

The in Vitro Antifungal Activity of some Nigeria Medicinal Plants against *Cryptococcus gattii*

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Abstract: The rise in resistance to the few available antifungals has led to increased search for more antifungals from plant sources. In this study, the leaves of *Swietenia mahogani*, *Senna siamea*, *Cassia alata* and the root and bark of *Smilax kraussiana* were extracted using four different extracting solvents of varying polarity (*hexane, dichloromethane, acetone and methanol*) and susceptibility tested against species of *Cryptococcus*. Dichloromethane extract of *C. alata* was the highest with 264mg from 15g of powdered samples. Antifungal activity of these plants extracts were determined using microtitre dilution assay, acetone extract of *C.alata* had the best antifungal activity against *Cryptococcus gattii* with MIC of 0.016mg/ml and total activity of 1042ml/g. Methanol extract of *C.alata* and root of *S. kraussiana* showed consistency in MIC 0.032mg/ml after 24hours and 48hour incubation with moderate total activity value of 500ml/g and 354ml/g respectively. Hence, were also selected for further study. Bioautography assay was used to determine the number of active compound in the 3 plants extracts (acetone and methanol extract of *C.alata* and dichloromethane extract of the roots of *S.kraussiana*). Five clear zone of inhibition was observed on the acetone extract of *C.alata*, two on methanol extract of *C.alata* and only the spot of crude application of roots of *S.kraussiana* showed clear zone of inhibition. *C.alata* was the most promising plant species with at least five distinct antifungal compounds. Plants extracts with low MIC value could be good source of bioactive components with antimicrobial potency.

Key words: Antifungal activity, total activity, minimum inhibitory concentration (MIC), fingerprinting, bioautography, phytochemical analysis .

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I. INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases. They constitute an effective source of both traditional and modern medicine. The acceptance of traditional medicine as an alternative form of health care has led researchers to further investigate antimicrobial activity of medicinal plants. Most developing countries use traditional medicine to help meet some of their primary health care needs (Sule et al., 2010). The use of plant compounds to treat infections is an age-old practice in large parts of the world, especially in developing countries, where there is dependence on traditional medicine for a variety of diseases since most lack access to modern medicine (Runyoro, Ngassapa, Matee, Joseph, & Moshi, 2006; Shibata et al., 2005).

Interest in plants with antimicrobial properties has revived as a result of current problems associated with the abusive use of antibiotics (Shiota et al., 2004). Herbal medicines have been important sources of products for the developing countries in treating common infections including fungal diseases. Some studies have demonstrated that the plant extract has been used traditionally to treat a number of infectious diseases caused by bacteria and fungi (Sadeghi Nejad, Rajabi, Zarei Mamoudabadi, & Zarrin, 2014). Plants are a valuable source of new bioactive compounds. Despite the availability of different approaches for discovery of medicines,

plants still remain as one of the best reservoirs of new structural types (Runyoro et al., 2006). This art of using natural products, mainly from plants to combat ailment caused by microorganism and to maintain good health has largely passed from generation to generation verbally, hence the importance of medicinal plant usage is not well documented in many cases (Van Wyk, 2015).

Cryptococcosis is a systemic potentially fatal disease that is worldwide in distribution. It is caused by members of the genus *Cryptococcus*, namely *Cryptococcus neoformans*, which also includes the two biovarieties *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* and *Cryptococcus gattii* (Chowdhary et al., 2013). This rare but emerging pathogen has shown to cause infection in both immunocompromised and immunocompetent individual (Byrnes et al., 2010). The current treatment of for cryptococcosis is not satisfactory, owing to the toxicity of existing therapies, their ability to clear infections completely and emergence of drug resistant strains (Smith et al., 2015) like the amphotericin B which shows toxic effects; ketoconazole, fluconazole and clotrimazole are limited in their spectrum and efficacy and the use may result in many strain resistance (Helmerhorst, Reijnders, van 't Hof, Veerman, & Nieuw Amerongen, 1999; Wakabayashi et al., 1998).

Hence it is important for researchers to source out new antimicrobial that can be used to curtail these uprising threat to the health status of the world. However, reports have shown that plants extracts possesses biological activities such as antimicrobial, antifungal, antioxidant activities (Kanatt, Chander, & Sharma, 2008; Soković et al., 2009) which they synthesize as a form of secondary metabolite to defend against microbial infections. These bioactive molecules can be good template for development of new drugs. It is estimated that up to a quarter of all prescriptions in industrialised countries contain one or more components derived from plants (Runyoro et al., 2006)

In this paper, antifungal activities of leaf extract of the following Nigerian plants were evaluated: *Swietenia mahogany*, *Senna siamea*, *Cassia alata* and the root and bark of *Smilax kraussiana*. These plants species were selected based on good preliminary activities of the extracts against human fungal pathogen amongst the population and its tradition method of use.

II. MATERIALS AND METHODS

Collection and preparation of plant material

Fresh leaves of *Swietenia mahogany*, *S. siamea* and *Cassia alata* and the roots and stem of *Smilax kraussiana* were collected from Vom and Barkin ladi axis of Plateau State, Nigeria. The plants were washed with clean tap water and allowed to shade-dry at room temperature (25°C). The dried leaves were crushed into powder. The roots were shredded into small pieces and allowed to shade-dry then pounded using mortar. The bark of the stem were scrapped and allowed to shade-dry before pounding in a mortar.

Extraction

Fifteen grams (15g) of powdered leaves of *S. mahogany*, *S. siamea*, *C. alata* and grounded root and bark of *S. kraussiana* were extracted with 150ml of four different solvents of increasing polarity (hexane, dichloromethane, acetone and methanol) in centrifuge tubes. The tubes were shaken thoroughly for some minutes and centrifuged at 2000rpm for 5 minutes and the supernatant was decanted into a pre-weighed glass beaker and the solvent allowed to evaporate. After evaporation, the beaker was weighed to determine the quantity extracted.

Phytochemical analysis.

Phytochemical screening test was carried out on all the plant samples according to the procedure of the Association of Official Analytical Chemist ("Association of Official Analytical Chemists (AOAC)," 2005) and (Harbone, 1998) for phytochemical analysis. Phytochemical was determined for A.) Alkaloids. B.) Saponins. C.) Phenol. D) Flavonoids E.) Terpenoid F.) Cardic Glycosides and, G.) Phlobatannins

Thin-Layer Chromatography Fingerprinting

Plant extract were re-suspended in acetone to give a concentration of 10mg/ml. Aliquot of 10µl of plant extract were loaded on each of three aluminium-backed thin-layer chromatography plate and developed in hexane: ethyl acetate (3:1) mobile system. After some minutes, the solvent was noticed that it was not moving, the plate was removed and the solvent front was marked and each spot on the TLC plate was circled using a pencil and the plate was allowed an hour for the eluting solvent to evaporate completely. The plate was then sprayed with vanillin spraying reagent and heated at 110°C for optimal colour development.

Fungal culture/Inoculum preparation

Fungal cultures of *Cryptococcus gattii* were obtained from the Microbiology laboratory (Department Of Microbiology, Faculty of Natural and Applied Science, Plateau State University, Bokkos). The cultures were

maintained in water and sub cultured onto SDA and incubated at 37°C to obtain colonies. The colonies were subcultured on a freshly prepared SDA and incubated at 37° C for 24 hours to obtain pure culture. Five colonies of approximately similar diameter from the pure culture plate after incubation were suspended in 5ml of fresh sterile water to form inoculum. Fungal inoculum was adjusted to approximately 2×10^6 cells/ml (0.5 McFarland standard) by adding sterile water to equal the absorbance using a spectrophotometer at 530nm wavelength. The inoculum was diluted 1:50 and followed by 1:20 which was used during microdilution and bioautography .

Microdilution assay

Hundred microliter (100µl) of SDB was dispensed into wells 1 to 11 but 200µl was dispensed into well 12 as media control. Residue of plant extracts were re-suspended into acetone to a concentration of 10mg/ml. Hundred microliter(100µl) of the plant extracts that were re-suspended into acetone were dispensed into well 1 of the microtitre plate and serially diluted to well 9 by taking out 100µl from one well to the next to reduce the concentration of the extract by half(this was done in duplicate set for all extracts). 100µl of acetone was added to well 11 as solvent control, and 100µl of 160µg/ml of fluconazole into well 10 as standard antifungal control. 100µl of 1:20 prepared *C.gattii* suspension (2×10^6 cell/ml) was added to each well. As an indicator of growth 40µl of 0.2mg/ml of TTC dissolved in water was added to each well and the covered microtitre plate was incubated at 37°C overnight to ensure adequate colour development. The MIC was recorded as the lowest concentration of the extract that inhibited fungal growth. The colourless tetrazolium salt acts as electron acceptor and is reduced to formazan product by biologically active organism. The MIC values were read after 24 hours and 48 hours. Where fungal growth is completely inhibited (lethal concentration), the solution in the well remains clear after incubation with TTC ,but inhibition of growth is measured as the first concentration of plant extract that causes a decrease in colour intensity indicated by the formazan salt (inhibitory concentration). The experiment was repeated twice to confirm results.

Bioautography assay of the extracts

TLC plates prepared developed in Hexane: ethyl acetate (3:1) were left for 24 hours to allow the eluting solvent to evaporate from the plates before being sprayed with a day-old actively growing suspension of *Cryptococcus gattii* in SDB. The TLC plates were then incubated for 24 hours at 37°C under 100% relative humidity to allow the organism to grow on the plate. After overnight incubation the bioautograms were sprayed with aqueous solution of 2mg/ml TTC and incubated overnight for conservation of clear zone. Clear zones indicated where reduction of TTC to coloured formazan did not take place due to presence of compounds that inhibited the growth of the test fungi.

Total activity

Total activity indicates the degree to which the active compounds in 1g of plant material can be diluted and still inhibit the growth of the tested fungal microorganism .this takes into account the quantity extracted from plant material and its calculated as follows:

Total activity=quantity extracted (mg/g)/MIC (mg/ml),the units is ml/g.The higher the total activity of a plant extract, the more effective the original plant is(J. Eloff, 2000).If the total activity is calculated at each step of the bioassay-guided fractionation procedure it is easy to determine if there is loss of biological activity during isolation ,and also synergistic effects can be discovered very easily. This situation is equivalent to the terms efficacy and potency used in pharmacology (J. N. Eloff, 2004)

III. RESULTS AND DISCUSSIONS

Plants contains bioactive compounds with antifungal activity

The leaves of *S.mahogani*, *S.siamea* , *C.alata* and the root and bark of *Smilax kraussiana* plants were extracted using four different solvents with different polarity : Hexane, dichloromethane, acetone and Methanol.

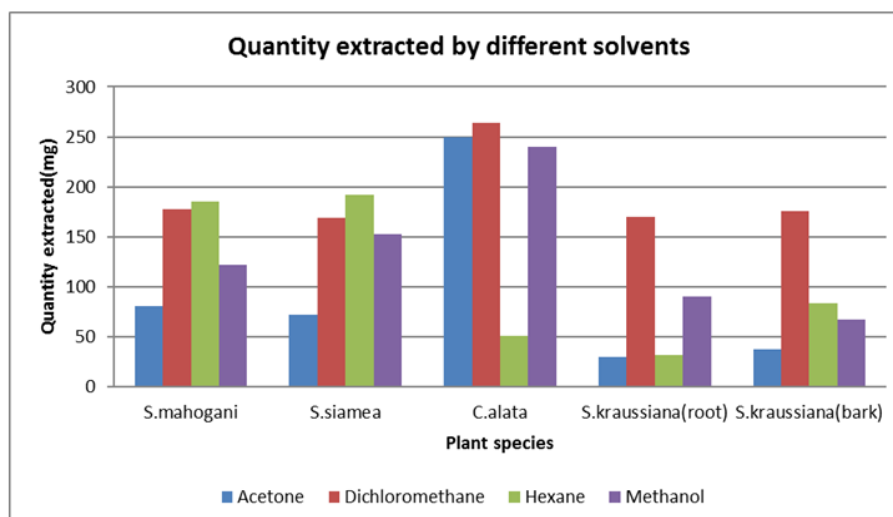


Figure 1. Quantity extracted from plant samples

For *S.mahogani*, acetone extracted the least quantity 81mg (0.54%) while hexane gave the highest 185mg (1.23%). Dichloromethane and methanol gave intermediate quantity but DCM was quite higher 178mg(1.19%) than methanol 122mg(0.81%). Similarly, *S.siamea*, acetone extract also gave the least extract of 72mg(0.48%) and hexane extracted 192mg(1.28%) which is the highest. DCM and methanol gave intermediate quantity but DCM is quite higher 169mg(1.13%) than methanol 153mg(1.02%). The results for *S.mahogani* and *S.siamea* showed consistency making hexane as the best extracting solvent for the two plants.

On the other hand, *C.alata* showed no consistency with that of *S.mahogani* and *S.siamea* as DCM extract of *C.alata* gave the highest quantity of 264mg (1.76%). Acetone and methanol gave almost the same quantity 250mg(1.67%) and 240mg(1.60%) respectively. And in this case, hexane gave the least quantity 51mg(0.34%).

For *S.kraussiana*, the root and bark were used instead of the leaves according to the traditional medical use. The roots of *S.kraussiana* gave the highest quantity of 170mg (1.13%) which showed consistency with that of *C.alata* but methanol gave a higher extract than acetone which is a reverse in the case of *C.alata* where acetone gave a higher yield. But hexane gave the least extracted quantity which makes it consistent to that of *C.alata*. The bark of *S.kraussiana* also showed consistency with that of the roots of *S.kraussiana* and leaves of *C.alata*; DCM having the highest extracted quantity of 176mg (1.17%) followed by hexane 83mg (0.55%) then methanol 67mg (0.45%) then acetone 37mg (0.25%) which is consistent with that of *S.mahogani* and the root of *S.kraussiana*. In general, DCM extract of *C.alata* gave the highest extraction of 264mg (1.76%) while acetone extract of *S.kraussiana* (root) gave the least extraction of 30mg (0.20%).

In summary, acetone gave lowest extraction in four different plant samples (*S.mahogani*, *S.siamea*, *S.kraussiana* root and bark). While DCM gave the highest extraction in 3 samples (*C.alata*, *S.kraussiana* (root), *S.kraussiana* (bark)). Interestingly, methanol extract and acetone extract of *C.alata* showed almost the same quantity which may be attributed to the degree of the polarity index of the two solvents which corresponds to the work of (Markom, Hasan, Daud, Singh, & Jahim, 2007) where the effect of polarity on extract were compared and water being more polar extracted more followed by ethanol and then other non-polar solvents. (Akowuah, Ismail, Norhayati, & Sadikun, 2005) also explained the relationship between quantity of extract based on polarity index of solvent; polar compounds are easier to be extracted compared to non-polar compounds. The difference in quantity extracted may be attributed to other factors including phytochemicals in plants, extraction temperature, extraction time and solvent to solid ratio.

All plants showed positive for the 3 test conducted for alkaloids, because almost all species of terrestrial plants have alkaloid but in varying concentrations. Ferric chloride test for phenols, terpenoid, cardiac glycosides and phtbatannins showed negative for all plants samples. The flavonoid test showed positive for all plants sample except *S.kraussiana* (root and bark). Interestingly, *C.alata* was the only plant species that showed positive for saponin test and according to (Yang et al., 2006), steroidal saponins have therapeutic potential for treating hepatitis and fungal infection. This suggest that some class of saponins do have a low toxicity and may become therapeutic agents with high therapeutic index (De Lucca, Boue, Palmgren, Maskos, & Cleveland, 2006).

Table1: Phytochemical analysis result of plants used in this study

Plant species	<i>S.mahogani</i>	<i>S.siamea</i>	<i>C.alata</i>	<i>S.kraussiana</i> (root)	<i>S.kraussiana</i> (bark)
ALKALOIDS					
a. Dragendroff' s test	-	+	--	--	--
b. Wagner' s test	+	+	++	++	++
c. Mayer' s test	+	+	++	++	++
SAPONINS					
	--	--	+	-	-
PHENOLS					
a. Ferric chloride test	--	--	--	--	--
FLAVONOIDS					
a. Lead acetate test	+	++	+	--	--
TERPENOIDS					
a. Salkowsk' s Test	--	--	--	--	--
b. Harbone' s Test	--	--	--	--	--
CARDIAL GLYCOSIDE					
Keller Kilhiamic Test	--	--	--	--	--
PHTOBATANNINS					
	--	--	--	--	--

Key: +=Presence; -=Absence

Plant extracts possesses antifungal effect against *Cryptococcus gattii*

C.alata , *S.kraussiana* (root and bark) had a very low MIC of 0.016mg /ml but after 24 hour incubation *S.kraussiana* (root)and *S.kraussiana* (bark) showed a drastic change in MIC value from 0.016mg/ml to 0.064mg/ml and 0.016mg/ml to 0.128mg/ml respectively after 48 hours incubation but *C.alata* gave just a slight change change from 0.016mg/ml to 0.032mg/ml after 48hours incubation which makes it a better option over the *S.kraussiana*. Table 2 shows the MIC values of the plants extracts.

Table 2. Minimum inhibitory concentration (MIC) of six plant species against *Cryptococcus gattii* using different extracting solvent;acetone(A),dichloromethane(D),hexane(H),methanol(M).

Plant Species	Time(H)	MIC(mg/ml)			
		A	D	H	M
<i>S.mahogany</i>	24	0.064	0.064	0.032	0.032
	48	0.064	0.064	0.064	0.064
<i>S.siamea</i>	24	0.032	0.064	0.128	0.032
	48	0.064	0.128	0.128	0.064
<i>C.alata</i>	24	0.016	0.064	0.064	0.032
	48	0.032	0.064	0.064	0.032
<i>S.kraussiana</i> (Root)	24	0.032	0.032	0.016	0.032
	48	0.064	0.032	0.064	0.064
<i>S.kraussiana</i> (Bark)	24	0.032	0.016	0.128	0.032
	48	0.128	0.128	-	0.128

Total activity is also an important parameter because it gives the concentration of the extract that can be in a specific volume of diluent which can still be potent against the test organism. MIC is a function of total activity; (Total activity =quantity extracted(mg)/MIC(mg/ml)) .Acetone extract of *C.alata* showed the highest total activity of 1042ml/g implying that 1g of acetone extract diluted in 1042ml will still inhibit the growth of fungi. DCM extract of *S.kraussiana* also showed high total activity of 733ml/g but because of the drastic drop in MIC after 48 hours incubation, dropping its total activity to from 733ml/g to 92ml/g. This makes *C.alata* a better option over *S.kraussiana* (bark) which shows just slight decrease in total activity after 48 hours incubation(1042ml/g to 521ml/g) .

Table 3. Total activity in ml/g of four plant species extracted with acetone(A), dichloromethane(D), hexane(H), methanol(M)

Plant Species	Time(H)	Total Activity(ml/g)			
		A	D	H	M
<i>S.mahogany</i>	24	84	185	385	245
	48	84	185	193	127
<i>S.siamea</i>	24	151	176	102	319
	48	75	88	100	159
<i>C.alata</i>	24	1042	275	53	500
	48	521	275	53	500
<i>S.kraussiana</i> (Root)	24	63	354	133	188
	48	31	354	33	94
<i>S.kraussiana</i> (Bark)	24	77	733	43	140
	48	19	92	-	35

Another interesting observation is the consistency of the methanol extract of *C. alata* with MIC of 0.032mg/ml and total activity of 500ml/g after 24 hours and 48 hours incubation which is similar to that of DCM extract of *S.kraussiana* (root) with MIC of 0.032mg/ml after 24 hours and 48hours but had a lower total activity of 354ml/g compared to that of methanol extract of *C.alata*. On these basis, selection of acetone and methanol extract of *C.alata* and DCM extract of the root of *S.kraussiana* were for further analysis; fingerprinting and bioautography.

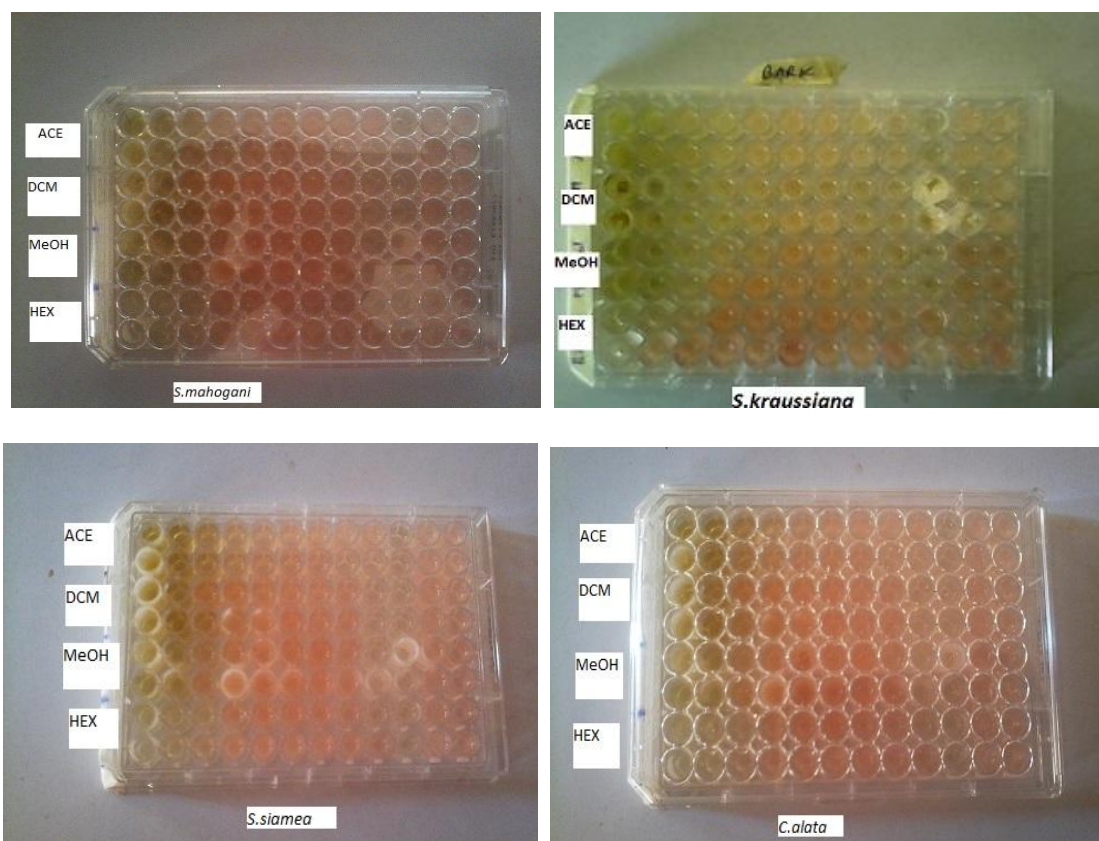


Figure 2. Pictures of some microtitre plate of plants extracts on *Cryptococcus gattii* after incubation. Pink color shows formazin production, this signifies fungal activity

Plant extracts shows bioactive rf on Thin-layer chromatography plate

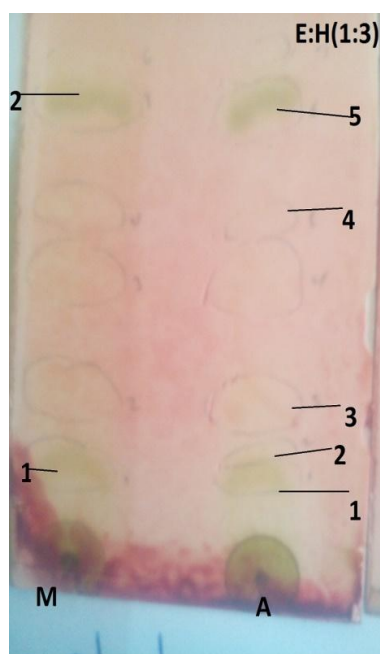
Since acetone and methanol extract of *C.alata* and DCM extract of *S.kraussiana* (root) showed exceptional bioactivity compared to that of other plants samples. They were used for further analysis: finger printing and bioautography. After eluting the plate with Ethyl acetate: Hexane (1:3) because this solvent system gave higher number of components on the plate of *C. alata* extract than other systems that were tried. The acetone extract of *C.alata* which had the highest total activity, the methanol extract of *C.alata* and DCM extract of *S.kraussiana* which showed consistency in MIC value after 24 and 48 hours incubation with relative

moderate total activity were used for finger printing. Both extract of *C.alata* showed four visible spots on the vanillin sprayed plate as shown on Figure 2 below.



Figure 2: Finger printing of methanol and acetone extract developed by vanillin spraying reagent.

The bioautography of methanol and acetone extract of against *C.gattii* are shown in figure 3. After incubating the plates for 24hour and sprayed with TTC and incubated for another 24 hours at 100% relative humidity, the methanol extract showed only two clear zones which inhibited the growth of *C.gattii*. These zones have the following R_f values 0.16 and 0.64 but the acetone extract showed five clear zones with R_f values of 0.16, 0.20, 0.27, 0.34, 0.64. It was observed that components with R_f values of 0.16 and 0.64 from both extract inhibited the growth of *C.gattii*.



C.alata

Figure 3. Bioautogram of methanol (M) and acetone (A) extract of *C.alata*. (the numbers represents the R_f of the components)

The root of *S.kraussiana* was not left out, bioautography was carried out on it because of the consistency in MIC of the DCM extract of *S.kraussiana*. although the plate showed no separation of components in the E:H solvent system but the crude spot (where extract was added on the plate) showed a clear zone after bioautography(Figure 3). It could be possible that the bioactive component compound only had a very low R_f value which agrees with the work of (Mahlo, Chauke, McGaw, & Eloff, 2016) where DCM extract of *S.kraussiana* had a low R_f value of 0.04.



Figure 4: TLC bioautogram of DCM extract of *S.kraussiana* root indicating the clear zone(zone of inhibition)

In summary, Acetone and methanol extract of *C.alata* and DCM extract of roots of *S.kraussiana* indicated the presence of antifungal constituents using bioautography, and relatively low MIC values in the microdilution assay against *C.gattii*.

IV. CONCLUSION

The evaluation of antifungal compounds from plant extract appears to be an important research activity (Hostettmann, Marston, Ndjoko, & Wolfender, 2000) because plants are considered as important source of bioactive molecule (Sharma,2002) synthesized as secondary metabolites. The presence of these compounds provides an invaluable resource that has been used to find new drugs molecules (Gurip-Fakim,2006).

This research shows that different solvents are needed for extraction of different types of plant constituents owing to their difference in polarities. DCM extract of *C.alata* extracted the highest quantity followed by acetone and methanol which had almost the same quantity of extract; which could be attributed to the degree of the polarity index of the two solvents. On the other hand, hexane yielded high extract in *S.mahogany* and *S.siamea* and in these plants acetone were relatively low, hence it can be concluded that polarity of extracting solvents have a contributing effect on the quantity extracted; as polar solvents extracts more polar constituents and non-polar solvents extract more non-polar constituents (Pin et al., 2009).

The antifungal activity of the plant species achieved during microdilution and well-defined fungal inhibition on bioautograms indicated that acetone extract of *C.alata* is a good extract with effective bioactivity against *C.gattii* because of its low MIC and high total activity, making *C.alata* a good source of antifungal agent.

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