Determination of the Growth Rate and Susceptibility Pattern of Fungi Using Agro-Waste Formulated Media

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Abstract: Management of wastes especially agro wastes has been a major problem experienced in developed and developing countries. There is a serious need to convert these agro-wastes into useful forms. This study was undertaken to determine the growth rate and susceptibility pattern of fungi using agro-waste formulated media. Sweet Potato and Watermelon peel wastes were used to formulate SPDA and WDA media respectively and their performance compared with Potato Dextrose agar (PDA). The proximate analysis of the peel wastes was done using standard methods. The methods used for fungi isolation, cultivation and susceptibility testing were spread plate, agar plug diffusion and agar well diffusion techniques respectively. Solvent extraction of scent leaf and Ginger was done using ethanol and the extracts were used for the susceptibility testing. The result of the proximate analysis of the agro wastes revealed the presence of carbohydrate, protein, crude fibre, moisture and ash. Nine fungal species were isolated and used for growth rate and susceptibility testing. The result showed that PDA (0.14- 0.44^{h-1}) performed better than the formulated media (0.14- 0.18^{h-1} and 0.13-0.22^{h-1})in growth rate of the isolates with a significant p value of 0.0020, while the formulated media(14-41mm and 14-34mm; 0-25mm and 0-25mm) performed far better than the PDA(0-15mm and 0-16mm)in susceptibility testing with p values of 0.0028 and 0.0007 for Scent leaf ethanol extract and Ginger ethanol extract respectively. Thus the formulated media can be utilized as alternative cheap sources of culture media for antifungal screening and other mycological assays.

Keywords: Agro-waste, Susceptibility testing, Sweet potato peeldextrose agar, Waste management and Watermelon peel dextrose agar.

INTRODUCTION

gro-wastes are materials generated from the food processing industries, household domestic activities and other human endeavours. They can also be regarded as leftovers from production and consumption. The disposal of these wastes into the environment has been of serious ecological hazard and environmental disorder (Smith etal., 1987; Lim etal., 2010).Improper disposal of these agrowastes leads to pollution of air and water bodies which will eventually lead to disease outbreak and destruction of aquatic lives (Ali etal., 2014; Lim et al., 2010). Waste dumping sites encourage the growth of pathogenic microorganisms and arthropod disease vectors which pose a serious risk in public health (Anbu et al., 2017), hence the need to address the problem of proper waste disposal.

Peel wastes emanating from fruits and crops are rich in sugars which are easily metabolized by microorganisms (Saheed *et*

al., 2013). They can be utilized as substrates for microbiological processes (Nwabueze and Otuwa, 2006) instead of being left as solid municipal waste. Moreover, the presence of carbon, nutrients and moisture also justifies the suitability of peel wastes for the growth of microorganisms.

Sweet potato (*Ipomoeabatatas*) peel waste and Watermelon (Citrulluslanatus) peel waste used in this study are rich in nutrients (Guechi and Hamdaoni 2016; Edwards et al., 2003). The major components of Sweet potato peel waste are usually water, starch, cellulose, hemicelluloses, fermentable sugar, lignin, proteins, vitamins, polyphenols and alkaloids (Izminlioglu and Dimirci 2012; Guechi and Hamdaoni 2016). The fruit Watermelon serves as a thirst –quencher owing to its high water content (92 %) and it is an excellent source of mineral (Hall 2004), lycopene, vitamins C, B₆ and A (Edwards et al., 2003). These contents in them provide conditions suitable for the development of microorganisms.

Fungi are a diverse group of microorganisms that range from unicellular forms (yeasts) to Moulds moulds and mushrooms. mushrooms are multicellular fungi that form thin threadlike structures called hyphae. They absorb nutrients from their environment including the organic molecules that they use as sources of carbon and energy. Fungi can be parasitic or saprophytic. The saprophytic fungi obtain their nourishment from the products of organic breakdown and decay. They are involved in the decomposition of plant chemical compounds such as cellulose, hemicellulose and lignin, thus contributing to the maintenance of the global carbon cycle (Itelima etal., 2014). Fungi grow on diverse habitats in nature and require several elements for growth and reproduction. They can also be cultivated in the laboratory using specific culture media that contained the requirements for their growth reproduction. Conventional media mostly used in the laboratory for the cultivation of fungi have been Sabouraud Dextrose Agar and Potato Dextrose Agar. Unfortunately, these conventional media have been very costly and are not readily available in most developing countries. There is a growing need for alternative cheap and available media for routine laboratory isolation of microorganisms the since known conventional media are not generally affordable.It is worthy to note that agrowaste peels are readily available, obtained at little or no cost and do not need further enhancement with supplements to give maximum fungi growth. Hence, utilization of agro-waste in media formulation will indeed help to alleviate the problem of shortage of culture media in the laboratory. Several types of research have been geared towards achieving this pursuit for cheap alternatives. It is in line with this pursuit that the present study was carried out. This study aimed to formulate cheap and efficient culture media from agro-wastes, cultivate fungi on the composed media and compare the formulated media with conventional media (Potato Dextrose Agar) in fungal

growth and antifungal susceptibility test quality.

MATERIALS AND METHODS Collection and Preparation of Samples

The Sweet Potato peels and Watermelon peels were collected from fruit and food vendors in Uli, Ihiala Local Government Area of Anambra State. The Peel wastes were obtained by cutting off the outer surface of Sweet potato and Watermelon. The wastes were collected in clean polythene bags and taken to the Microbiology Laboratory of Chukwuemeka Odumegwu Ojukwu University, (COOU) where they were washed, sundried and then milled into powdery form. The milled peels were stored in sterile plastic airtight containers until when used.

Source of test organisms

The test organisms used for the experiment were isolated from soil samples from different farmlands, contaminated water, air, cow dung and pig dung around Uli and different Abattoirs in Ihiala Government Area of Anambra State. The samples were collected aseptically with a sterile spatula, placed in sterile polythene bags and 1 L plastic containers and transported to Microbiology Laboratory of Chukwuemeka Odumegwu Ojukwu University, Uli for analysis.

Proximate Analysis of Agro-Wastes

The proximate compositions of the Sweet Potato Peel waste (SPP) and Watermelon Peelwaste (WP)were determined using the standard methods of Association of Official Analytical Chemists (AOAC, Moisture contents of the samples were determined by air oven (Gallenkamp)method at 105°C for 3h, while the protein contents were determined using the micro-Kjeldahl method. The ash contents were determined using a muffle furnace set at 550°C for 4 h until the constant weight of ash was obtained. The Crude fibres were determined using a multimetre, while the carbohydrate contents were obtained using anthrone method.

Media Formation using Agro-Wastes

Twenty grams (20g) of each powdery sample (SPP and WP) were weighed and mixed with 200 ml of 100°C boiled sterile water, stirred with a glass rod and allowed to cool. Each mixture was filtered using muslin cloth and a filter paper. Thirty-eight grams (38g) of glucose and 5 g of agar were added to 150 mL of each sample filtrate. The mixtures were sterilized by autoclaving at 121°C for 15 minutes. The media were allowed to cool to about 45 °C and 1ml of 0.005% Chloramphenicol was added to inhibit bacterial growth (Itelima et al., comparative analysis, 2014). For conventional mycological media Potato Dextrose Agar (PDA) was also prepared according to manufacturer's (Oxoid) specification and used as a control.

Fungi Isolation

One gram (1g) each of the soil samples, cow dung and pig dung were measured into different sterile tubes containing 9 mL of distilled water to produce a stock suspension (10⁻¹). 1 mL of the contaminated water was also measured into the sterile tube containing 9 mL of distilled water. The stock suspensions were then shaken vigorously for five seconds to dislodge the organisms. Tenfold serial dilution was carried out up to 10⁻⁵ dilution and sterile plates of the Potato Dextrose Agar (PDA) inoculated using standard spread plate technique. Passive air sampling was performed using settle plates. Petri dishes containing Potato Dextrose Agar were left open to air for 1 h, 1 m from the floor and 1 m away from walls or any obstacle so that microbes carried by inert particles will fall onto the surface of the plates, with an average deposition rate of 0.46 cm/s as reported byNdimele et al., 2015and the culture plates were incubated at a laboratory temperature of 25 °C for 72 h (Akharaiyi and Abiola, 2016; Onome and Ejale, 2018; Sah, 2019). Nine pure cultures were obtained after incubation by subculturing in Bijou bottles containing PDA medium and kept at 4 °C refrigeration temperature for further microbiological examination.

Identification of Fungal Cultures

A small amount of aerial growth of each fungus was removed using mounted sterile needle and transferred to a drop of cotton blue lactophenol on a clean slide. The hyphae were teased apart with the needle and a cover slip was placed over the preparation taking care to prevent air bubbles. The preparation was viewed under a digital camera compound microscope (Stereo OF0533, China) for observation of features such as nature of hyphae, fruiting structures, spore types and spore attachment. The descriptions of the nine fungal isolates were compared with those of known taxon described in the identification key of fungal atlas (Itelima et al., 2014; Sah, 2019).

Determination of Fungal Radial Growth

The suitability of the formulated media (Sweet Potato peel Dextrose Agar and Watermelon peel Dextrose Agar) for fungal growth was estimated by culturing the test fungi on them. The test organisms from the pure culture plates were introduced into the prepared sterile agar plates using agar plug diffusion technique. A 9 mm sterile cork borer was used to introduce the agar plugs of the nine fungal isolates that were previously cultured in Potato Dextrose Agar for 7 days to both the formulated media and Potato Dextrose Agar (control medium) and then incubated at 25 °C for 72 h. After incubation, the mean radial colony growth of each of the test fungus was measured in millimeters (mm), recorded, and growth rate was compared in both the formulated media and PDA (control medium)

Preparation of Plant Extracts

The plants used in this study were *Ocimum* gratissimum (scent leaf) and Zingiber officinale (Ginger) bought at Afo Egbu Market, Uli, Anambra State. They were thoroughly washed with running tap water and dried at laboratory temperature of 25 °Cfor 14 days. They were ground into smooth powder with electric blender. Ten grams of both powders were weighed and placed into separate 250 mL conical flasks containing 100 mL of ethanol for 24 h.

After 24 h, they were double filtered with a muslin cloth and Whatman no. 1 filter paper and left for the ethanol to evaporate. The crude extracts were finally stored at 4 °C refrigeration temperature for further analysis.

Screening for Antifungal Activity using Formulated Media and PDA

The antifungal activities were determined using agar well diffusion technique. Nine Petri dishes of the formulated Sweet Potato peel Dextrose agar (SPDA) and Watermelon peel Dextrose Agar (WDA) in triplicates were evenly seeded with the nine test organisms and were allowed to stand for 1 h. Thereafter, a 9 mm sterile cork borer was used to create 4 wells on each of the inoculated plates. Two milliliter(2mL) of each crude extract (Scent leaf ethanol extract and Ginger ethanol extract), 0.01 % nystatin (positive control), sterile distilled water (negative control) and ethanol (negative control) were aseptically dispensed into each of the respective wells with their labels on them and left for 30 min to allow proper diffusion of the antimicrobial agents. The plates were then incubated at laboratory temperature of 25 °C for 72 h and the zone of inhibition was measured in mm (Akharaiyi and Abiola, 2016).

Analysis of Biodata

The results were expressed as mean \pm standard deviation (SD) and were subjected to one factor analysis of variance (ANOVA) with Dunnett comparative test using GraphPad Prism software version 7.00 to determine their significant levels at 95 % confidence interval. Values were considered statistically significant if p< 0.05.

RESULTS

The proximate analysis of the agro-waste materials revealed that Sweet Potato Peel and Watermelon Peel samples had the same carbohydrate content of 26.00mg/100g. However, Sweet Potato Peel had higher protein content (9.16mg/100g) and ash content (4.70mg/100g), while Watermelon Peel had higher moisture content

(6.31mg/100g), and crude fibre content (6.31mg/100g) (Table 1).

Nine Fungi species were isolated from this study. They were identified as Aspergillus flavus, Fusarium sp, Aspergillus terreus, Aspergillus niger, Aspergillus fumigatus, Candida albicans, Cladosporium sp, Geotrichum candidum and Rhizopus stolonifer. Aspergillus genera were the major fungal isolates characterized and described (Table 2)

The result of the Mean Radial ColonyGrowth (MRCG) of the test fungi on the formulated media and PDA is shown in Fig.1. Amongst nine the tested, Rhizopus stolonifer had the highest radial colony growth of 32 mm on PDA, followed by Aspergillus Fumigatus with radial colony growth of 20mm on PDA, while the lowest value of 9mm was shown by Fusarium sp on WDA, Aspergillus terreus on SPDA, and Candida albicans on SPDA. In general, the MRCG on the control medium (PDA) was higher in five of the test fungal isolates, but Aspergillus terreus, Aspergillus niger and Candida albicans gave higher MRCG values of 16mm,18mm and 11mm on WDA formulated medium respectively. Moreover, Geotrichum candidum gave the same MRCG value of 16mm on both the PDA (Control medium), and WDA (Formulated medium). Similarly, Fig. 2 illustrated the Growth Rates (GR) of the test fungi on the formulated media and PDA. The result showed that Rhizopus stolonifer hadthe highest growth rate value of 0.444 h⁻¹ on PDA medium, while Fusarium sp., Aspergillus terreus, and Aspergillus fumigatus gave the least growth rate of 0.125h⁻¹ on WDA, SPDA, and SPDA media respectively. Comparatively, PDA had higher MRCG and GR than WDA followed by SPDA in most of the test fungal isolates. Statistically, the MRCG and GR between PDA and the formulated media were significant at p=0.0020. The MRCG and GR of the fungal isolates were higher on WDA than SPDA with significant p values of 0.0104 and 0.0105 respectively.

Figs. 3,4 and 5 illustrated the susceptibility patterns of the test fungal isolates to Scent leaf ethanol extract, Ginger ethanol extract, positive control antifungal drug (Nystatin) respectively. The Scent leaf ethanol extract inhibited the growth of all the test fungal isolates on the formulated media (SPDA and WDA) with inhibitory zone diameters ranging from 13mm-41mm except Candida albicans that was resistant to the extract on WDA medium. Three of the isolates (Aspergillus fungal Cladosporium sp. and Rhizopus stolonifer) cultivated on PDA were resistant to the Scent leaf ethanol extract, while the other five isolates were susceptible to the extract with inhibitory zone diameters ranging from 10mm-15mm. The comparison between the formulated media(SPDA and WDA), and PDA for susceptibility testing of the fungal isolates using Scent leaf ethanol extract was statistically significant at p =0.0028, the formulated media gave better results for the susceptibility test than the PDA (Fig. 3).

The Ginger ethanol extract inhibited all the test fungal isolates on SPDA and WDA with an inhibitory zone diameter range of 12mm -34mm except*Candida albicans*that was resistant to the extract on WDA. Six of the fungal isolates were resistant to the Ginger ethanol extract on PDA, while, the three namely Candida others albicans. Cladosporium sp. and Rhizopus stolonifera were susceptible to the extract on PDA with inhibitory zone diameters of 12mm, 12mm, and 16mm respectively. The formulated media performed better than the PDA with a statistical significant p value of 0.0007 (Fig. 4)

The known antifungal drug (Nystatin) inhibited all the test fungal isolates on the formulated media and PDA except *Candida albicans* that was resistant to the antifungal drug on WDA. The formulated media was better than the PDA for the antifungal susceptibility testing with a significant p value of 0.0114(Fig. 5). Generally, there was no statistical significance at p> 0.05 in the inhibition zones diameters of the extracts and Nystatin on the formulated media and PDA.

DISCUSSION

Management of waste especially agro-waste has been a major problem experienced in developed and developing Countries. There has been a growing interest regarding the conversion of organic waste generated by the food processing sector, household domestic activities and other human endeavours into useful forms. In addition, the expensive nature of the conventional media used in the laboratories for microbial isolation and cultivation has prompted the need to embark on a search for alternative cheap sources of culture media. In this study, the agro-waste formulated media (SPDA and WDA) supported the growth of the test fungal isolates at varying degrees and this agrees with the findings of Akharaiyi and Abiola (2016), Umedum and Enejekwute (2017), Anbu etal., (2017) and Okove etal., (2020) who reported the use of agro-waste materials as alternative culture media for the cultivation of fungi.

The growth of the fungal isolates on the agro-waste formulated media implies that the media formulation contained essential nutrients for microbial growth. The proximate analysis of the agro-wastes revealed the presence of nutrients like carbohydrate, protein, crude fibre and ash contents. This observation is in line with other previous works on the nutritional composition of some agro-waste materials (Itelima etal., 2014; Uwa etal., 2018). Protein constitutes a significant portion of microbial cells and thus is necessary for the growth of microorganisms (Prescot and Harley, 2002). Moreover, Nitrogen and carbon are key requirements for fungal growth and these nutrients may have been sourced from the proteinand carbohydrate contents of the agro-wastes.

The result of the Mean Radial Colony Growth (MRCG) and the Growth Rate (GR) of the fungal isolates revealed that Watermelon peel Dextrose Agar (WDA) supported the growth of the fungal isolates more than the Sweet Potato peel Dextrose Agar (SPDA).

This was quite different from the findings of Itelima *etal.*, (2014) and Anbu *etal.*, (2017) who reported that Sweet Potato peel Dextrose Agar was the best media for growing their fungal isolates. The higher moisture and crude fibre contents of WDA may have contributed to the high MRCG and GR of the isolates since Growth rate is dependent on the capacity of medium and culturing conditions that will support the microbial growth. Furthermore, there was higher MRCG and GR on the control medium (PDA)than the formulated media. This was also at variance with the research studies of Adesemove and Adedire (2005), Gabriel-Ajobiewe et al., (2012) and Anbu et al., (2017) which reported higher growth rates of fungal isolates on their formulated media than the conventional control media (SDA or PDA).

The susceptibility testing of the fungal isolates on formulated media and PDA using Scent leaf ethanol extract and Ginger ethanol extract showed that all the fungal isolates were susceptible to the extracts on the formulated media except Candida albicans which was resistant to the extracts on WDA medium. The extracts showed laudable inhibitory activities against the fungal isolates and the antifungal activities could be attributed to the presence of phytochemicals such as flavonoid, saponins, tannins et cetera seen in Scent leaf (Ocimum gratissimum) and Ginger (Zingiber officinale)as previously reported by Uba et al., (2016). The observation of no statistical significance (p> 0.05) on the inhibition zone diameters of the extracts and the nystatin against the tested fungal isolates implies that the extracts have potent antifungal activities comparable to the known antifungal drug

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(Nystatin). Hence, they could be sources of antifungal remedies especially in these days multidrug resistance with antimicrobials. It is also credible to note that our formulated media were better than PDA in terms of susceptibility testing suggesting the possibilities of our formulated media (SPDA and WDA) being used as ideal reference testing media for antimicrobial testing and other mycological assays. These unique features of our formulated media are in line with the valid criteria established by Akharaiyi and Abiola (2016) which stated that an ideal medium for reference testing should be totally defined, reproducible, free of antagonists or boosters of antimicrobial action, well buffered to maintain pH and available in both liquid and solid formulations.

CONCLUSION

This study revealed that the agro-wastes (Sweet potato peels and Watermelon peels) contained nutrients that can meet the nutritional conditions for fungi cultivation. Our formulated media supported the growth of the fungal isolates cultivated on them. Theyperformed better than PDA with regards to fungal susceptibility testing and thus can be utilized as an alternative, cheap and ideal media for antifungal screening and other mycological assays. Furthermore, the ethanol extracts of Ocimum gratissimum (scent leaf) and Zingiber officinale (ginger) exhibited laudable antifungal activities against the isolates and were comparable to the antifungal activities of the known Nystatin drug. Hence, the extracts could serve as possible cheap sources of antifungal remedies in these days of multiple resistance with known conventional antimicrobials.

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Table 1. Nutritional composition of the agro-waste materials

Waste	Carbohydrate content (mg/ 100g)	Crude protein (mg/ 100g)	Crude fibre (mg/ 100g)	Moisture content (%)	Ash content (%)
Sweet potato peel	26.00	9.16	0.50	4.58	4.70
Watermelon peel	26.00	6.79	1.50	6.31	1.00

Legend: mg = Milligram; g = Gram; % = Percent

Macroscopic appearance	Microscopic appearance	Identity of Isolate	
Colonies are yellow green on the surface, pale or yellowish on the reverse, downy to powdery texture with a rapid growth.	Conidial heads mostly radiate, with conidial masses splitting into blocky columns at maturity. Conidiophores with roughened walls, especially near the vesicle; Philades uniseriate and biseriate. Some strains produce	Aspergillus flavus	
	brownish sclerotia.	Fusarium sp.	
Colonies growing slowly; purface usually orange to deep pricot due to confluent conidial slime; aerial mycelium cometimes floccose and whitish.	branched, with a scarcely discernible collarette at the apex.	Aspergillus terreus	

Т	hla	2	Continue

Colonies are typically pseudolike and cinnamon-buff to sand-brown in colour with a yellow to deep dirty brown reverse.

Colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads.

conidiophores hyaline, smooth walled; phialides biseriate, limited mainly to the upper part of the vesicle surface.

Conidia heads radiate, splitting into loosely structured columns in age; conidia brown, rough, rough walled, in chains; conidiophores with smooth walls; phialides biserate, covering the entire surface of the vesicle.

Aspergillus niger

Aspergillus fumigatus

Candida albicans

Colonies are typically bluegreen with a suede-like surface, rapid growth and downy to powdery texture.

Colonies are white to creamcoloured smooth, glabrous, yeast-like.

Colonies are slow growing to rapid, mostly olive brown to blackish-brown on the surface and reverse with a velvety texture.

Colonies are fast growing, flat, white to cream, dry and finely suede-like with no reverse pigment.

Colonies are very fast growing, about 5-8 mm high, white cottony at first becoming brownish grey to blackish-grey on the surface and pale on the reverse.

Conidial head in the form of compact columns; conidiophores smooth- walled, often tinted greenish; phialides uniserate, concentrated on the upper surface of the vesicle; conidia round, with finely roughened walls, in chains.

Spherical to subspherical budding blastoconidia, 2-7 x 3-8 µm in size

Hyphae septate, brown; conidiophores brown, often septate; blastoconidia brown, in a very fragile branching chains, bicellular and shield shaped at the base of the chains, unicellular and ellipsodial to round at the tip, prominent black scars are visible at the point of attachment.

Hyphae septate, hyaline;

conidiophores absent; arthroconidia rectangular, not alternating, liberated by the fission of double walls; blastoconidia absent.

Hyphae broad, not or scarcely septate, rhizoids and stolons present; sporangiospores brown, solitary or in tufts on the stolons, diverging from the point at which the rhizoids form; sporangia rather round; sporangiospores ovoid.

Cladosporium sp.

Geotrichum candidum

Rhizopus stolonifers

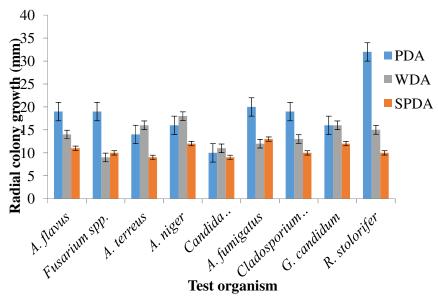


Fig. 1. Mean radial colony growth of the fungal test organism on the PDA and formulated media.

Legend:PDA = Potato dextrose agar; SPDA = Sweet potato peel dextrose agar; WDA = Watermelon peel dextrose agar; mm = Millilitre; sp = Species; Error bar = Standard error in mean

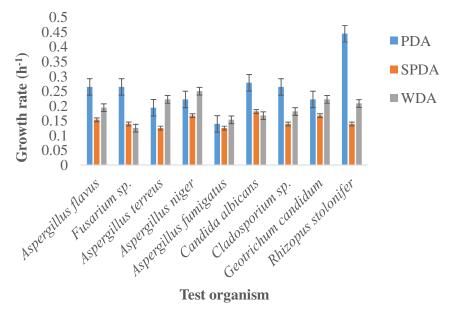


Fig. 2. Growth rate of the fungal test organism on PDA and formulated media. Legend: PDA=Potato dextrose agar; SPDA= Sweet potato peel dextrose agar; WDA =Watermelon peel Dextrose agar; h⁻¹ = Per hour; sp = Species; Error bar = Standard error in mean.

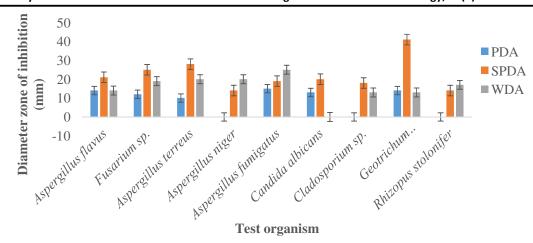


Fig. 3. Sensitivity pattern of the fungal test organisms exposed to Scent leaf ethanol extract. Legend:PDA = Potato dextrose agar; SPDA = Sweet potato peel dextrose agar; WDA = Watermelon peel Dextrose agar; sp = Species; mm = Millilitre; Error bar = Standard error in mean.

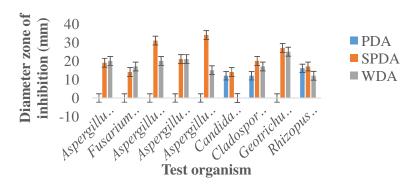


Fig.4. Sensitivity pattern of the fungal test organisms exposed to Ginger ethanol extract. Legend:PDA = Potato dextrose agar; SPDA = Sweet potato peel dextrose agar; WDA = Watermelon peel Dextrose agar; sp = Species; mm = Millilitre; Error bar = Standard error in mean.

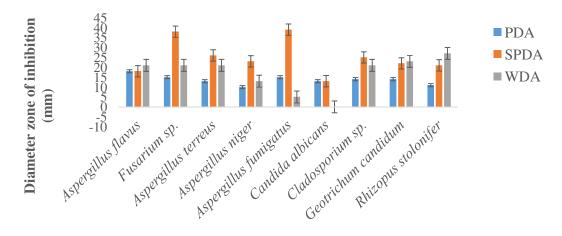


Fig.5. Sensitivity patterns of the fungal test organism exposed to nystatin antifungal drug. Legend:PDA = Potato dextrose agar; SPDA = Sweet potato peel dextrose agar; WDA = Watermelon peel Dextrose agar; sp = Species; mm = Millilitre; Error bar = Standard error in mean.

Test organism