



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

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Authors' contributions

This work was carried out in collaboration among all authors. Author FG designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors FG and GS carried the antimicrobial properties. Authors RK and AAA prepared the final draft, performed overall analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

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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 was an effort to identify, environment friendly antifungal compounds from these locally available plants against fungal pathogen *Curvularia lunata*.

Study Design: An experiment based study done to identify antifungal property and phytochemical identification of *C. lunata*.

Place and Duration of Study: Plants were collected from the coastal areas of Guyana and identified at the Biodiversity Center, University of Guyana, 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 properties. 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.

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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: The finding of this study indicates, the possible use of *P. calomelanos* extract as an antifungal agent against *C. lunata* and its potential to control fungal plant diseases.

Keywords: Antifungal; phytochemical; poison food technique; well diffusion technique; *Curvularia lunata*.

1. INTRODUCTION

Fungal damages to crops are enormous and affects agricultural productivity [1]. One such fungus is *Curvularia lunata*. It is mainly an opportunistic leaf spot fungus and affects grains and appears as a floccose (wooly tufts), brown, dark brown to black, and often zonate [2]. It causes leaf spots on grasses as well as storage molds on grains. *Curvularia* species are recorded to be the cause of plant diseases in various parts of the world [3]. 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 [4]. Use of fungicides are not only expensive but can be very harmful to living beings. Biological control of plant diseases has been one of the most sustainable method, since years [4,5]. Biological control is the inhibition of growth, infection or reproduction of one organism using another organism [5,6]. United States National Research Council (USNRC) referred the term biological control as the use of natural or modified organisms, genes, or gene products, to reduce the effects of undesirable organisms and to favor desirable organisms such as crops, beneficial insects and microorganisms [7]. Bio control agents that exhibit antifungal properties have the potential to be a substitute for fungicides and are environment friendly [8]. Bio pesticides are less toxic, and effective at small doses and are known to decompose easily with less toxic to health [9].

These three species are commonly used by the local Guyanese for treating many ailments. *S. alata* is used in Guyana for the treatment of ringworm [10], and *S. bicapsularis* leaves are macerated and used externally to treat rashes, skin eczema, ringworm and thrush. *P. calomelanos* is known for its anti-candidal property [11].

S. alata is a shrub, and belongs to Fabaceae family. It is commonly known as the "carrion crow bush" or "canny crow bush" in Guyana. This

grows up to 5 meters in height [12]. *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 bears 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 the tropical America. It is cultivated widely as a silver fern and this Asiatic plants are usually considered as those naturalized from cultivation [13].

Plant diseases should be controlled so as to maintain food, feed and fiber grown around the world with biocontrol agents. Biocontrol agents against fungal plant diseases include hyperparasitism, predation, antibiosis, cross protection, competition for site and nutrient and plant inducing resistance [7]. Therefore, this study focused on accessing the antifungal property of the selected plant extracts against *C. lunata*.

2. MATERIALS 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 process [14].

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 [15]. 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 a 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°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 a 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 per 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 [16].

2.6 Culture Technique

Positively identified fungal culture of *C. lunata* was obtained. A 5 mm fungal discs were then placed upside down, on the freshly plated Potato Dextrose Agar (PDA). Antifungal activity was performed by Well Diffusion and Poisoned Food technique. Measurements for Poisoned Food Technique [17] were taken between five to seven

days or once the control was completely covered. Fluconazole and distilled water were used as positive and negative control.

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 done and 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 the presence of Anthraquinone glycosides and Flavonoids however the presence of alkaloids, saponins and steroids were noted 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 the hexane extract. Alkaloids were present only in the water extract of *P. calomelanos*.

3.2 Poisoned Food Technique

Table 2 illustrates Mean±SE for all plant extract. *P. calomelanos* methanol extract (Fig. 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% at a concentration of 300 µl, 400 µl and 500 µl respectively. The water and methanol extracts of *P. calomelanos* were not equally effective. *S. alata* water extract (Fig. 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 were inhibitive against *C. lunata*.

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
<i>S. alata</i> (methanol)	-	+	+	-	+	+	+
<i>S. alata</i> (hexane)	+	-	+	-	+	-	+
<i>S. alata</i> (water)	-	+	-	-	-	-	-
<i>S. bicapsularis</i> (methanol)	-	-	-	-	+	-	+
<i>S. bicapsularis</i> (hexane)	-	-	+	-	+	-	+
<i>S. bicapsularis</i> (water)	-	+	-	-	-	-	-
<i>P. calomelanos</i> (methanol)	-	-	+	-	+	-	+
<i>P. calomelanos</i> (hexane)	-	-	+	-	+	-	+
<i>P. calomelanos</i> (water)	-	+	-	+	-	+	-

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

Conc (µl)	<i>S. alata</i>			<i>S. bicapsularis</i>			<i>P. calomelanos</i>		
	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

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

Conc (µl)	<i>P. amboinicus</i>			<i>C. citratus</i>			<i>C. cajan</i>		
	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

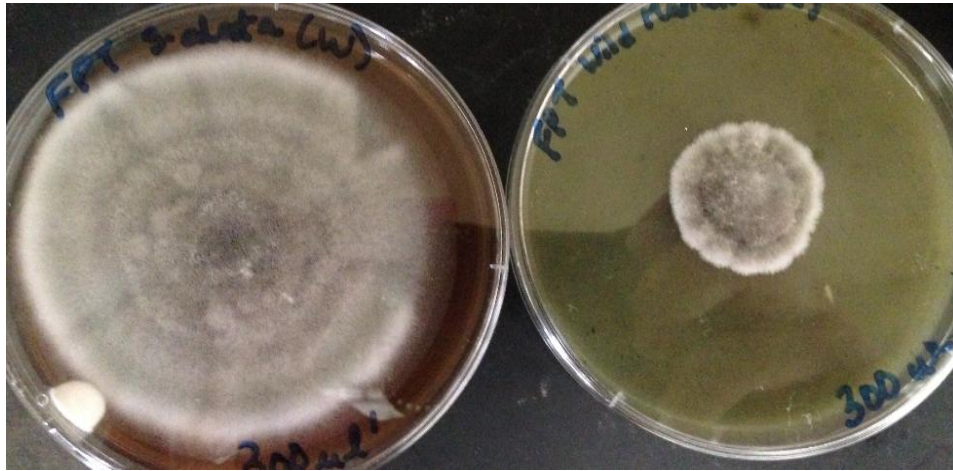


Fig. 1. Plates showing the end result with poisoned food technique, *S. alata* water extract (left), compared to the most effective extract, *P. calomelanos* methanol extract (right)

Boxplot showing mean+SD for different Plant extracts and Percent inhibitor

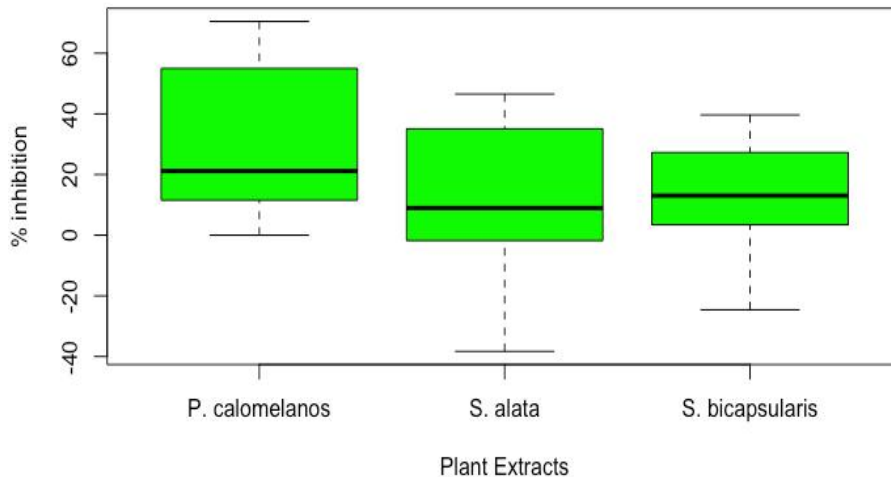


Fig. 2. Boxplot (Mean±SD) for different plants and percent of inhibition

Tukey tests (Post-hoc tests) showed a significant statistical difference against the fungus ($p=0.02$). Individual analysis 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* ($p=0.04$).

Mean percentage of inhibition with *P. calomelanos*, *S. bicapsularis* and *S. alata* were 28.2%, 13.5% and 13.1% respectively. This correlates with the most effective extract being the methanol extract of *P. calomelanos*.

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 ($p<0.0001$) (Fig. 3).

Post-hoc tests such as the Tukey test was performed to pinpoint the differences between concentrations for different extracts and boxplots were constructed (Fig. 4). There were (9) Tukey tests results that were relevant. *P. calomelanos* water and methanol extracts showed significant differences (500 μ l) ($p<0.05$). *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 lower than 50.3% mean of *P. calomelanos*.

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 with 300 µl, 400 µl and 500 µl concentration respectively. *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* showed the least antifungal activity.

No statistically significant differences were observed between *S. bicapsularis* and *S. alata* as well as between *S. bicapsularis* and *P. calomelanos*. However, *S. alata* and *P. calomelanos* showed a statistically significant difference ($p < 0.05$).

Mean size observed with *P. calomelanos*, *S. bicapsularis* and *S. alata* were 12.7 mm, 19.8 mm and 22.2 mm respectively as shown in Figs. 5 & 6.

Boxplot showing mean+SD for different Solvents for % inhibition

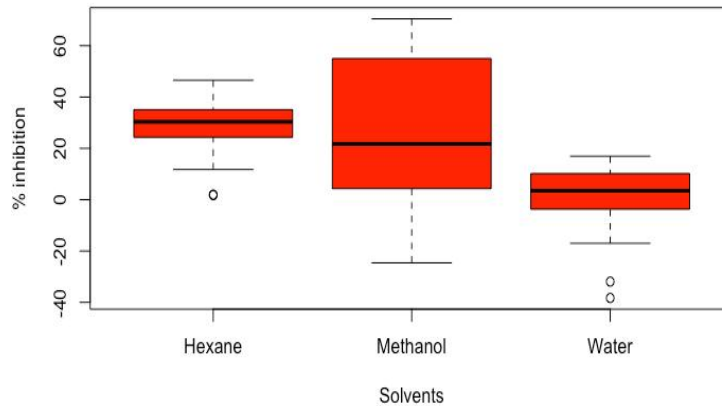


Fig. 3. Boxplot (Mean±SD) for different solvents and percent inhibition

Boxplot showing mean+SD for different Concentrations for % inhibition

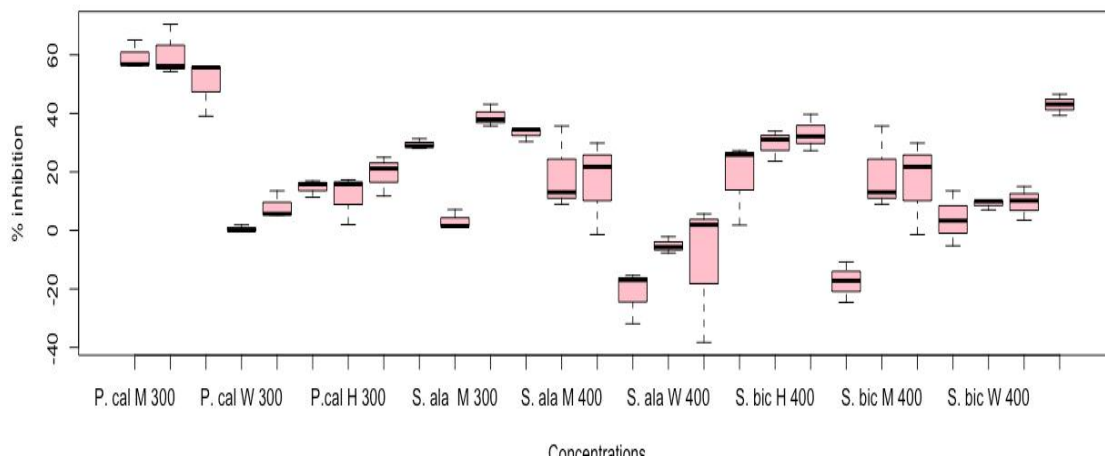


Fig. 4. Boxplot (Mean±SD) between concentrations and percentage inhibition

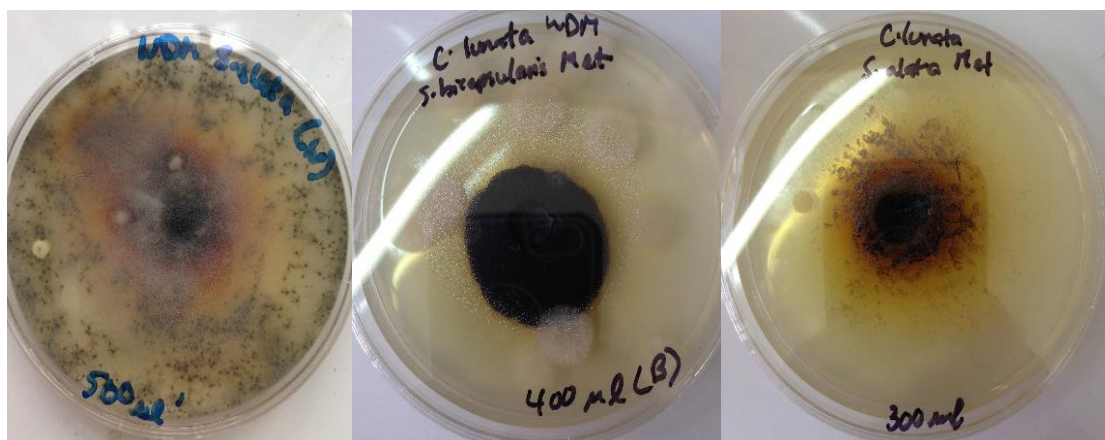


Fig. 5. Result of well diffusion technique with least effective plant extract compared to most effective plant extract (*S. alata* water extract (left) at 500 µl, *S. bicapsularis* methanol extract (center) at 400 µl and *S. alata* methanol extract at 300 µl)

Boxplot showing mean±SD for different Plant extracts and Zol

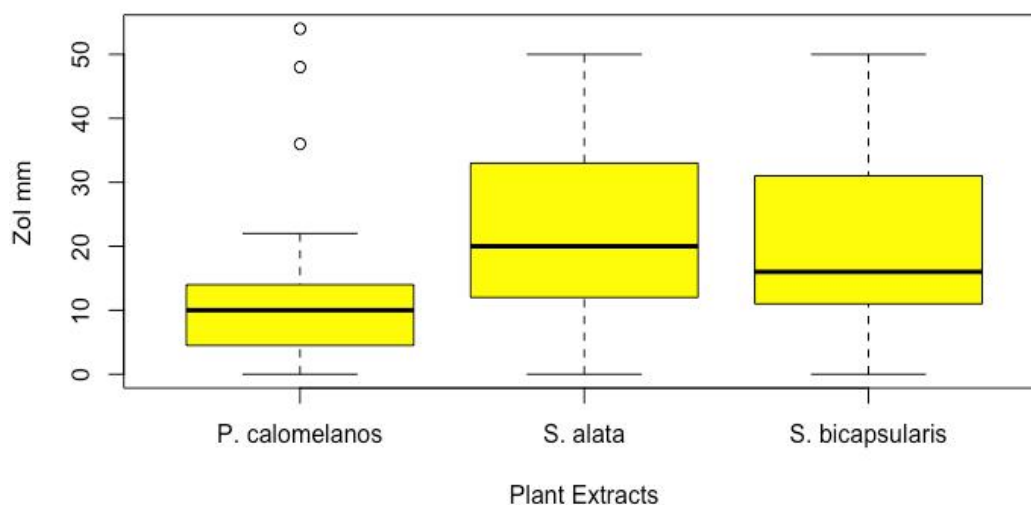


Fig. 6. Boxplot showing the Mean±SD of different plants for zZone of inhibition with Well Diffusion technique

The post-hoc Tukey tests indicated no statistically significant difference between methanol and hexane, however a significant statistical differences were observed between water and hexane ($p < 0.005$) as well as between water and methanol ($p < 0.0001$) (Fig. 7).

Fig. 8 shows antifungal properties of *P. calomelanos* methanol extract significantly different from hexane extract ($p < 0.001$) as well as with methanol extracts of *S. alata* and *S. bicapsularis* at a concentration of 500 µl. 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 ($p < 0.05$).

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 [18]. This study showed the

presence of flavonoids and glycosides in all plant extracts, however, the presence of alkaloids, saponins and steroids were observed in the methanol and hexane extracts. This was in agreement with the study done by Owoyale, Olatunji, Oguntoye, [19] which highlighted the presence of phenols, tannins, saponins, alkaloids, steroids, flavonoids and carbohydrates in the *S. alata* ethanol leaf extract.

The flowers of *S. bicepsularis* has shown the presence of phenols, flavonols, flavonoids, and anthocyanins [20]. However, this study identified flavonoids only with water extract of *S. bicepsularis*. Methanol and hexane extracts exhibited the presence of steroids, glycosides and saponins similar to the findings from Souza et al [11].

P. calomelanos methanol extract was the most effective plant extract against *C. lunata*. A study conducted by Souza et al. [11], stated that the methanol fraction of *P. calomelanos* showed good activity against *Candida albicans*, *Candida krusei* and *Candida tropicalis* but only when associated with antifungals.

Methanol extract identified the presence of saponins, steroids or glycosides. These

secondary metabolites exert antimicrobial activity through different mechanisms and have been previously reported by Owoyale et al [19], to have inhibitory effect or antifungal activity on *Mucor*, *Rhizopus* and *Aspergillus* fungi. The same study by Owoyale et al [19], 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 this 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 [21]. It had also been observed that the 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 [19] Although, several studies established antifungal activity of *S. alata* and *S. bicepsularis* on human pathogenic fungi, this study did not corroborate an overall effective antifungal activity [19,22].

Boxplot showing mean±SD for different Solvents for Zol

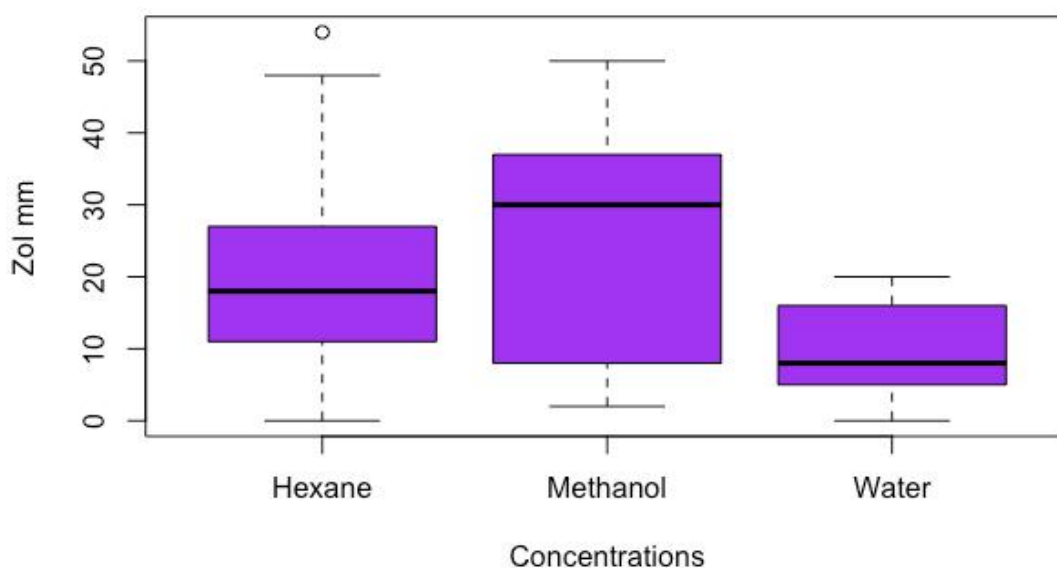


Fig. 7. Boxplot showing the Mean ±SD for different solvents for Zone of inhibition with Well Diffusion technique

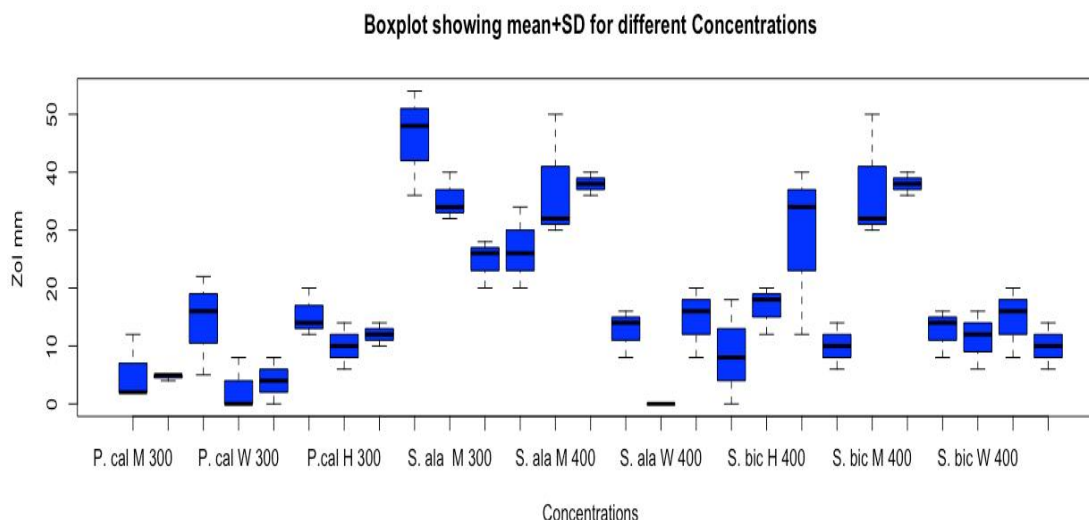


Fig. 8. Boxplot showing the Mean±SD of different extracts at different concentrations for the Well Diffusion Technique

5. CONCLUSION

Natural fungicides could be the best option to replace harmful chemical fungicides. The study identified optimum antifungal property with methanol extract of *P. calomelanos* using Poisoned Food Technique. Water extract of *P. calomelanos* also demonstrated optimum antifungal property with well diffusion technique. However, extracts of *S. alata* and *S. bicuscularis* were not effective against the fungus, *C. lunata*. *P. calomelanos* has showed a great potential as an antifungal agent and can be an alternative to control plant diseases caused by *C. lunata*.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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