Original Article

Antifungal Activity of *Senna alata, Senna bicapsularis and Pityrogramma calomelanos*

ABSTRACT

Aims: *Senna alata* (Carrion crow bush), *Senna bicapsularis* (Money bush) and *Pityrogramma calomelanos* (Wild maran) are known for their value in traditional medicine. The study analyzed the antifungal properties of these plants against the plant fungal pathogen *Curvularia lunata.*

Study design: Experiment based study.

Place and Duration of Study: Plants were collected along the coastal areas of Guyana and identified at the Biodiversity Center, University of Guyana, Georgetown, Guyana between January 2017- May 2017.

Methodology: Phytochemical extraction was conducted using soxhlet and rotovap apparatus. Hexane, methanol and aqueous extracts of plants were analyzed for antifungal property. Antifungal activity was tested using the poisoned food and well diffusion technique. Data analysis of the study was done using R-Studio Program for statistical computing and graphics. A Tukey test was done along with ANOVA and Boxplots.

Results: Methanol extract of *P. calomelanos* showed maximum antifungal activity with an inhibition percentage of 60.3% at 400 µl with poison food technique. With well diffusion method, *P. calomelanos* showed an inhibition zone of 54 mm at 500 µl. Saponins, steroids and glycosides were identified with methanol extract of *P. calomelanos.*

Conclusion: In conclusion, *P. calomelanos* was most effective with higher zone of inhibition with poisoned food technique.

Keywords: antifungal, phytochemical, poison food technique, well diffusion technique, Curvularia lunata

1. INTRODUCTION

Fungal damages to crops are enormous and affects agricultural productivity**.** One such fungus is *Curvularia lunata.* It is mainly an opportunistic leaf spot fungus and affects grain and appear as floccose (wooly tufts), brown, dark brown to black, often zonate [1]. It causes leaf spots on grasses as well as storage molds of grains. *Curvularia* species are recorded to be the cause of plant disease disasters in various parts of the world. These include *Curvularia* blight of turf, fading out of turf, *Curvularia* leaf spot of Oyster plant and Hibiscus, grain mold of sorghum, Kernel smudge of wheat, and *Curvularia* leaf spot of corn. Use of fungicides are not only expensive but can be very harmful to living organisms. Bio control agents that exhibit antifungal properties have the potential to be a substitute for fungicides and are environment friendly [2]. Bio pesticides are less toxic, and effective at small doses and are known to decompose easily with less toxic to health [3].

The three species selected for analyzing antifungal properties against *C. lunata* was due to the fact that these plants are used as traditional medicine by local Guyanese since many decades. *Senna alata* is used in Guyana for the treatment of ringworm De Filips *et al* [4], and *S. bicapsularis* leaves are macerated and used externally to treat rashes, skin eczema, ringworm and thrush. *P. calomelanos* is known for anti-candidal property [5].

S. alata is a shrub in the Fabaceae family and it is commonly known as the “carrion crow bush” or “canny crow bush” in Guyana. This grows up to 5 metres in height [6]. *S. bicapsularis* is a sprawling ever-green shrub and it is commonly known as the money bush in Guyana. This grow up to 4 metres tall and bear yellow or yellow green flowers. *P. calomelanos* is a fern and commonly called as wild maran. Pityrogramma is a genus with about 17 species occurring mainly in tropical America. It is cultivated widely as silver fern and the Asiatic plants are usually considered as those naturalized from cultivation [7].
Plant diseases should be controlled so as to maintain food, feed and fiber grown around the world with biocontrol agents. This study therefore analyzed and compared the effectiveness of selected plant extracts for antifungal property against a known fungal plant pathogen *C. lunata*.

2. MATERIAL AND METHODS

2.1 PLANT COLLECTION

Plants were gathered along the coastal areas of Guyana and identified at the Biodiversity Center, University of Guyana, Georgetown, Guyana. The collected leaves of each plant were washed under running tap water, dried and placed in brown paper bags. The paper bags were weighed and placed in a hot air oven, drying at a constant temperature of 55˚C until a constant weight was recorded. The dried leaves were powdered using a mixer grinder at GuySuCo Laboratory and stored for the next step [8].

2.2 SOXHLET APPARATUS

Adhering accordingly to the method followed at the Pesticides and Toxic Chemicals Control Board (PTCCB), 64 grams of dried plant material were weighed and placed in a thimble. The thimble (a thick porous cellulose container) was placed into the extraction chamber. The selected solvent was slowly poured through the condenser opening. The boiling flask was heated by a heating mantle. The boiling flask collected the extracted phytochemicals with each evaporation that passed through the siphon arm and the solvent vapor was rapidly cooled in the condenser’s cooler. Each plant used a new thimble and fresh solvent.

2.3 ROTARY EVAPORATOR (ROTOVAP)

The rotary evaporator reduces the solutions down to a solid state. The extract containing either hexane or methanol as a solvent were collected in the boiling flasks from the soxhlet apparatus. Each flask with residue were labelled; depicting solvent used and plant species.

2.4 AQUEOUS EXTRACTION

The aqueous extraction was carried out using the standard method [9]. About 15 grams of grounded leaves from each plant species were extracted by successive soaking for 3 days using 35 ml of distilled water in separate container. The extracts were filtered using Whatman No. 1 filter paper using a vacuum. The filtrates were concentrated by evaporation at low temperature of 30^{o}C using a water bath. The concentrated samples were used to make a 50% stock solution from which the tested concentrations were created. Phytochemical residue of 5 grams was added to 5 ml of the indicated solvent to make 50% stock solution. From the stock solution, 300µl, 400µl and 500µl were used to check the antifungal property. Each test was done in triplicate per plant and solvent.

2.5 QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

Phytochemical analysis was done to identify the presence of the phytochemicals; tannins, alkaloids, glycoside, saponins, flavonoids, terpenoids and steroids [10].

2.6 CULTURE TECHNIQUE

Positively identified fungal culture of *C. lunata* was obtained, fresh Potato Dextrose Agar (PDA) was made and poured into petri plates to solidify. After the PDA had solidified, a 5 mm cork borer was used to punch discs of the primary fungal culture. The 5 mm fungal discs were then placed upside down, on the freshly plated PDA. At room temperature the fungal cultures were left to grow and fresh fungal cultures of *C. lunata* were then prepared. Antifungal activity was performed by Well Diffusion and Poisoned Food technique. Measurements for Poisoned Food Technique was done in accordance to [11] between five to seven days or once the control was completely covered.

Percentage of mycelial growth inhibition was calculated from the formula

$I = C – T/C \times 100$
Where I is the inhibition percent, C is the colony diameter in the control (mm) and T is the colony of the diameter in the treatment (mm).

2.7 ANALYSIS

Data analysis of the study was done using R-Studio Program for statistical computing and graphics. A Tukey test was tested along with ANOVA and Boxplots were constructed. A Tukey test was used to compare concentration, plant and solvents versus techniques. Box and whiskers plot also called boxplots were used as visual representations of the replicates.

3. RESULTS AND DISCUSSION

3.1 PHYTOCHEMICAL ANALYSIS

Table 1 shows the result of phytochemical screening in different plant extracts. Leaves of *S. alata* showed presence of Anthraquinone glycosides and Flavonoids however it also noted the presence of alkaloids, saponins and steroids in the methanol and hexane extracts. Other phytochemicals noted were steroids and glycosides in both methanol and hexane extracts of all three plant extracts and additionally saponins were predominantly present in hexane extract. Alkaloids were present only in the water extract of *P. calomelanos* in this study.

Table 1 Results on the qualitative tests indicating presence or absence of phytochemicals in each plant solvent.

Extract	Tannins	Flavanoids	Saponins	Terpenoids	Steroids	Alkaloids	Glycosides
S. alata (methanol)	-	+	+	-	+	+	+
S. alata (hexane)	+	-	+	-	+	-	+
S. alata (water)	-	+	-	-	-	-	-
S. bicapsularis (methanol)	-	-	-	-	+	-	+
S. bicapsularis (hexane)	-	-	+	-	+	-	+
S. bicapsularis (water)	-	+	-	-	-	-	-

P. calomelanos (methanol)	-	-	+	-	+	-	+
P. calomelanos (hexane)	-	-	+	-	+	-	+
P. calomelanos (water)	-	+	-	+	-	+	-

3.2 POISONED FOOD TECHNIQUE

Table 2 illustrates mean±SE for every plant extract. *P. calomelanos* methanol extract (Figure 1) as shown in Table 2 was the only plant extract that was effective against *C. lunata* with an inhibition percentage of 59.4%, 60.3% and 50.3% with a concentration of 300 µl, 400 µl and 500 µl respectively. The water and methanol extracts of *P. calomelanos* were not as effective. The *S. alata* water extract (Figure 1) was the least effective with inhibition percentages of -21.4%, -5.2% and -10.2%, the hexane and methanol extracts of *S. alata* were not effective. None of the *S. bicapsularis* extracts was inhibitive against *C. lunata*.

Table 2 Measurements of fungal growth millimeters (mm) and standard error for the Poisoned Food Technique (mean±SE).

Conc (µl)	*S. alata*			*S. bicapsularis*			*P. calomelanos*		
	Methanol	**Hexane**	**Water**	**Methanol**	**Hexane**	**Water**	**Methanol**	**Hexane**	**Water**
300	2.4±2.5	42.9±2.1	-21.4±9.1	-17.5±4.0	18.3±8.3	3.9±5.4	59.4±2.9	11.7±4.9	0.6±0.6
400	19.2±8.3	21.6±5.0	-5.2±1.6	-15.4±3.0	29.5±3.1	9.1±1.0	60.3±5.1	21.6±5.0	8.2±2.7
500	-10.2±14.1	33.0±3.6	-10.2±14.1	17.0±4.1	33.0±3.6	9.6±3.3	50.3±5.6	29.4±1.0	14.7±1.7

Figure 1: Plates showing the end result with poisoned food technique, *S. alata* water extract (left), compared to the most effective extract, *P. calomelanos* methanol extract (right).

Tukey tests (Post-hoc tests) showed statistically different in terms of the effect against the fungus (p=0.02). The post-hoc Tukey test indicated no statistically significant difference between *S. bicapsularis* and *S. alata* but significant difference was observed between *S. alata* and *P. calomelanos* (p=0.002) and between *S. bicapsularis* and *P. calomelanos* (p=0.04). *P. calomelanos* demonstrated inhibition with a mean of 28.2% compared to *S. bicapsularis* and *S. alata,* which had, means of 13.5% and 13.1% respectively as shown in Figure 2. This correlates with the most effective extract being the methanol extract of *P. calomelanos.*

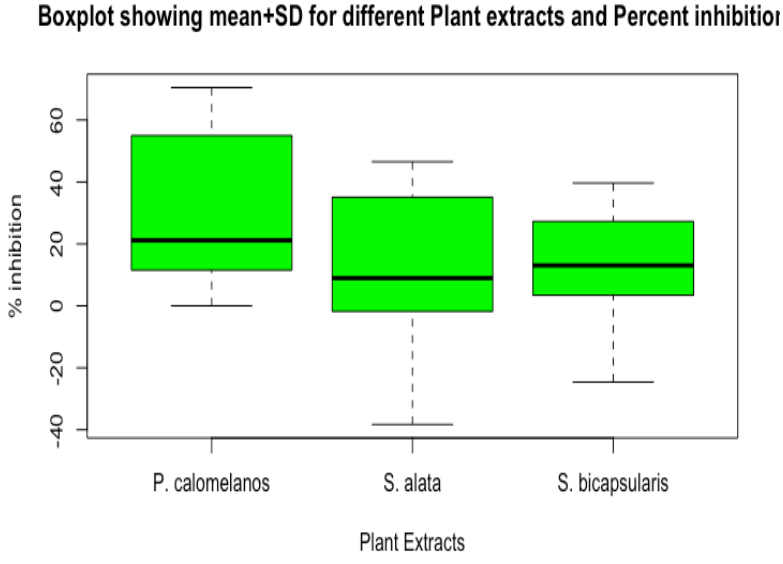

Figure 2. Boxplot (mean±SD) for different plants and percent inhibition.

The post-hoc Tukey tests indicated no statistically significant difference between methanol and hexane but significant difference was observed between water and hexane ($p<0.0001$) as well as between water and methanol ($p<0.0001$) (Figure 3).

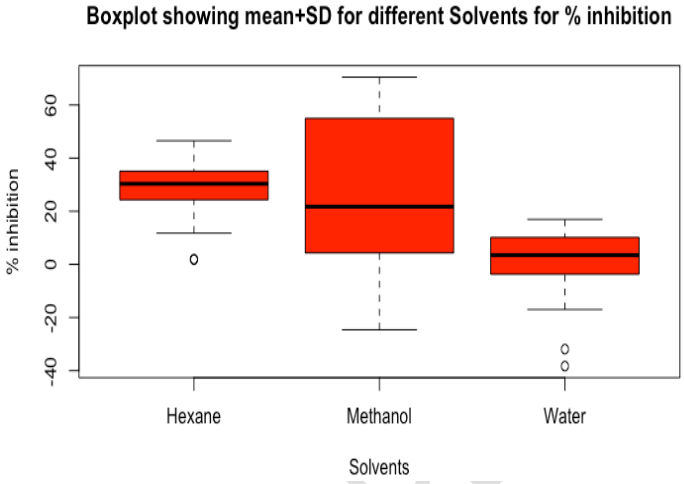

Figure 3 Boxplot (mean±SD) for different solvents and percent inhibition.

Post-hoc tests such as the Tukey test was performed to pinpoint the differences between concentrations for different extracts and boxplots were constructed (Figure 4). There were (9) Tukey tests results that were relevant. *P. calomelanos* water and methanol extracts showed significant differences (500 µl) ($p<0.05$). *P. calomelanos* which had a mean of 50.3% indicated high statistical difference between different solvent extract and with other plant extracts. This correlates with the most effective extract being the methanol extract of *P. calomelanos.* The mean inhibition percentages for the methanol and water extracts of *S. alata* were 16.7% and -10.2% respectively which were statistically lower than 50.3% mean of *P. calomelanos.*

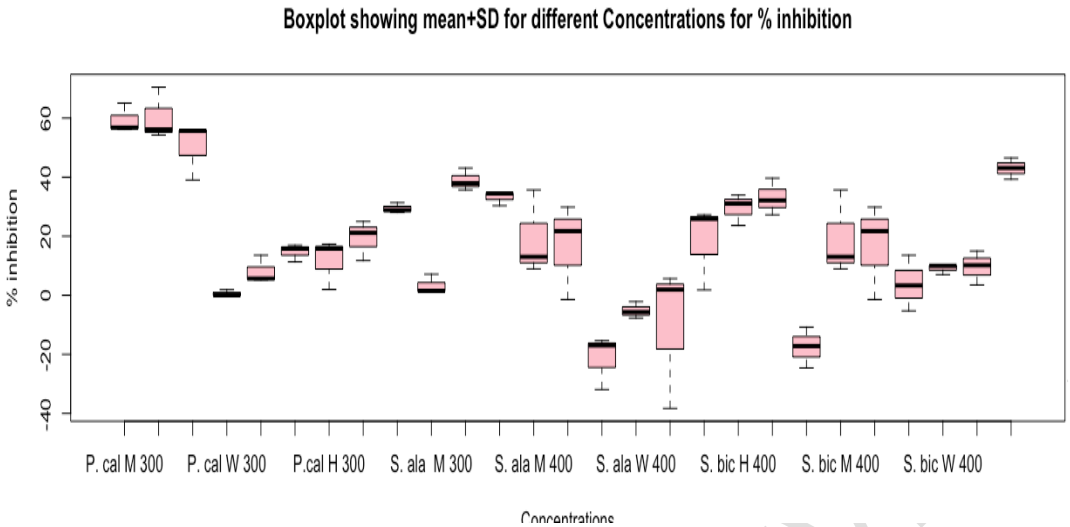

Figure 4: Boxplot (mean±SD) between concentrations and percentage inhibition.

3.3 WELL DIFFUSION TECHNIQUE

P. calomelanos hexane extract was most effective against *C. lunata* with inhibition zones of 10 mm, 12 mm and 46 mm for 300 µl, 400 µl and 500 µl concentration respectively. The *S. alata* water extract was the least effective with inhibition zones of 0 mm or no inhibition zones for all three concentrations. Overall, *S. alata* plant showed least antifungal activity.

Table 3: Zone of inhibition (mean±SE) in millimeters (mm) with well diffusion technique.

Conc (µl)	*P. amboinicus*			*C. citratus*			*C. cajan*		
	Meth	**Hex**	**Water**	**Meth**	**Hex**	**Water**	**Meth**	**Hex**	**Water**
300	35.3±2.4	16.7±4.8	0	10.0±2.3	8.7±5.2	12.7±2.4	5.3±3.3	10±2.3	2.7±2.7
400	37.3±6.4	24.7±2.4	0	22.0±4.2	16.7±2.4	11.3±2.9	4.7±0.3	12±1.2	4±2.3
500	38.0±1.2	26.7±4.1	0	27.3±7.5	28.7±8.5	14.7±3.5	14.3±4.9	46±5.3	15.3±2.4

No statistically significant differences were observed between *S. bicapsularis* and *S. alata* as well as with *S. bicapsularis* and *P. calomelanos*. However, *S. alata* and *P. calomelanos* showed a significant difference ($p<0.05$). *P. calomelanos* showed a mean size of 12.7 mm when compared to *S. bicapsularis* and *S. alata* with mean size of 19.8 mm and 22.2 mm respectively as shown in Figure 6.

Figure 5: Result of well diffusion technique with least effective plant extract compared to most effective plant extract *(S. alata* water extract (left) at 500 µl, *S. bicapsularis* methanol extract (center) at 400 µl and *S. alata* methanol extract at 300 µl).

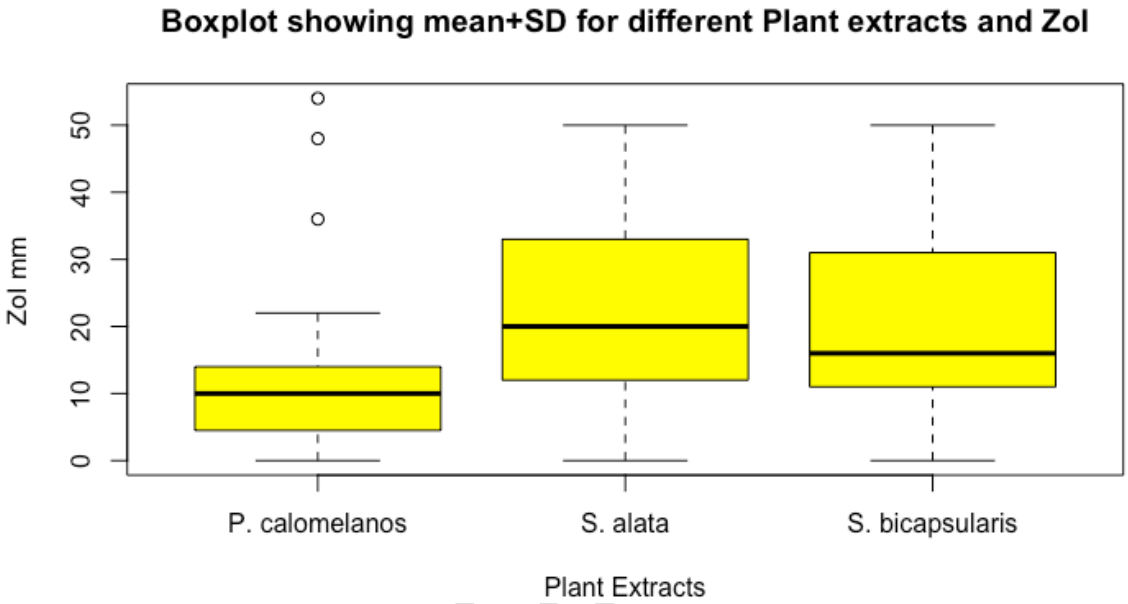

Figure 6: Boxplot showing the mean±SD for the different plants for Zone of inhibition with Well Diffusion technique

The post-hoc Tukey tests indicated no statistically significant difference that between methanol and hexane however a significant difference was observed between water and hexane ($p < 0.005$) as well as water and methanol ($p < 0.0001$).

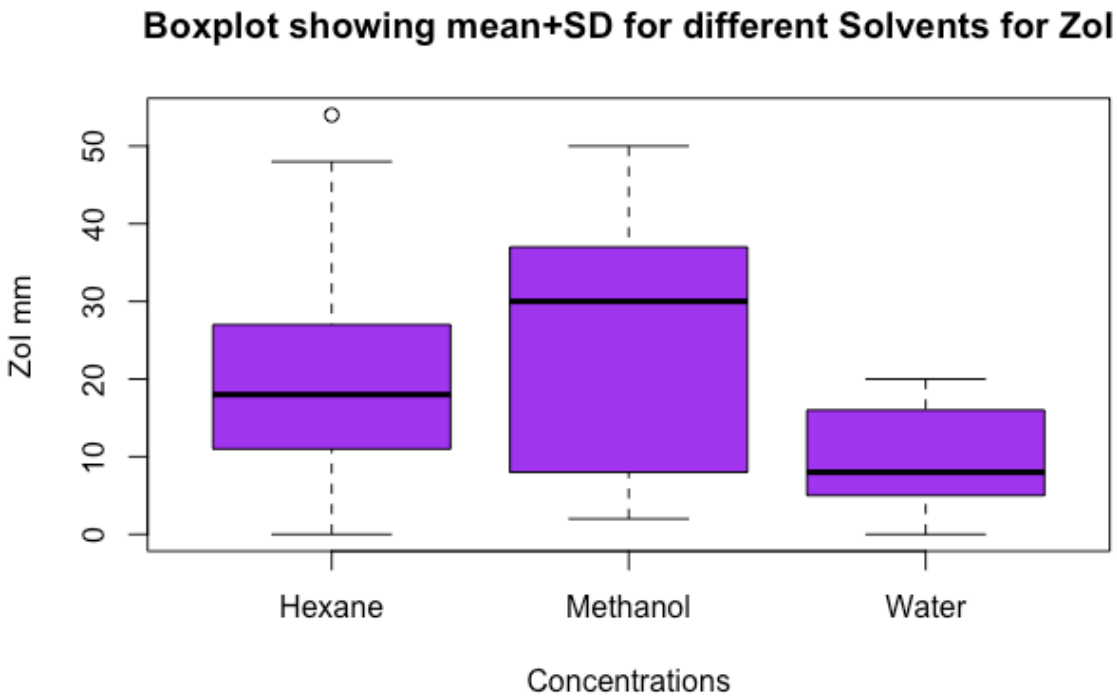

Figure 7: Boxplot showing the mean ±SD for different solvents for Zone of inhibition with Well Diffusion technique

Figure 8 shows *P. calomelanos* methanol extract significantly different than hexane extract ($p < 0.001$) and methanol extracts of *S. alata* and *S. bicapsularis* at a concentration of 500 µl in terms of antifungal effect. Water extract of *P. calomelanos* showed significant difference with both *S. alata* and *S. bicapsularis* methanol extracts ($p<0.05$). *S. alata* methanol extract (500 µl) was statistically different from *S. alata* water extract and *S. bicapsularis* water extract ($p<0.05$).

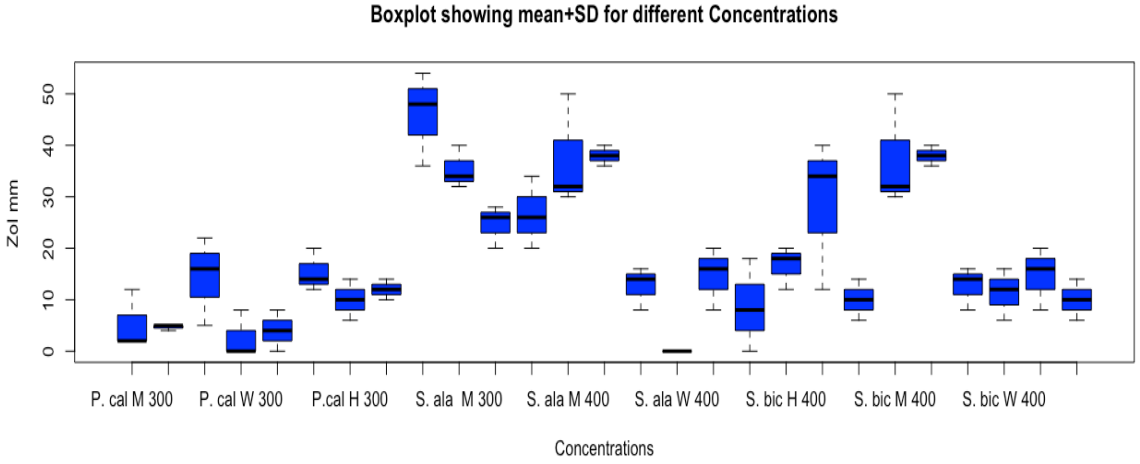

Figure 8: Boxplot showing the mean±SD of different extracts at different concentrations for the Well Diffusion Technique

4 DISCUSSION

The Anthraquinone glycoside includes rhein, emodine, physion, chrysophanol (marker), obtusin, chryso- obtusin, chryso-obtusin-2-O-β-D-glucoside, obtusifolin and chryso-obtusifolin-2-O- β -D- glucoside [12]. Our study showed presence of flavonoids and glycosides were present in all plant extracts however it also noted the presence of alkaloids, saponins and steroids in the methanol and hexane extracts which correlates with the study by Owoyale, Olatunji, Oguntoye, [13] which highlighted the presence of phenols, tannins, saponins, alkaloids, steroids, flavonoids and carbohydrates in the *S. alata* ethanol leaf extract.
The flowers of *S. bicapsularis* has shown the presence of phenols, flavonols, flavonoids, and anthocyanins [14]. Our study identified flavonoids only with water extract of *S. bicapsularis*. Methanol and hexane extract exhibited steroids, glycosides and saponins similar to findings from Souza *et al* [5] also recorded the presence of alkaloids, aurones, catequines, chalcones, flavones and flavonones.
P. calomelanos methanol extract was the only plant extract that was effective against *C. lunata* having large inhibition percentages. The research conducted by Souza *et al.* [5], stated that the methanolic fraction of *P. calomelanos* showed good activity against *Candida albicans, Candida krusei* and *Candida tropicalis* but only when associated with antifungals.

Based on the phytochemical analysis, possible phytochemicals associated with the effective antifungal activity for the methanol extract (the most effective extract) were saponins, steroids or glycosides. These secondary metabolites exert antimicrobial activity through different mechanisms and have been previously reported by Owoyale et al [13], to have inhibitory effect or antifungal activity on *Mucor*, *Rhizopus* and *Aspergillus* fungi. The same study by Owoyale et al [13], indicated that *S. alata* leaves had the presence of phenols, tannins, saponins, alkaloids, steroids, flavonoids and carbohydrates resulting in the ethanol extract being effective, the presence of these metabolites were similar in this study. However, the effectiveness of the *S. alata* extract was not the same in our study. Sule et al in a study on the in vitro antifungal activity of *S. alata* crude leaf extracts showed stem bark was fungicidal for all tested dermatophytes at concentrations of 10.0 and 5.0 mg/mL except *E. floccosum* which was only fungicidal at concentration of 10.0 mg/mL [15]. It had also been observed that antimicrobial activity of *S. alata* is associated with the presence of some chemical components such as phenols, tannins, saponins, alkaloids, steroids, flavonoids and carbohydrates. These secondary metabolites exert antimicrobial activity through different mechanisms and have been previously reported by Owoyale et al [13], to have inhibitory effect or antifungal activity. Although several authors demonstrated antifungal activity on human pathogenic fungi our study did not corroborate an overall effective antifungal activity of *Senna alata* and *Senna bicapsularis* [13, 16].

5. CONCLUSION

The maximum antifungal activity with Poisoned Food Technique was shown by the methanol extract of *P. calomelanos* against the fungus *C. lunata* with an inhibition percentage of 60.3% at 400 µl. With the well diffusion technique, maximum antifungal activity was shown by the water extract of *P. calomelanos* against the fungus *C. lunata* with an inhibition zone of 56 mm at 300 µl. The extracts of *S. alata* and *S. bicapsularis* were not as effective against *C. lunata* in either of the two techniques used for the antifungal testing.

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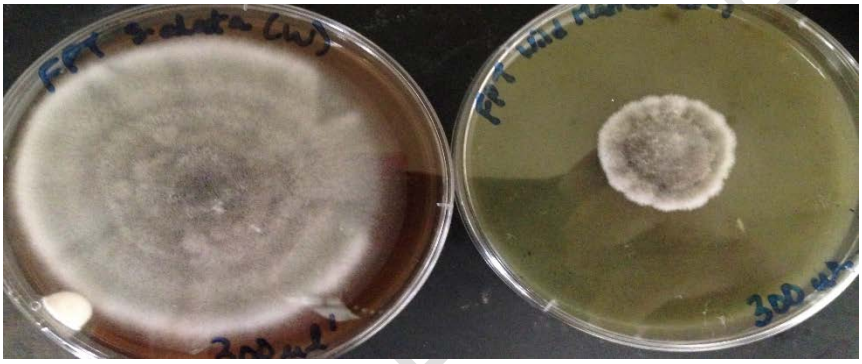

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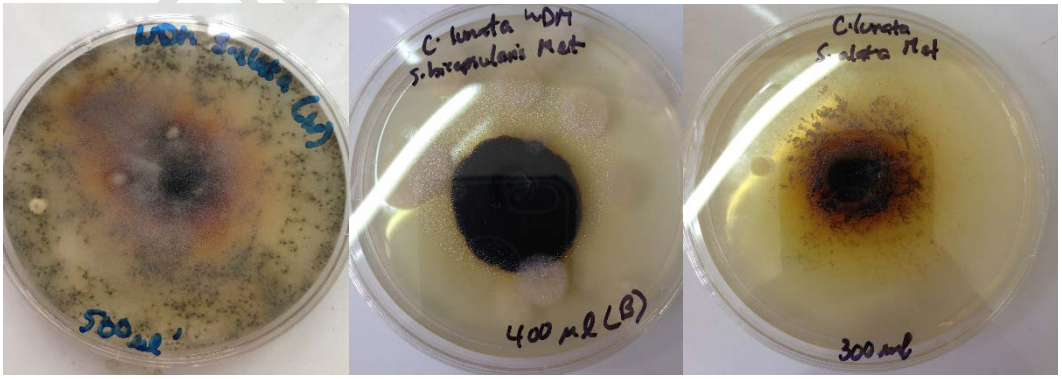

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