other trace compounds	Compound of interest with a Rf value of 0.4. Compound of interest present in sample with a Rf value of 0.39. Additional compound with Rf value of 0.84 along with other trace compounds.
Mycelia Extract (EtOAc)	Compound of interest with a Rf value of 0.42. Compound of interest present in sample with a Rf value of 0.40. Additional compounds present with Rf values of 0.17, 0.51, and 0.79 along with traces of minor compounds.	Compound of interest with a Rf value of 0.38 present in sample Additional compounds with Rf values of 0.53 and 0.83 present with other trace compounds.	Compound of interest with a Rf value of t 0.38. Compound of interest present in sample with a Rf value of 0.35. Additional compounds with Rf values of 0.50 and 0.83 present with other trace compounds.	Compound of interest with a Rf value of 0.40. Compound of interest present with a Rf value of 0.39. Additional compound present with a Rf value of 0.84 along with other trace compounds.

Table 7. Rf values for TLC spots in the extract of *Geopyxis* sp. nov.

Eluent: 8% MeOH/DCM Adsorbant: Silica Gel 60 RP-18 F_{254} S

	PDB	**PDB + resin**	**PDB+$CuSO_4$**	**PDB +$CuSO_4$ + resin**
Filtrate	Compound of interest with a Rf value of 0.29. Compound of interest present in sample with a Rf value of 0.27 along with other trace compounds.	Compound of interest with a Rf value of 0.27. Compound of interest present in sample with a Rf value of 0.29. Additional compound found with a Rf value of 0.44 along with other trace compounds.	Compound of interest with a Rf value of 0.27. Compound of interest present in sample with a Rf value of 0.33 along with other trace compounds.	Compound of interest with a Rf value of 0.33. Compound of interest present in sample with a Rf value of 0.32. Additional compounds present with a Rf value of 0.44 along with other trace compounds.
Mycelia (MeOH)	Compound of interest with a Rf value of 0.25 present in sample. Additional compounds with Rf values of 0.63 and 0.88 present along with other trace compounds.	Compound of interest with a Rf value of 0.20. Compound of interest present in sample with a Rf value of 0.22. Additional compounds with Rf values of 0.80 and 0.84 present along with other trace compounds.	Compounds of interest with a Rf value of 0.34. Compound of interest present in sample with a Rf value of 0.32. Additional compounds with Rf values of 0.60 and 0.84 present along with other trace compounds.	Compound of interest with a Rf value of 0.31 present in sample. Additional compounds with Rf values of 0.48 and 0.88 present along with other trace compounds.
Mycelia (EtOAc)	Compound in interest with a Rf value of 0.38 present in sample. Additional compounds with Rf values 0.86 and 0.60 present along with other trace compounds.	Compound of interest with a Rf value of 0.25. Compound of interest present in sample with a Rf value of 0.24. Additional compounds with Rf values of 0.88 and 0.39 present along with other trace compounds.	Compounds of interest with a Rf value of 0.30. Compound of interest present in sample with a Rf value of 0.28. Additional compound with a Rf value of 0.86 present with other trace compounds.	Compounds of interest with a Rf value of 0.29 present in sample Additional compound with a Rf value of 0.49 present with other trace compounds.

3. Discussion

3.1 Introduction

Overall, mycelia extract yields greatly exceeded the filtrate extract yields. Investigation of mycelia extracts also revealed a higher number of compounds present than the filtrate extracts. The yields of extracts obtained suggest that resin did not increase the extract yields as expected. Results obtained for the fungal strains investigated are presented below.

3.2 *Verticillium chlamydosporium*

The filtrate extract of the strain *Verticillium chlamydosporium* derived from the PDB medium with no $CuSO_4$ and no resin proved to have a higher yield which was nearly more than three-fold greater than that derived from the cultures with resin. The mycelia extracts had more comparable yields across the four media but the compound of interest was found to be absent when investigated with thin-layer chromatography (TLC). TLC revealed that the compound of interest, radicicol (figure 1), was only present in the filtrate extracts in samples with no resin. This suggests that the resin could have adsorbed radiciol as expected but the compound was not removed from the resin during the extraction process. The lack of radicicol in the mycelia extract could also suggest that the compound is primarily present in the extract derived from the filtrate.

3.3 *Paraphaeosphaeria quadriseptata*

The strain *Paraphaeosphaeria quadriseptata* was cultured and extracted twice due to the low yield of the first extraction. The PDB + resin, PDB + $CuSO_4$, and PDB + $CuSO_4$ + resin media were depleted of glucose and extracted two weeks before the PDB media was depleted of glucose. This difference in growth rate is likely why the PDB media extract exhibits at least a five-fold higher yield than the extracts derived from the other three culture media. The second innoculation proved to have similar results but with a much higher extract yield of the PDB + $CuSO_4$ media which exceeded the extract yield of the PDB culture by nearly three-fold. Interestingly the two cultures containing resin in their media took ten days longer than the PDB and PDB + $CuSO_4$ culture media to deplete their

glucose levels. This suggests that a longer incubation time will not always result in a higher extract yield. This strain seemed to suggest that the incorporation of resin into the culture media reduced the potential extract yield. It is also important to note that *Paraphaeosphaeria quadriseptata* when compared to the other fungal strains had significantly lower yields of extract in most of culture media. TLC revealed that all four culture media contained the compound monocillin I in both the first and second cultures.

3.4 *Ulocladium sp.*

The strain *Ulocladium sp.* proved to have a much higher filtrate extract yield with the PDB media following similar trends as *Verticillium chlamydosporium* extract yields and *paraphaeosphaeria quadriseptata* extract yields. For the filtrate the PDB extract yield was greater than two-fold when compared to the extract yields of the PDB + resin, and PDB + $CuSO_4$ culture media. The mycelia yield had more varying results with PDB + resin + $CuSO_4$ culture media having the higher extract yield. The compound of interest, Abscisic Acid, was present in all of the filtrate extracts but only present in the PDB+ resin, and PDB + $CuSO_4$ + resin mycelia extracts. This suggests that although the filtrate extract yields with resin were low, it is possible that the compound Abscisic Acid may be more efficiently obtained from mycelia extractions when the fungi are cultured with resin.

3.5 *Greletia reticulosperma*

The strain *Greletia reticulosperma* has no apparent growth when cultured with in PDB media containing $CuSO_4$ after four months of incubation. The filtrates for the fungi cultured in PDB and PDB +resin media were extracted and investigated. The extract derived from the culture without resin showed a significantly higher yield by more than six-fold. Mycelia extracts also proved to have higher yields by nearly two-fold in the cultures lacking resin. The TLC for both the mycelia extract and the filtrate extract reveal the presence of the active compound, sphaeropsidin A.

3.6 *Phaeosphaeria/ Phaeosphaeriopsis*

The strain *Phaeosphaeria/ Phaeosphaeriopsis* also showed a higher yield without the presence of resin in the extracts derived from the filtrate while the extracts derived from mycelia proved to have the highest extract yield when cultured in PDB media with resin. This is significant because the mycelia extracts all had a strong presence of the *Phaeosphaeria* compound. The extracts derived from the filtrate of the resin cultures also showed a weak presence of the active compound during TLC analysis suggesting that the low filtrate yield is likely not relevant to the presence of the *Phaeosphaeria* compound.

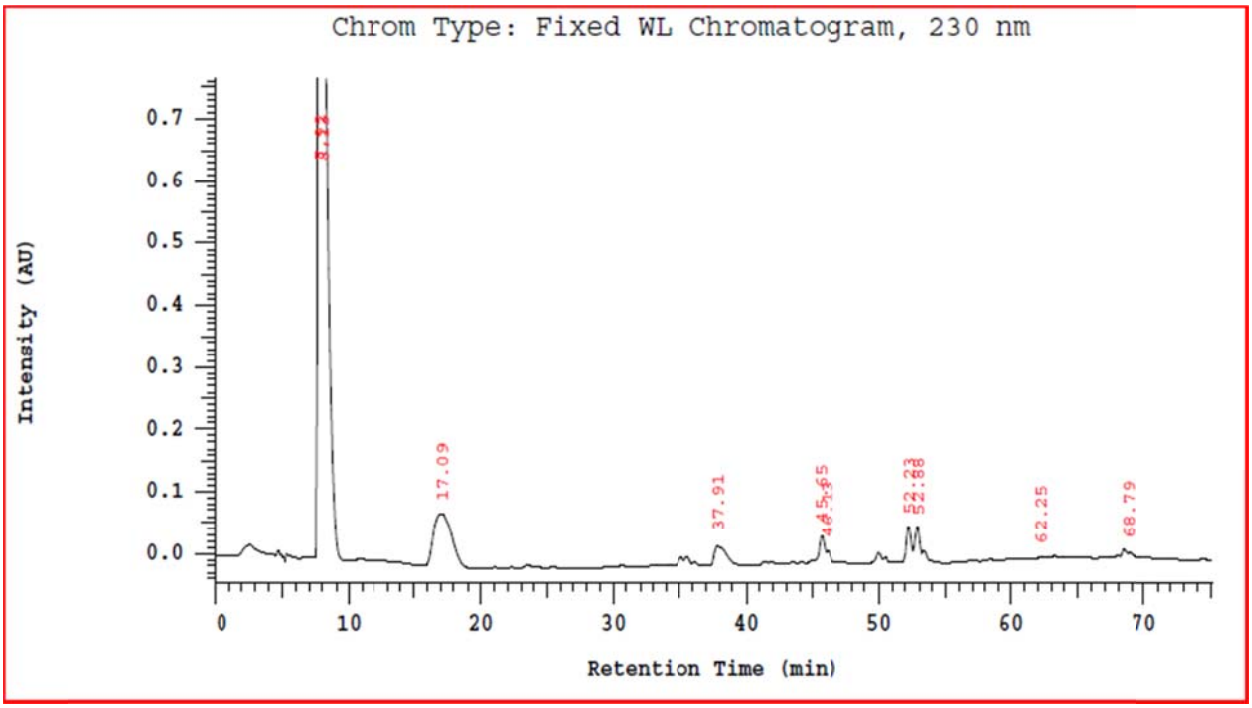
3.7 *Geopyxis sp.*

Geopyxis sp. nov. also proved to have a higher filtrate extract yield in the media cultured without resin. The highest yield for the fungal strain was the extract derived from the PDB media cultured with neither resin nor $CuSO_4$. The opposite was true for the mycelia extractions where the cultures containing resin have extract yields more than four-fold greater than the extract yields derived from the PDB and PDB + $CuSO_4$ culture media. The total extract yield for *Geopyxis sp. nov*. was over two-fold greater for the cultures with resin than the highest-yielding culture without resin. This is significant considering that the TLC for both mycelia extractions and the filtrate extraction showed the strong presence of the compound of interest.

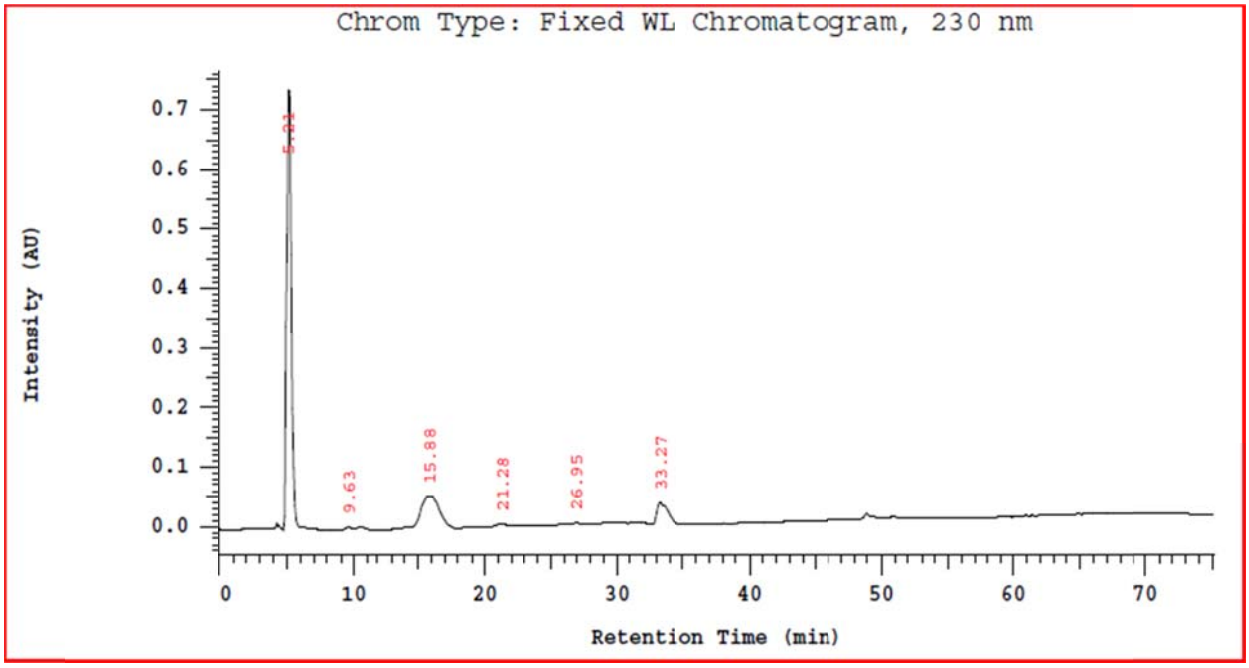
Although TLC investigation suggested the presence of the compound of interest high performance liquid chromatographic analysis (HPLC) revealed otherwise. HPLC was performed on the mycelia extracts (see figure 2). For the extract derived from PDB culture media the presence of the *Geopyxis* compound is apparent at a retention time of 17.09 min. For the extract derived from PDB and $CuSO_4$ culture media the compound of interest appears at a retention time of 15.88 min. The extracts derived from media cultured with resin show a little to no presence of the active compound (see figure 2C. and 2D.) There is peak in the extract from the media culture with resin at 16.23 min but this suggest a very weak presence when compared to the intensity of the peaks from the other extracts.

Figure 2. HPLC data for the Mycelia Extract of *Geopyxis Sp.*

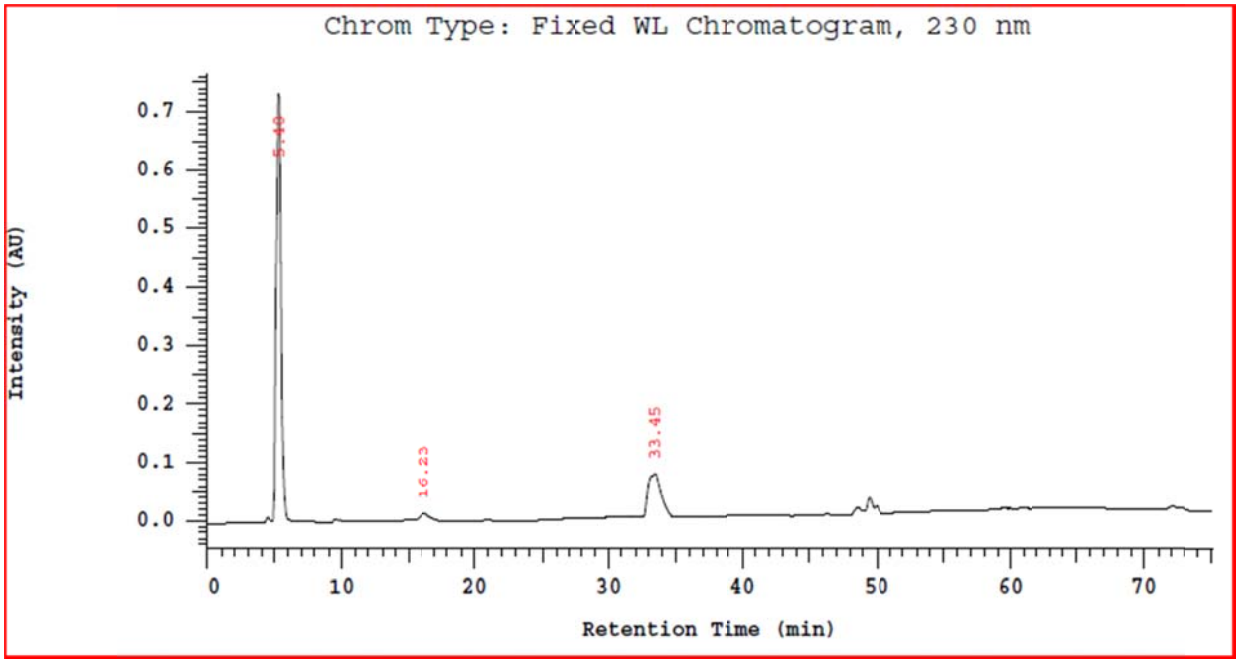
a. PDB



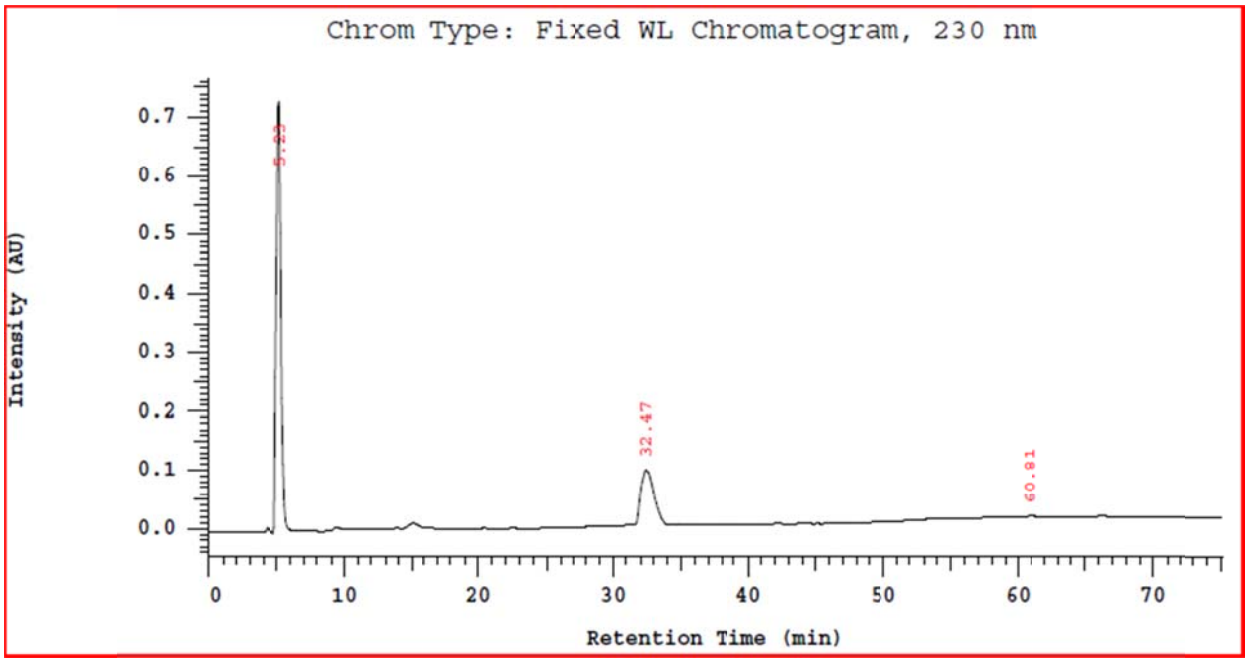
b. PDB +$CuSO_4$



c. PDB + Resin



d. PDB + Resin + $CuSO_4$



4. Conclusions

In the control, *Geopyxis* sp. nov., resin proved to significantly increase yield in the mycelia extract but the compound of interest was not verified for these extracts according to HPLC analysis. In addition to the control, all five fungal strains: *Paraphalasphaeria quadriseptata, Verticillium chlamydosporium, Ulocladium sp.,Gretetia reticulosperma, Phaeosphaeria/ Phaeosphaeriopsis* revealed a higher filtrate extract yield in strains cultured without resin. The mycelia extract yield was more varied among the fungi which is likely due to the presence of a higher number of compounds in the mycelia extract as revealed by TLC. For many of the fungal strains the presence of resin seems to decrease extract yield especially in the extracts derived from the filtrate. Overall resin incorporation did not prove to significantly increase the extract yield. It is important to note that the adsorptive nature of the resin may have prevented the extraction of the compounds of interest resulting in a lower yield. This is supported by the HLPC analysis of *Geopyxis* sp. nov. which revealed the absence of the *Geopyxis* compound in the extracts derived from media cultured with resin. This suggests that the resin was successfully adsorbing compounds of interest from the media but was not allowing for the total extraction of these compounds from the resin using the extraction techniques discussed in the experimental section.

5. Experimental

5.1 Activation of resin HP-20

Dianion HP-20 resin (60g) was weighed into a 500 mL flask and 120 mL of MeOH was added. The solution was stirred for one hour using a stir bar and plate. Excess MeOH was decanted and discarded. The resin was washed 6 times with distilled water and stored immersed in water at 4^{o} C.

5.2 Preparation of media (per fungal strain)

Difco Potato Dectrose Broth was weighted into 4 erlenmeyer flasks using 2.4 grams per flask. 100mL of distilled water was added to all flasks. Wetted resin (3% W/V of wet HP-20) was added to two of the four flasks and all were autoclaved with a cotton stopper and foil. Flasks were then allowed to cool before inoculation.

5.3 Inoculation (per fungal strain)

$CuSO_4$ (25 µL) were added to two of the previously prepared PDB media (one with resin and one without). The flasks were labeled appropriately. The spore solution was prepared by adding 10mL of autoclaved water to a centrifuge tube. 1-2 plates of fungi grown on Potato Dextrose Agar (PDA) were added, avoiding addition of the PDA media. The solution was vortexed in the centrifuge tube for 3-5 minutes. 1mL of spore solution was removed and placed in a cuvet to record the absorbance using a spectrophotometer. If necessary, the spore solution was adjusted to ensure all samples had an absorbance in the range of 0.3-0.5 abs. The spore solution (0.6mL) was used to inoculated the 4

different media (PDB, PDB +resin, PDB + $CuSO_4$, and PDB+ resin + $CuSO_4$). Flasks were labeled appropriately, dated, and placed in an incubator at 160 RPM and 28 ^oC.

Note: *Geopyxis* sp. nov. was inoculated with an absorbance of 0.8 abs. This adjustment was made to generate growth with $CuSO_4$.

5.4 Cultivation of samples

Samples were checked regularly for the depletion of glucose using Uriscan glucose strips

5.5 Extraction and isolation of metabolites

5.5.1 Filtrate Extraction

Once the glucose was depleted from the media the samples were prepared for extraction. The media was neutralized and checked with a pH meter. The fungi was filtered using a vacuum and funnel. The mycelium was placed in a centrifuge tube which was labeled, dated, and frozen for extraction. The filtrate was collected and placed in a separatory funnel. EtOAc (50mL) was added and the solvent was shaken vigorously. Separation of sample and solvent was allowed. When emulsification occurred centrifugation was performed to encourage separation. The bottom aqueous layer was decanted and the EtOAc layer was placed in an Erlenmeyer flask. The aqueous layer was placed back into the funnel and 50mL of EtOAc was added again and the solvent layer separated. 75mL of EtOAc was added for the third and last solvent extraction. The aqueous layer was decanted, leaving the EtOAc in the funnel. The collected EtOAc was added to the funnel and 50mL of water was added. The sample was shaken and the bottom aqueous layer was decanted. The sample was washed two more times with water, separated, and placed in a 250mL evaporation bulb. The solvent was evaporated using rotovapor. 3:1

EtOAc/MeOH was used to transfer the sample to a 50mL evaporation bulb and evaporated. The extract was transferred from the bulb to a pre-weighted and labeled vial using 3:1 EtOAc/MeOH. The extract was dried with compressed nitrogen and placed in a vacuum dryer.

5.4.2 Mycelia Extraction

The mycelium was allowed to dry using a freeze dryer. The mycelium was removed from the filter paper and placed in the centrifuge tube. EtOAc (15mL) was added and the centrifuge tube was sonicated for 30 minutes. The solvent was filtered through a pipet into a 150mL centrifuge tube. Centrifugation was performed if necessary to decant the solvent. This was done two more times using 30mL of EtOAc for the last sonication. The sample was evaporated using a rotovapor and transferred to a pre-weighed and labeled vial using 3:1 EtOAc/MeOH. The sample was dried using compressed nitrogen and place in a vacuum dryer. The process of sonication and filtration was performed again three times using MeOH as the solvent.

5.5 Investigation of metabolites

5.5.1 Thin-layer Chromatography Investigation

TLC silica gel (60 F_{254}) was cut into 3 1/2cm x 6 2/3 cm strips. Solvent (3:1 EtOAc/MeOH) was added to the extracts. The purified compound was obtained from extract library and solvent was added. The TLC strips were appropriately labeled and three marks were made. Capillary tubes were used to add pure compound to the left-most mark, both pure compound and extract to the middle mark, and only extract was added to the right-most mark. The strip was placed in a developing jar with solvent, usually 8% MeOH (solvent is specified in the TLC supplementary data). Once the solvent front was about 1cm from the top the strip was removed and the solvent front was marked with a pencil. The strip was placed under a UV light and the active compounds were marked. The strip was then sprayed with

anisaldehyde reagent and dried with a blow dryer to visualized compounds. The strips were then scanned and the Rf values were calculated.

5.5.2 High-Performance Liquid Chromatography Investigation

High-Performance Liquid Chromatography was performed successfully on the Mycelia extract of *Geopyxis* sp. nov with EtOAc. 2mg of the sample was weighted out and 500 microliters of acetone was added. The sample was filtered and placed into the chromatograph. The appropriate solvent ratio was determined , the sample was run, and the data was collected and analyzed.

Acknowledgment
I would like to thank Dr. Leslie Gunatilaka, my project advisor, for giving me the opportunity to pursue this independent research project. I have learned many invaluable lessons and skills throughout the year which will benefit my future lab research. I would also like to thank Dr. Kithsiri Wijeratne and Patricia Espinosa-Artiles for assistance and guidance throughout the project. In addition I would like to thank and acknowledge all other personnel of the Gunatilaka Lab.

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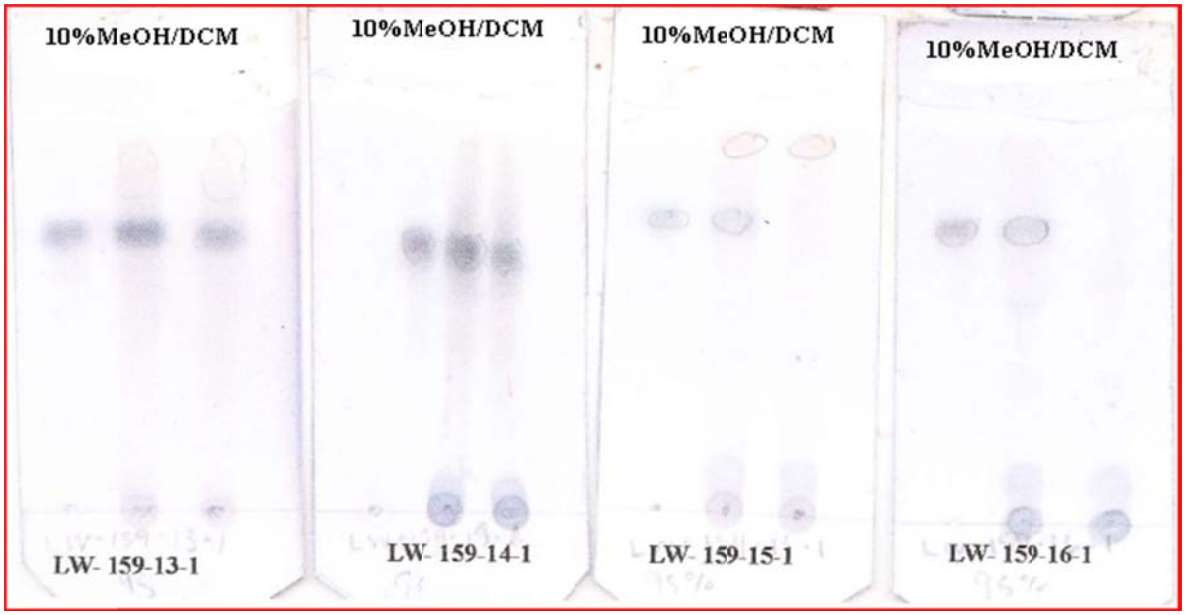
Supplementary Information

Thin-layer Chromatography

Figure S1. TLC for *Verticillium chlamydosporium*

From right to left. PDB, PDB with .0.25 mM $CuSO_4$, PDB with resin, and PDB with resin and 0.25mM $CuSO_4$.

a. Filtrate Extract



b. Mycelia Extract

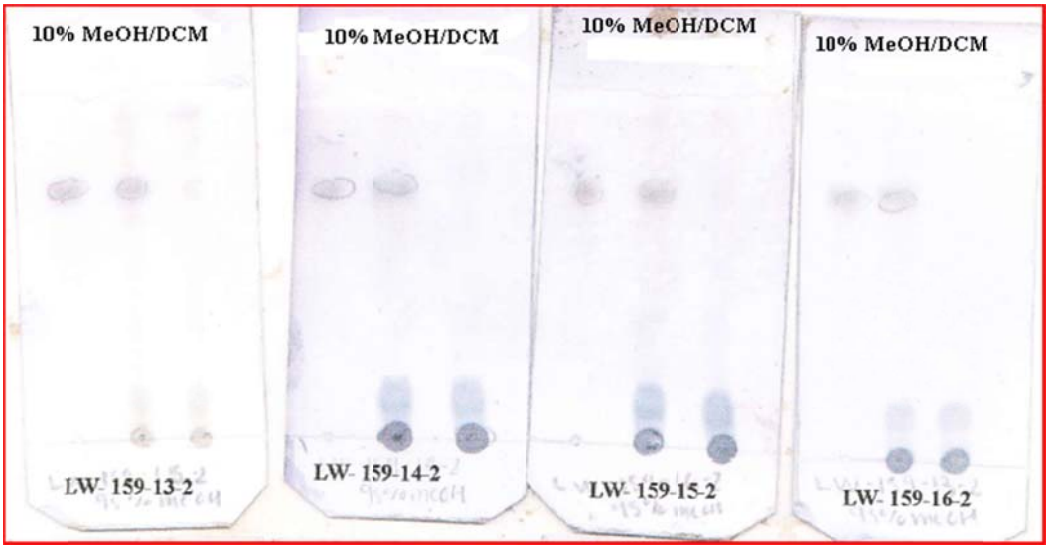
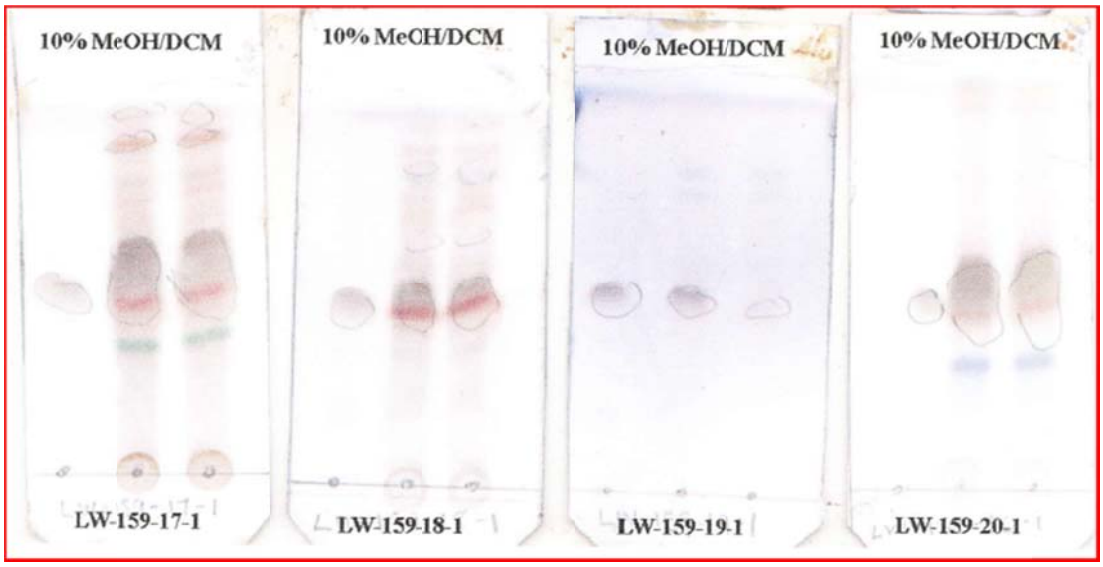


Figure S2. TLC for *Ulocladium sp.*

From right to left. PDB, PDB with .0.25 mM $CuSO_4$, PDB with resin, and PDB with resin and 0.25mM $CuSO_4$.

a. Filtrate Extract



b. Mycelia extract

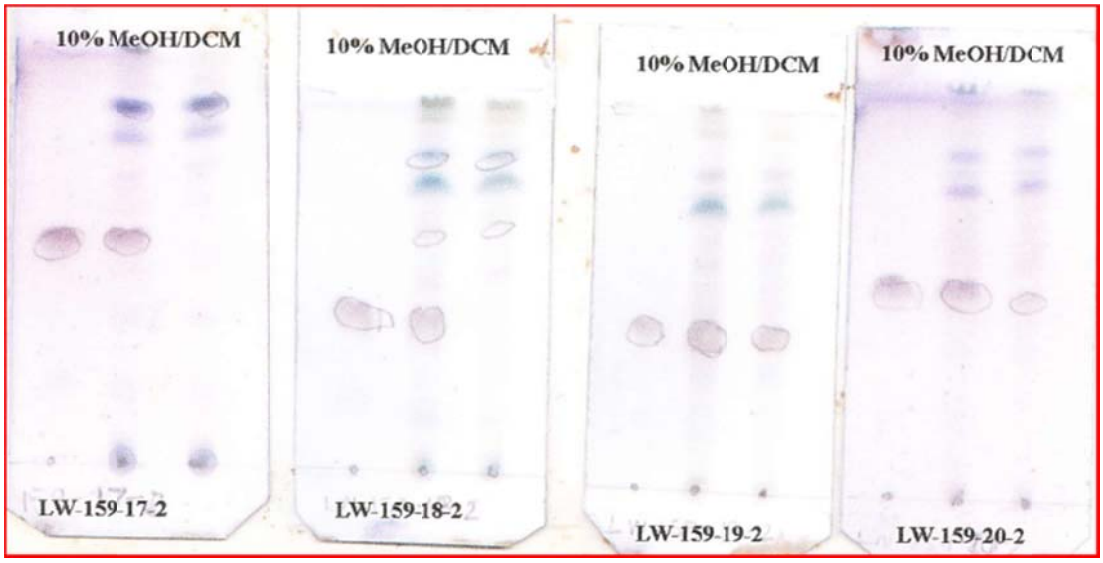
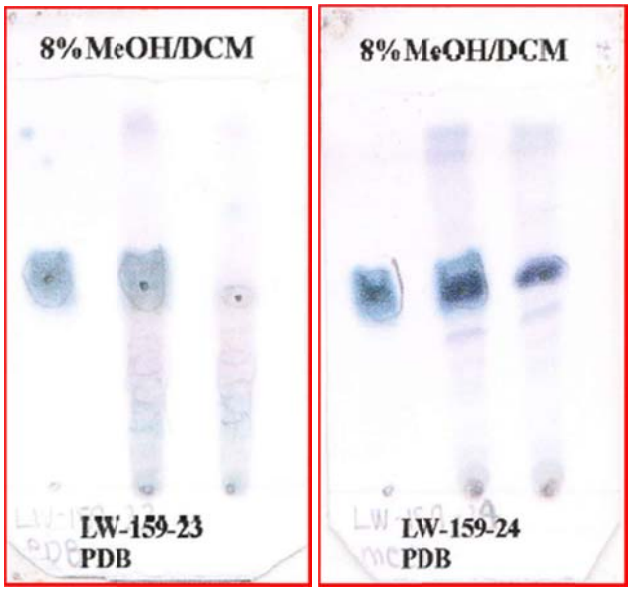


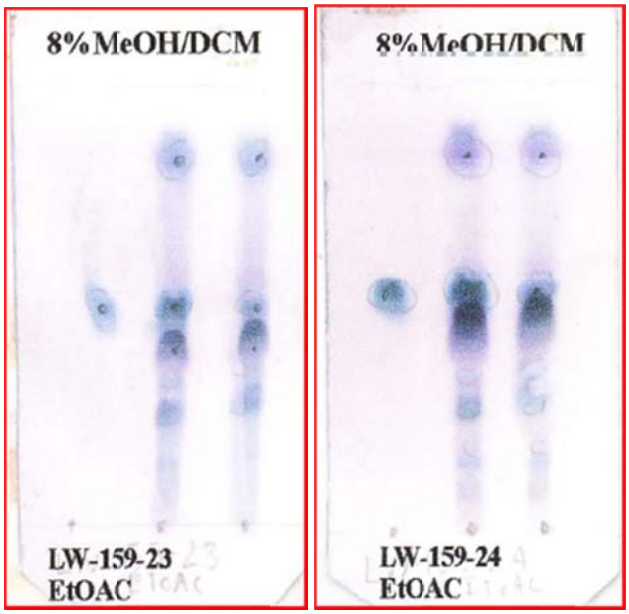
Figure S3. TLC for *Gretetia reticulosperma*

From right to left. PDB, PDB with resin (no growth with $CuSO_4$)

a. Filtrate



b. Mycelia extraction with EtOAc



c. Mycelia extraction with MeOH

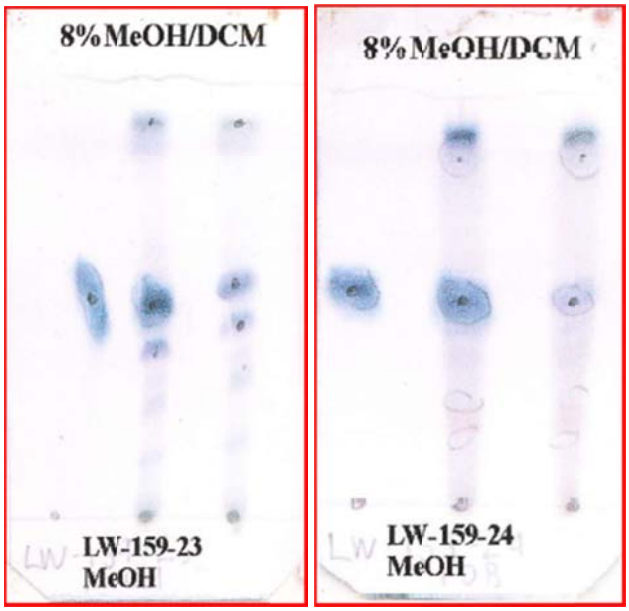
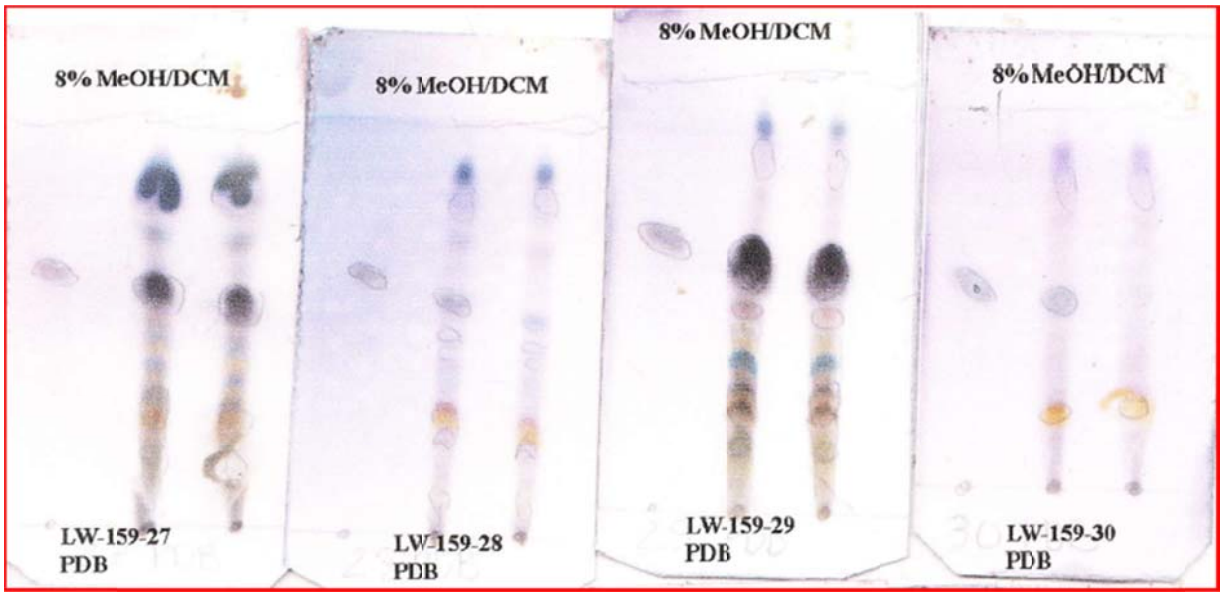


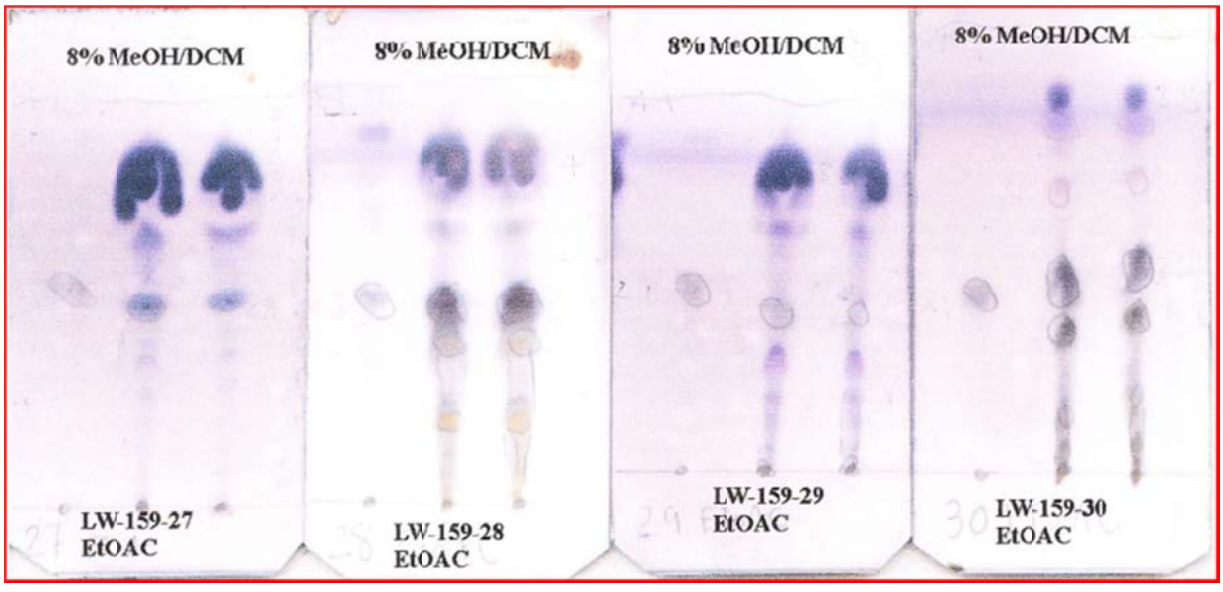
Figure S4. TLC for *Paraphaeosphaeria quadriseptata* (first culture)

From right to left. PDB, PDB with resin , PDB with .0.25 mM $CuSO_{4,}$, and PDB with resin and 0.25mM $CuSO_4$

a. Filtrate Extract



b. Mycelia Extract with EtOAc



c. Mycelia Extract with MeOH

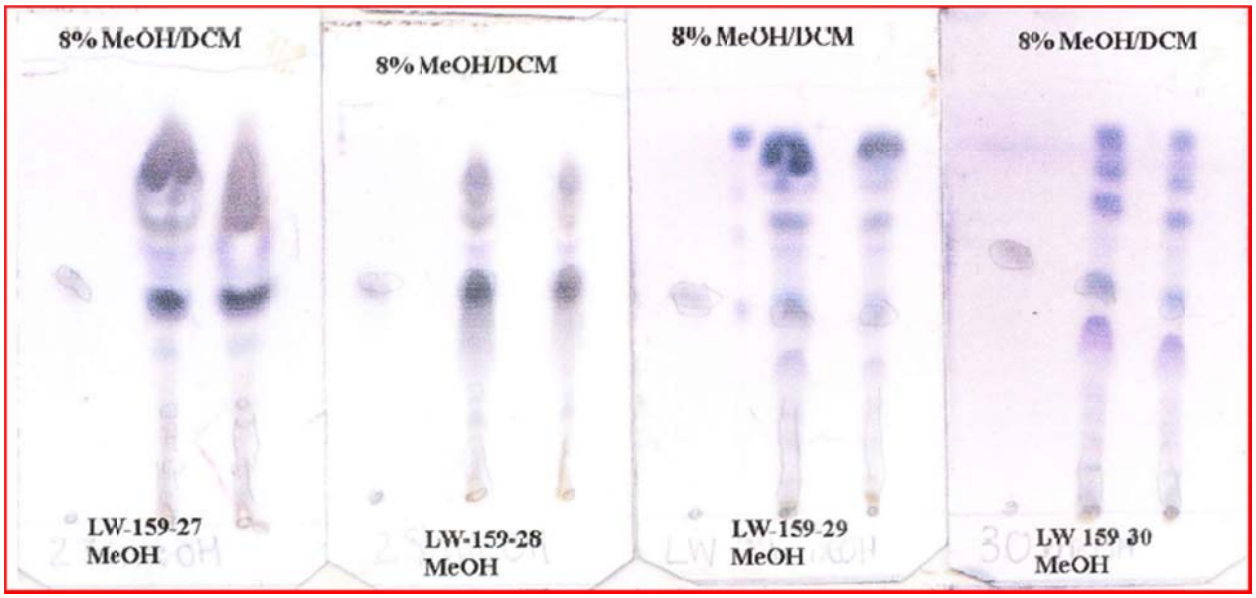
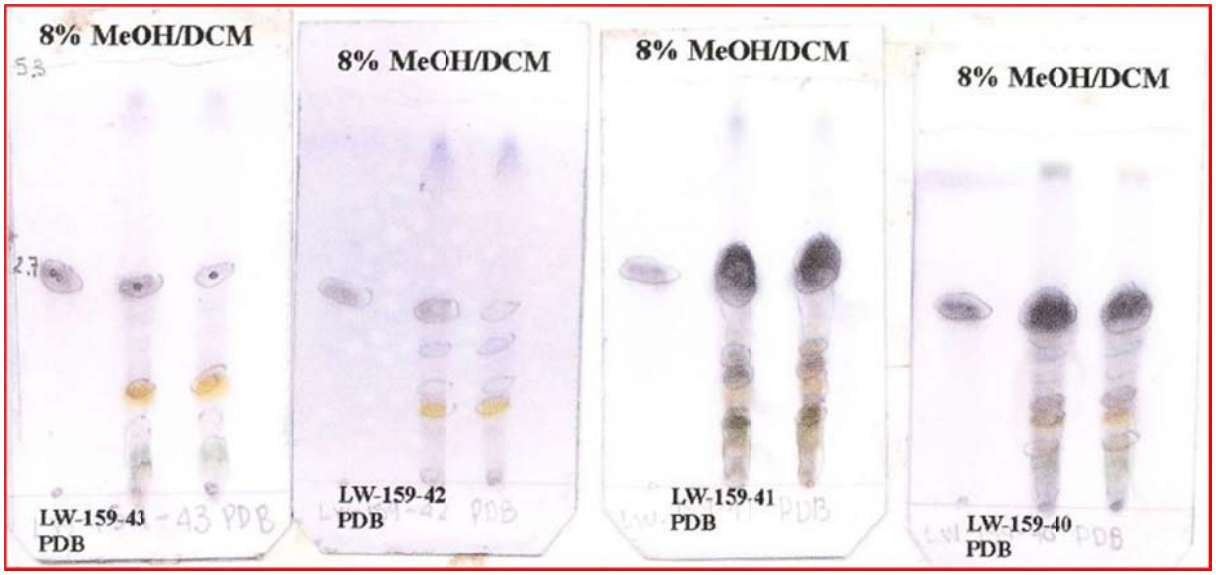


Figure S5. TLC for *Paraphaeosphaeria quadriseptata* (second culture)

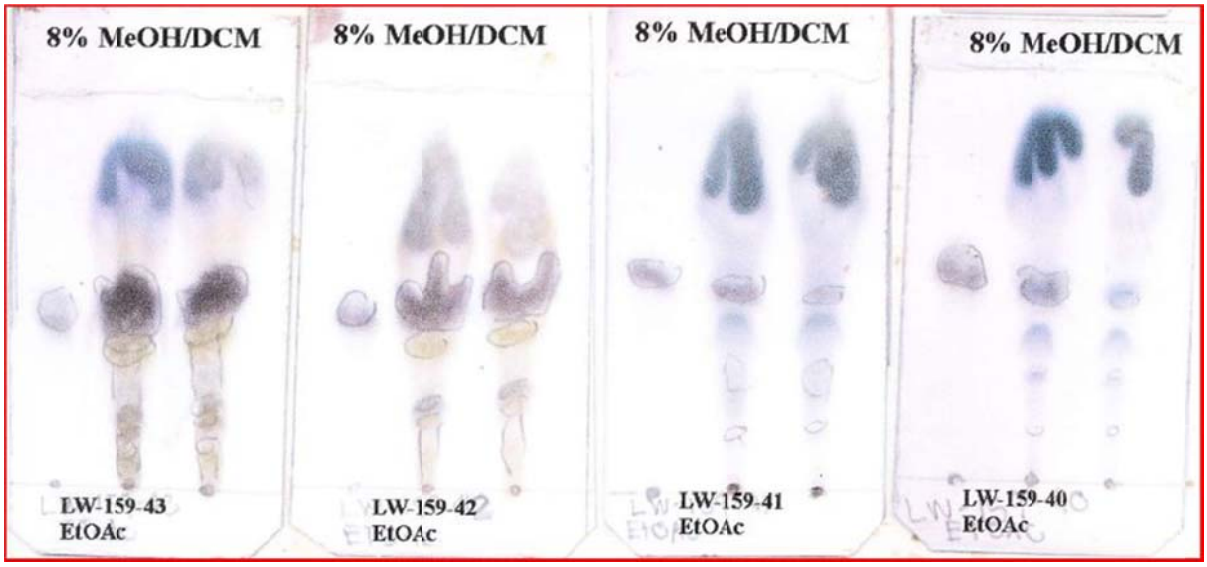
Note: This strain was cultured twice due to low yield. TLC data was obtained for both. Below is the TLC images for the second strain which were not used to calculate the TLC Rf values

From right to left. PDB with resin and 0.25mM $CuSO_4$, PDB with resin, PDB with .0.25 mM $CuSO_4$,, and PDB .

a. Filtrate Extract



b. Mycelia Extract with EtOAc



c. Mycelia Extract with MeOH

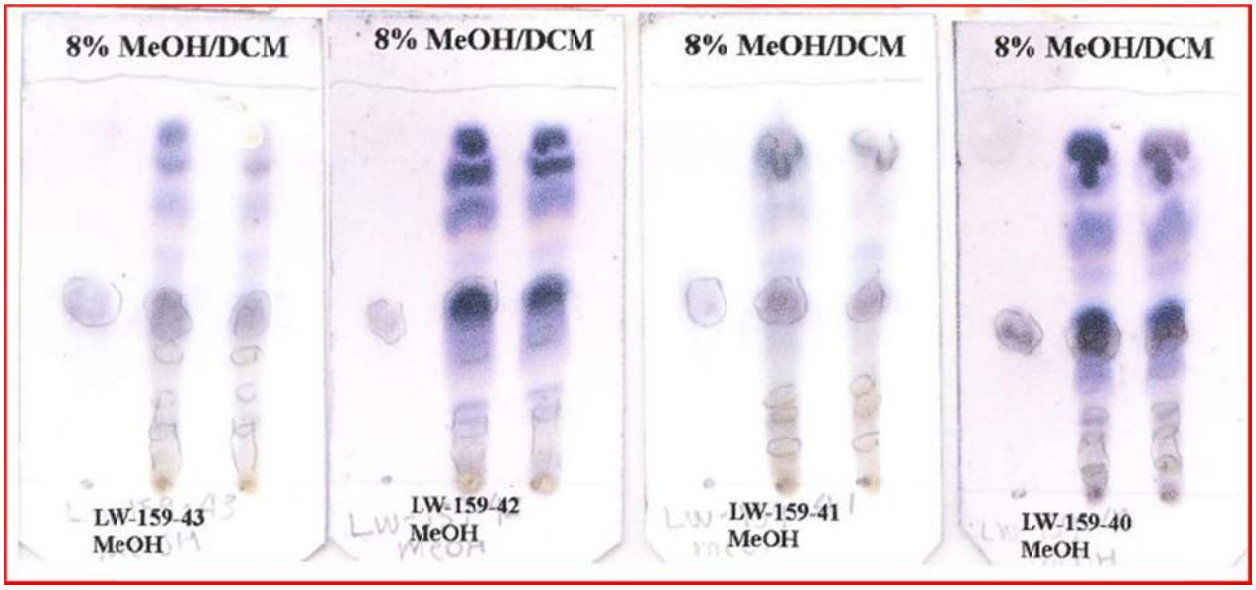
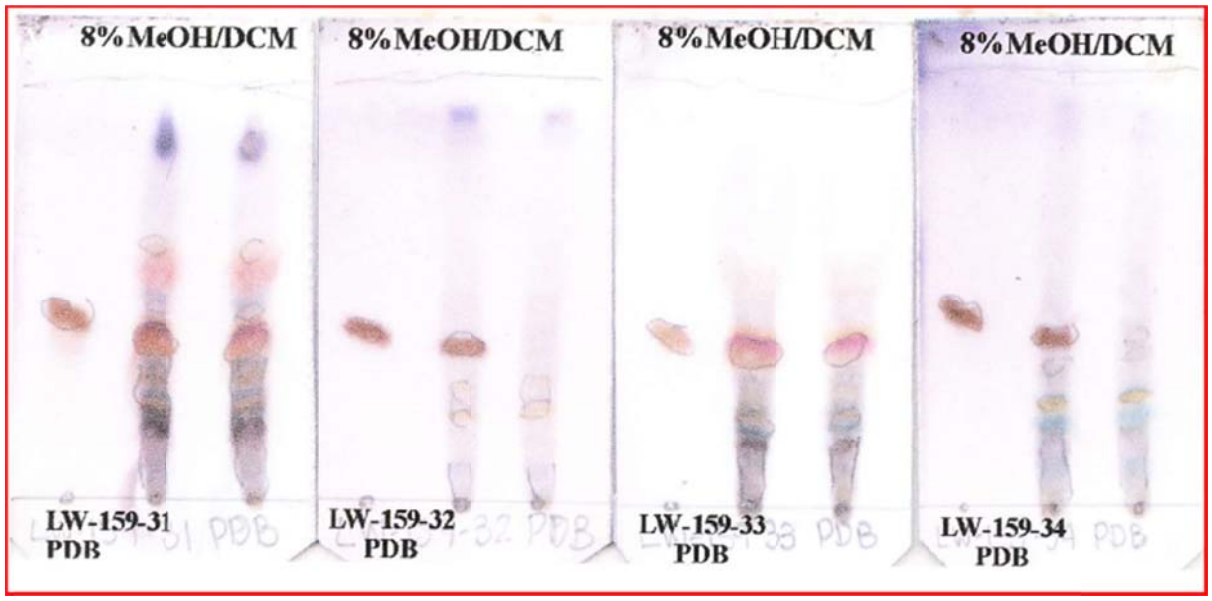


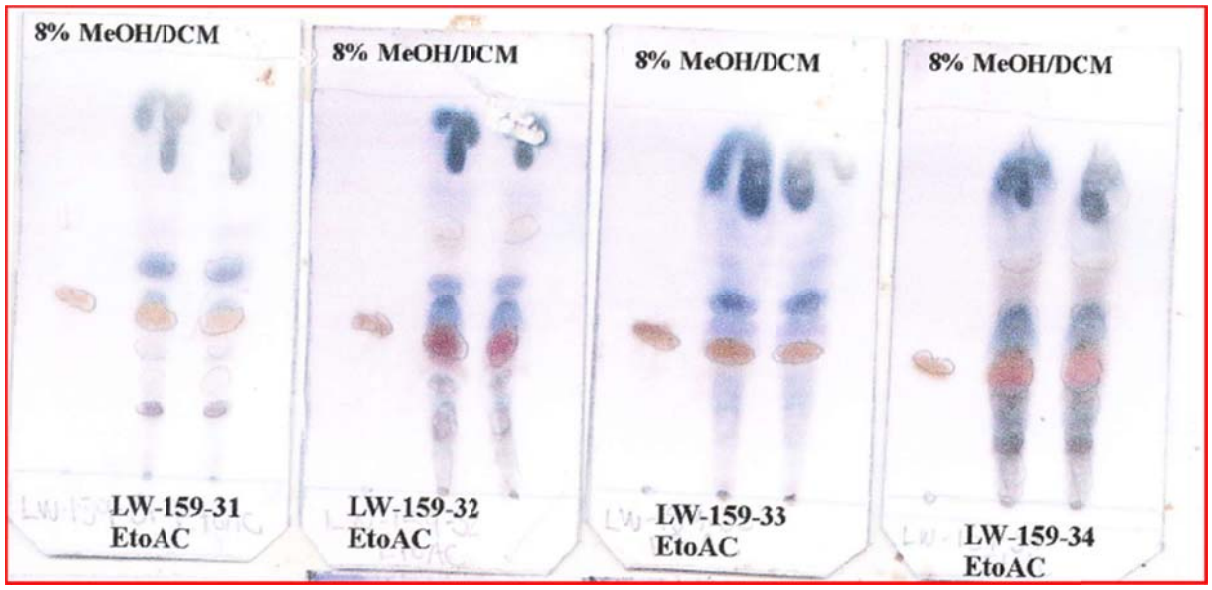
Figure S6. TLC for *Phaeosphaeria/ Phaeosphaeriopsis*

From right to left. PDB, PDB with resin , PDB with .0.25 mM $CuSO_4$,, and PDB with resin and 0.25mM $CuSO_4$

a. Filtrate Extract



b. Mycelia Extract with EtOAc



c. Mycelia Extract with MeOH

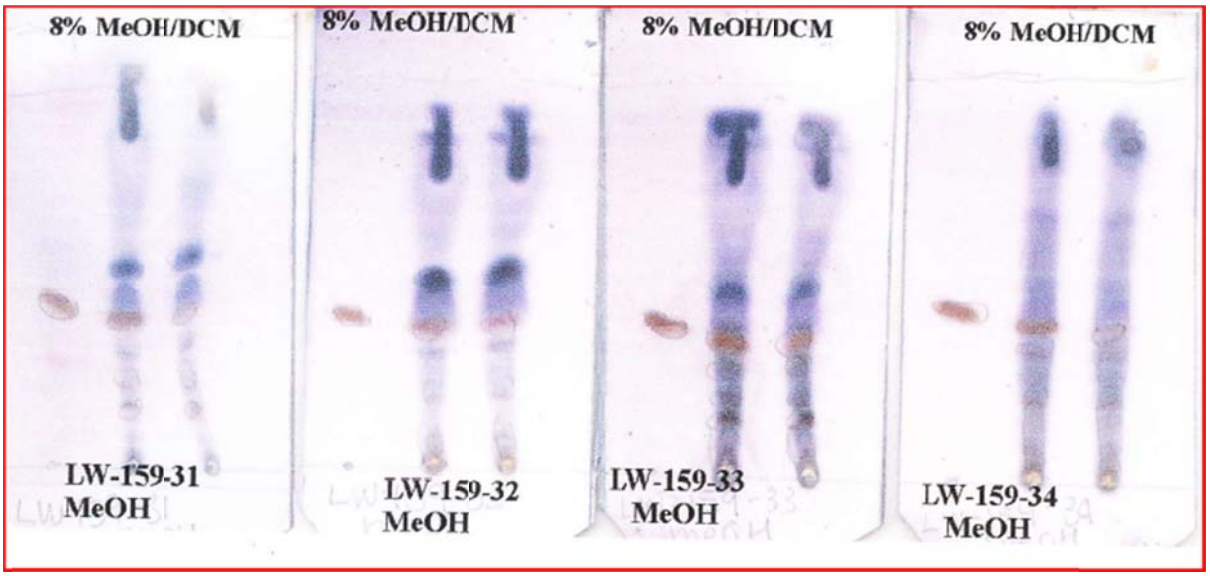
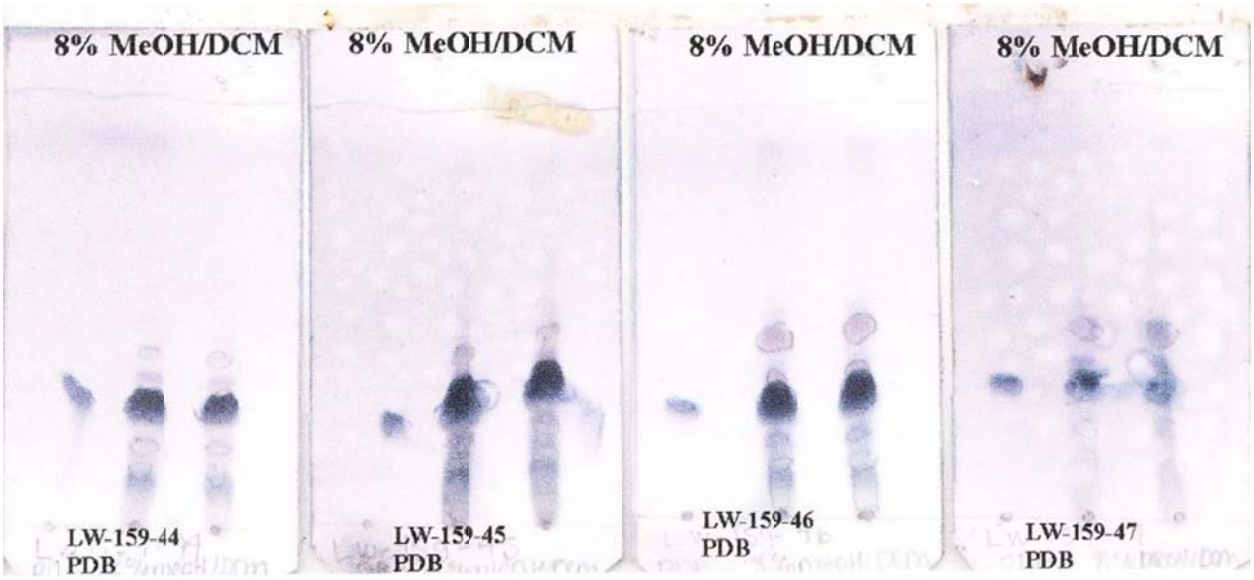


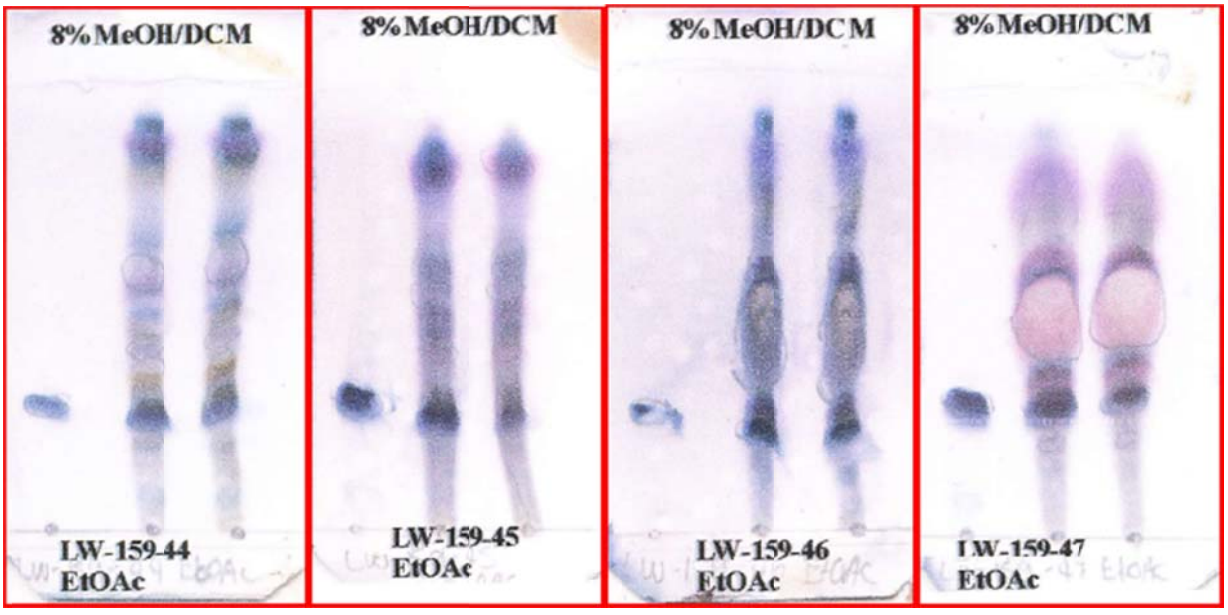
Figure S7. TLC for *Geopyxis* sp. nov.

From right to left. PDB, PDB with .0.25 mM $CuSO_4$, PDB with resin, and PDB with resin and 0.25mM $CuSO_4$.

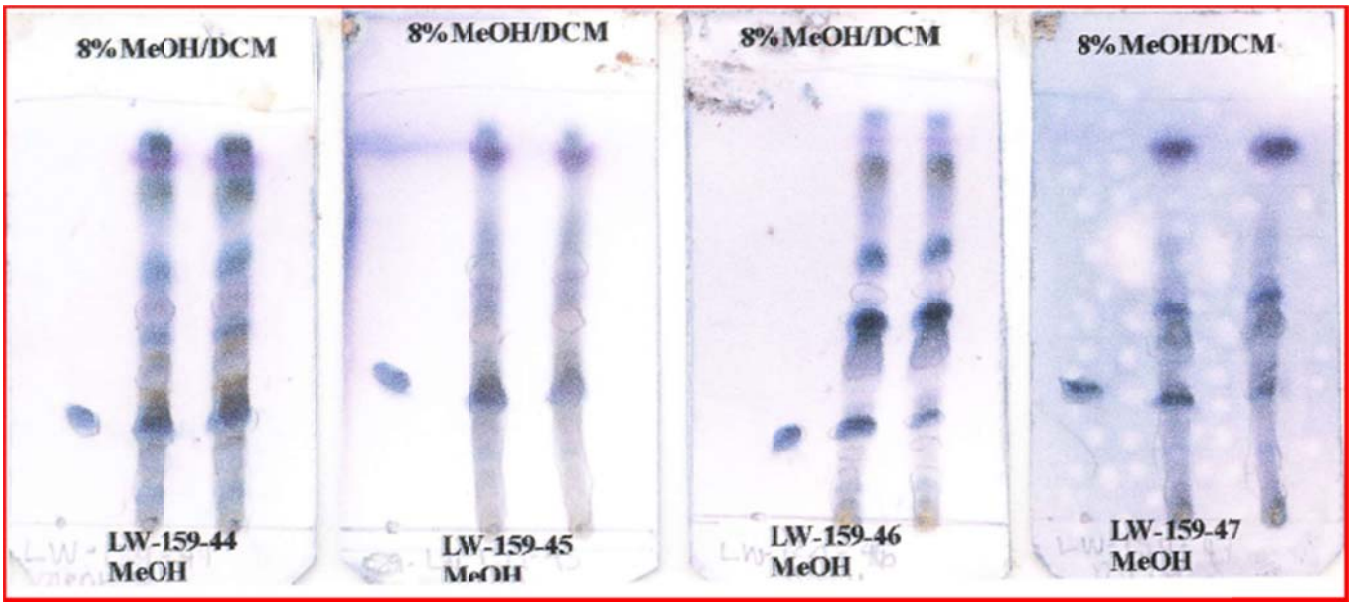
a. Filtrate Extract



b. Mycelia Extract with EtOAc



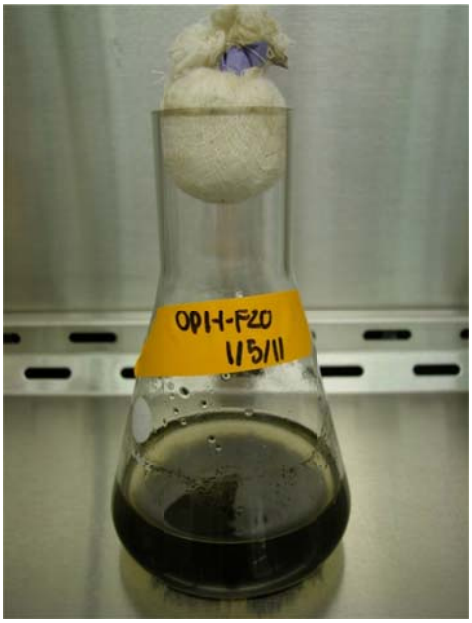
c. Mycelia Extract with MeOH



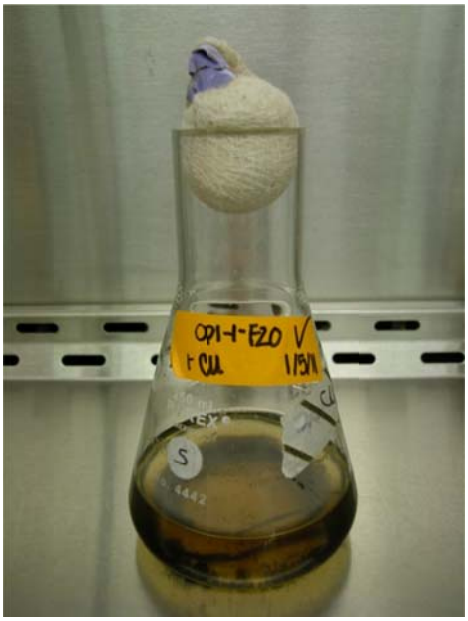
Cultures

Figure S8. *Paraphalasphaeria quadriseptata*

a. PDB



c. PDB + $CuSO_4$



b. PDB + Resin

b. PDB + Resin + $CuSO_4$

Figure S9. *Verticillium chlamydosporium*

a. PDB

c. PDB + $CuSO_4$

b. PDB + Resin

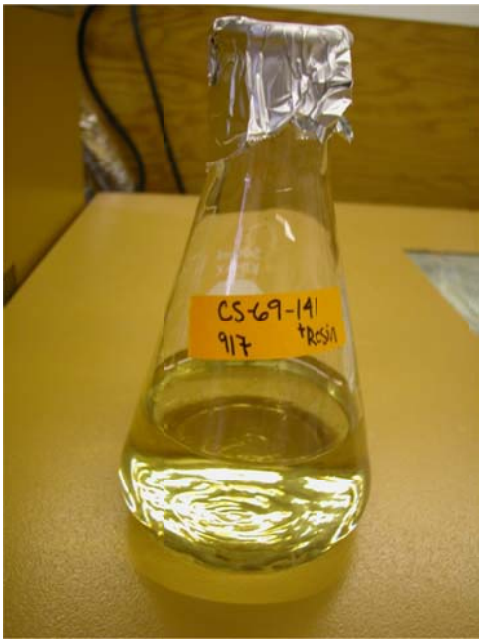
b. PDB + Resin + $CuSO_4$

Figure S10. *Ulocladium sp*

a. PDB

c. PDB + $CuSO_4$

b. PDB + Resin



d. PDB + Resin + $CuSO_4$

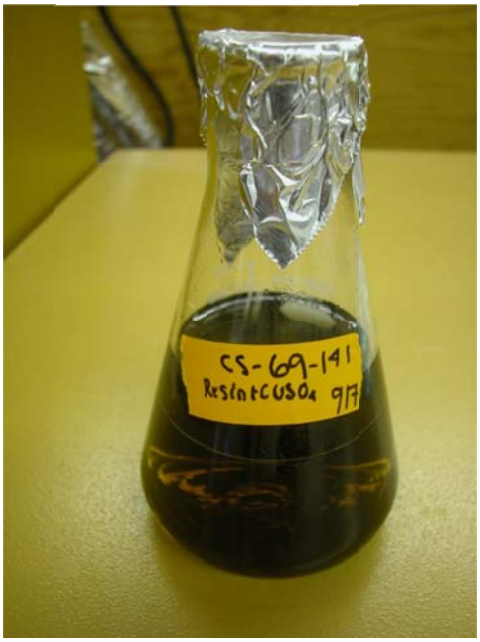


Figure S11. *Gretetia reticulosperma*

A. PDB

B. PDB + Resin

Figure S12. *Phaeosphaeria/ Phaeosphaeriopsis*

a. PDB

c. PDB + $CuSO_4$

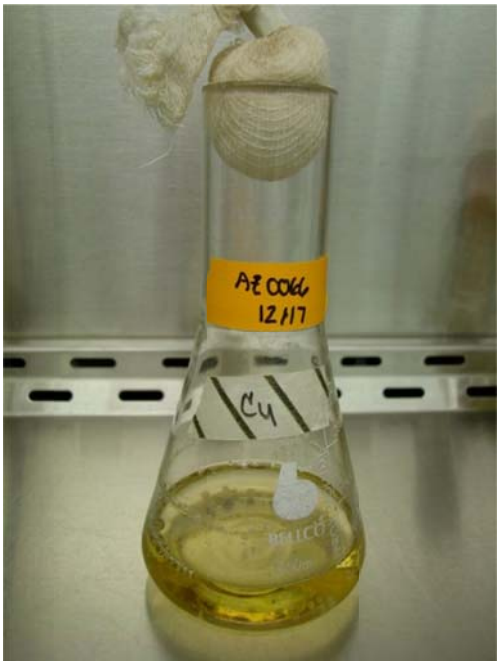
b. PDB + Resin

d. PDB + Resin + $CuSO_4$

Figure S13. *Geopyxis* sp. nov.

a. PDB

c. PDB + $CuSO_4$



b. PDB + Resin

d. PDB + Resin + $CuSO_4$