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Antifungal effects of two botanical extracts and honey on fungi associated with sunflower (*Helianthus annuus* L.) seeds

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Abstract
The antifungal efficacy of *Phyllanthus amarus* and *Trianthema portulacastrum* ethanolic leaf extracts, and honey were investigated *in-vitro* against fungi of sunflower; *Helianthus annuus* L. seeds. The study tested four varieties of sunflower seeds namely SAMSUN 1, SAMSUN 2, SAMSUN 3 and SAMSUN 4. The culture and isolation of the seed-borne fungi were carried out using the Agar Plate method while the inhibitory assessment was done by using the agar well diffusion method. Fungi identification was done through observation of colony appearance and microscopic evaluation. Ethanol extract of the leaf sample was obtained, filtered, and evaporated at 65 °C under a reduced pressure. The result showed that *Aspergillus*, *Penicillium* and *Fusarium* genera were isolated from the seeds. The honey treatment produced the highest inhibition response of 9.3% (4.2mm) and 8.0% (3.6 mm) against *Penicillium* spp. and *Aspergillus niger*, respectively. This was followed by the honey combination with ethanolic leaf extract of *T. portulacastrum* and the combination with ethanolic leaf extract of *T. portulacastrum* which both inhibited *Penicillium* spp. at 7.1% (3.2 mm). The extract of *P. amarus* recorded the least inhibition of the radial growth at 3.8% (1.7 mm), 3.3% (1.5 mm) and 2.6% (1.2 mm) against *A. niger*, *F. oxysporum* and *A. flavus* respectively, five days after inoculation (DAI). From this study, honey only or when combined with *T. portulacastrum* ethanolic leaf extract could serve as promising agents for producing seed dressing botanical fungicides.

Keywords: *Aspergillus niger*, *Penicillium* spp., *Phyllanthus amarus*, *Trianthema portulacastrum*, honey, sunflower seeds

1. Introduction
Sunflower (*Helianthus annuus* L.) is commonly grown as a crop for its edible oily seeds which its propagation plays essential secondary roles in ecology and the lifecycle of organisms. The increased occurrences of seed-borne diseases such as sunflower verticillium wilt, sunflower fusarium wilt and a host of other phytopathogenic diseases were often experienced by most farmers as well as seed-producing companies, have increasingly affected sunflower production in major farming regions [1, 2]. Thus, there is a need to efficiently manage seed-borne fungi in sunflower production.

Seed-borne fungi are one of the major infectious agents that affect crops when they release various potent toxic chemicals such as aflatoxins, fumonisin, patulin, deoxynivalenol, etc. on stored food products or during harvest which cause changes at the various stages of plant growth and development, resulting in major postharvest losses of various seeds and grains [3, 4, 5]. Also, [3, 4] reported 48 species of fungi belonging to 19 genera associated with sunflower seeds. *A. niger*, *A. flavus*, *A. fumigatus*, *Penicillium expansum*, *P. brevicompactum*, *F. oxysporum*, *F. solani*, *Rhizopus* were the most frequent species. Earlier in a study reported by [6], among the dominant phytopathogenic fungi species identified were *Alternaria helianthi*, *Rhizoctonia bataticola*, *A. alternata*, *A. flavus*, *A. niger*, *Penicillium* spp., *Rhizopus stolonifer* and *Cladosporium* spp.

Over the long time, synthetic fungicides have been used to manage the undesirable effects of phytopathogenic fungi. However, their applications were limited by various factors such as the high cost of most available chemicals, development of resistance, excessive use and improper handling.

Synthetic fungicides were also found to contaminate farm produce and make food unsafe for human consumption, reduce market value with adverse effects being linked to chronic human illnesses when injected in or human exposure incident, ozone layer depletion leading to detrimental impacts on the environment, lives or non-target organisms which has a negative influence on biodiversity [7, 8]. Thus, plant extracts were found to have a beneficial combination of compounds that may suppress the growth of a variety of phytopathogenic fungi without negative side effects. Importantly, the modes of action of these antifungal substances may reduce the emergence of resistance to the fungicides and may prevent the emergence of antimicrobial chemical resistance [9, 10].

The increased demand for natural products with no chemical residues, and eco-friendly and sustainable alternatives to synthetic fungicides has made botanicals to gradually been explored and gained acceptability [11]. The objectives of this study are to identify mycobiota associated with sunflower seeds, determine the antifungal effects of *Phyllanthus amarus*, *Trianthema portulacastrum* and honey on the fungi associated with seeds of sunflower and also determine the combined antifungal effect of each botanical extract with honey on the fungi associated with sunflower seeds.

2. Materials and Methods

2.1 Source of sample

The seeds of sunflower (Plate I) used in this study were of four different varieties *viz.* SAMUN 1, SAMSUN 2, SAMSUN 3 and SAMSUN 4. These new hybrids were developed through a combined effort of researchers in the Institute of Agricultural Research and Training (IAR&T), Ahmadu Bello University, Zaria and Nigeria Seed Initiative to produce sunflower hybrids and new genotypes adapted to the climate of different regions with Nigeria [12].

2.2 Collection of seeds

They were collected from a seed farmer in Zaria, Nigeria. The Sabouraud Dextrose Agar (SDA) and other reagents used were of analytical grade and were purchased from a commercial laboratories store in Suleja, Niger State. The natural honey (Plate II) of about 0.1L was obtained from a beekeeper shop in Gwagwalada area of FCT and was maintained aseptically in a well-capped plastic container. Fresh leaves of *Phyllanthus amarus* (Schum. & Thonn.) and *Trianthema portulacastrum* (Plates III and IV) were plucked near a farm at Sheda village, in Kwali Area Council, FCT, Abuja and were taken aseptically in polythene bags to the Sheda Science and Technology Complex (SHESTCO), Kwali Area Council, Abuja, FCT for ethanolic extraction. All solvents used in this work were of standard grades.

Plate 1: Sunflower seeds

Plate 2: Sample of pure honey

Plate 3: Leaf of *P. amarus* plant

Plate 4: Leaf of *T. portulacastrum*

2.3 Preparation of media

A measure of 39 g of Sabouraud Dextrose Agar (SDA) powder was dissolved in 1000 ml of distilled water. 0.5 g of streptomycin sulfate was added as an antibiotic against potential bacterial contamination of the media. The solution was autoclaved at 121 °C and 15 psi and then allowed to cool to a temperature of about 45 °C. The flask was then removed while the media were poured aseptically into the sterile 90mm disposable Petri dishes in a surface-sterilized inoculating chamber. The media were allowed to solidify

after about 20 minutes and then maintained upside down to prevent condensation water from collecting on the surface of the agar, at 4 °C until use.

2.4 Isolation of fungal pathogens

The agar plate method was used in isolating potential fungi from the seeds as displayed in plates V-VIII. Specifically, 120 seeds were taken at random from the available seed samples. The seeds were soaked in hypochlorite solution for about half an hour to break dormancy. They were washed with distilled water, air dried, and were then transferred aseptically to the plates containing solidified SDA medium. Four samples with three replications were placed at ten (10) seeds per plate, at equidistance. The plates were placed in cellophane untied and incubated at 25 °C for seven (7) days. The mycoflora observed around the seeds were isolated and recorded.

2.5 Identification of fungi

Identification was done through the aid of compound microscope observations of conidia growth. According to the standard described by Barnett and Barry (1999) and reported by [13] in determining the appearances of the colony such as a change in media color, conidia shape, conidiophores and growth rate as follows: *Aspergillus niger*- dark grey, *Fusarium oxysporum*- pinkish hairy, *Penicillium* spp.-bluish and isolate of *Aspergillus flavus* were identified. These four well-characterized *in-vitro* isolates were further confirmed using a compound microscope. The culture media *Aspergillus* spp. were examined in the lacto phenol blue (LPB) under the microscope for hyphae and sporing head.

2.6 Preparation of *P. amarus and T. portulacastrum* ethanolic extracts

The leaves of *P. amarus* and *T. portulacastrum* collected were taken to the Chemistry Advanced Laboratory, Sheda Science and Technology Complex, Abuja (SHESTCO) Kwali, FCT, for qualitative and quantitative analysis of both plant materials. They were washed separately under running water to remove the dust and dirt, then air dried aseptically and pulverized separately to obtain individual fine powdered form. According to the methods of Karuna as reported by [14], 10g each of the powdered samples of *P. amarus* and *T. portulacastrum* were extracted separately with 100 ml of ethanol using Soxhlet apparatus for 3 hours. Ethanol extract obtained for each leaf sample was filtered and evaporated in a rotatory evaporator at 65 °C respectively under reduced pressure. The prepared extracts were stored in sterile airtight bottles to prevent cross-contamination. Each bottle was labeled accordingly to include concentration and dates of preparation.

2.7 Evaluating the antifungal effects of botanical extracts and honey

The antifungal activity of *P. amarus* and *T. portulacastrum* extracts, honey and a combination of individual extracts with honey were evaluated against the isolated fungi using the agar well diffusion method. The plots was replicated thrice and a Completely Randomized Design was used. After inoculation, a sterile cork borer was used to make a small uniform hole referred to as a "well" of about 6 mm diameter on the surface of the agar. 0.5 ml of DMSO (5%v/v of 100 ml) was added to *P. amarus* extract (PHA) to produce 5 ml concentration aqueous solution of the botanical extract. The same concentration of *T. portulacastrum* extract (HSP) and honey (HNY) were obtained. A sterile micropipette was used to deposit 0.25 ml of *P. amarus* (PHA) extract, *T. portulacastrum* (HSP) extract, pure Honey (HNY), a mixtures of *P. amarus* + Honey (PA+H) and *T. portulacastrum* + Honey (HP+H) on the respective wells while the standard control check containing 0.25 ml of DMSO only as a negative control negative control (CON) were maintained. All the samples were then incubated at 25 °C for 3-4 days to test for efficacy and compare the treatments. After inoculation, the antifungal activities of the botanical extract were evaluated by noting the zone of inhibition around the wells, against the seed-borne fungi.

2.8 Zone of inhibition

The visibility of inhibition was observed on the surface of the media and through the bases of the plates when turned upside down. The growth-inhibited portion around the treatment "No growth" on the inoculated SDA media (cm in diameter) was measured with the aid of thread and then placed on a simple ruler to take the readings. The percentage inhibition is determined as the radius recorded in the treated plate divided by the colony radius in the control plate multiplied by 100. This is as given by the formula.

$$\text{i.e. \% RGI} = \frac{R2}{R1} x100$$

Where
R_1 = colony radius in control plate,
R_2 = colony radius observed and recorded

2.9 Data collection

The four samples were replicated three times making 12 plates in all. The six (6) treatments were represented as *P. amarus* (PHA), *T. portulacastrum* (HSP), Honey (HNY), *P. amaru* + Honey (PA+H), *T. portulacastrum* + Honey **(**HP+H) and negative Control (CON), for each of the four (4) fungal isolates namely *Aspergillus niger*, *Penicillium* spp., *Fusarium oxysporum* and *Aspergillus flavus*. They were replicated three (3) times to give a total of seventy-two (72) plates. The total number of fungi species isolated from the sample and the percentage occurrence of each species were obtained

2.10 Statistical Analysis

The means of the three replicates were computed using MS Excel software package. Data collected were analyzed using a one-way-Analysis of Variance (ANOVA). The means between the treatments were determined using Least Significant Differences LSD at a probability level of 0.05 and separated using Duncan's Multiple Range Test DMRT.

3. Results

3.1 Occurrence of fungi species on sunflower seeds

Seed-borne fungi associated with sunflower seeds of the 12 samples examined were found to contain fungi species from the three genera: *Aspergillus*, *Penicillium* and *Fusarium*, specifically identified as *Aspergillus flavus*, *Penicillium* spp., *Fusarium oxysporum* and *Aspergillus niger* as shown in Plates V-VIII

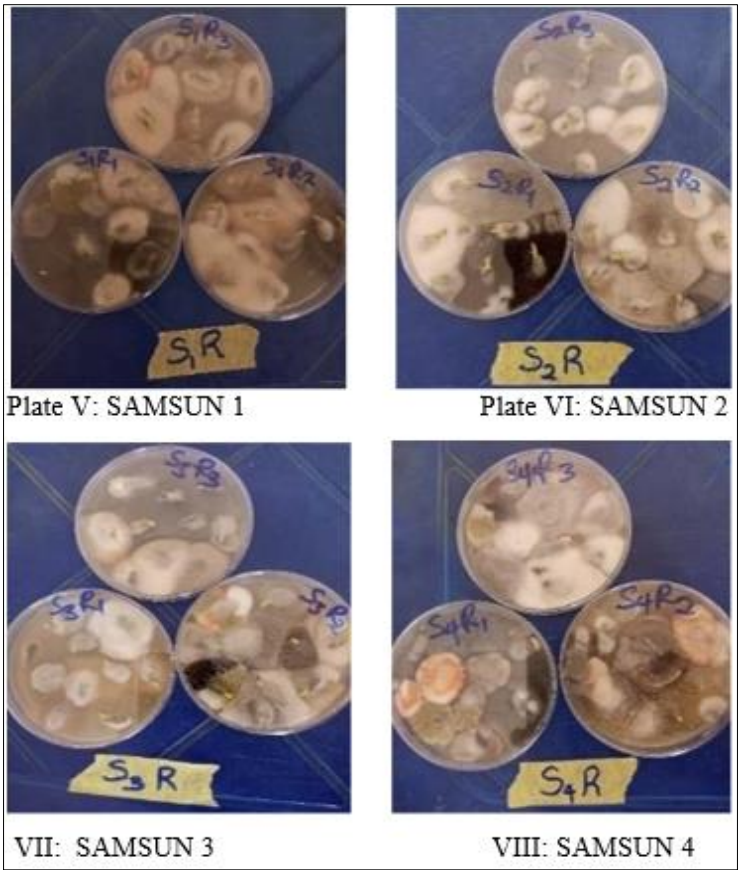

Plate 5-8: Isolation of phytogenic fungi from four varieties of sunflower seeds in agar media

The result as displayed in Table 1, *F. oxysporum* was the most occurring, having 68 present at 56.2% and occurring across the four varieties of sunflower seeds tested, followed by *Aspergillus niger* with 39 occurrences in almost all samples tested obtained at 32.2%. The fungi species with the least frequency of occurrence is *Penicillium* spp with 5 occurrences at 4.1% and present in only three out of the twelve samples which is next to the *Aspergillus flavus* occurring at 7.4% with 9 occurrences and present in six samples examined in the experiment. The result showed that there was a significant difference ($p \leq 0.05$) between fungi occurrence of *F. oxysporum* and the other three species; *A. niger*, *A. flavus* and *Penicillium* spp. Similarly, there was *a* significant difference between *A. niger* and *A. flavus*. However, no significant difference between *Penicillium* spp and *A. flavus* at 5 Day after Inoculation.

3.2 Radial growth inhibition of fungi isolates of sunflower seeds treated with botanical extracts and honey: In table 2, the result of the effects of different ethanolic botanical extracts, honey and negative control (CON) on radial growth of four fungi species of sunflower seeds, namely *F. oxysporum*, *A. flavus*, *P.* spp. and *A. niger* shows variations in inhibition in diameter of the radial growths of the fungi isolates against various treatments 5 days after inoculation (DAI). *Penicillium* spp. was most inhibited against honey at 4.2mm followed by *A. niger* at 3.6mm, then, the radial growth inhibition of *F. oxysporum* and *A. flavus* radial growth were the least inhibited fungi species at 1.5 mm and 1.2 mm respectively, 5 day after inoculation. The highest percentage radial growth inhibition of 9.3% is obtained from honey against *Penicillium* spp. Similarly honey inhibited radial growth of *A. niger* at 8.3%. Also, radial growth of *A. flavus* was inhibited at 6.9% by honey only and honey combination with *T. portulacastrum* extract respectively. There is no significant difference between the percentage inhibition of radial growth of *F. oxysporum* inhibited at 6.3% by both extracts of *T. portulacastrum* combination with honey and *P. amarus* extract combination with honey. However, the extract of *P. amarus* and *T. portulacastrum* has the least percentage inhibitory effect on the radial growth of *F. oxysporum* at 5 days after inoculation.

From the control treatment (CON), there was no inhibition of the radial growth of fungi isolates and it was significantly different ($p \leq 0.05$) from other treatments. In contrast, honey (HNY) had the highest radial growth inhibitory effect obtained at 4.2 mm, 3.6 mm and 3.1 mm against *Penicillium* spp., *A. niger* and *A. flavus* respectively. There was a significant difference in radial growth inhibition between the effects of each of the extracts of *P. amarus* (PHA), combined *P. amarus* + honey (PA+H), *T. portulacastrum* (HSP) and honey (HNY) treatments 1.7 mm, 2.0 mm 3.1 mm and 3.6 mm respectively against the fungus *A. niger*. There was no significant difference ($p \geq 0.05$) in the inhibitory effect of PA+H and HP+H recorded as 2.0mm and 2.5 mm as well as between honey and HSP against *A. niger* in the experiment. Also, there is no significant difference in the inhibitory effect of PAH, HSP and HP+H treated against *Penicillium* spp. obtained as 3.1 mm, 3.2 mm and 3.1 mm respectively. Similarly, the combined effect of HP+H, PA+H and HSP all obtained at approximately 2.9 mm, show no significant difference in radial growth inhibition against *F. oxysporum* isolates, but there is a significant difference in the inhibitory effect of HNY and PHA recorded as 3 mm and 1.5 mm respectively at 5 Days After Inoculation (DAI).

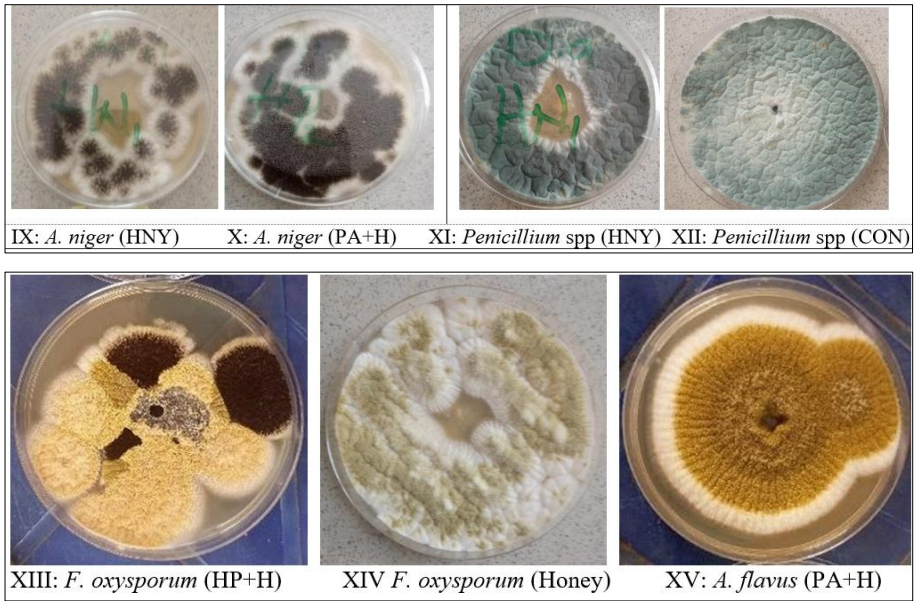

Plate 9-15: Radial growth inhibition of four fungi species against botanical extracts and honey at 5 days after inoculation

4. Discussion

The results of this study indicated the prevalence of *Aspergillus niger*, *Penicillium* spp., *Fusarium oxysporum* and *A. flavus* in sunflower seed *Helianthus annuus* L. from Abuja, Nigeria, The findings agreed with [15, 16, 17, 18]. The isolated fungi when cultured, their radial growth was inhibited by ethanolic leaf extracts of *P. amarus* and in combination with ethanolic leaf extract of *T. portulacastrum.* The *in-vitro* inhibitory effect of ethanolic leaf extracts of *these plants* on the fungi might be due to the presence of phytochemicals such as flavonoids tannins, alkaloids (Phyllantine) lignans, and tannins. This result aligns with the report of [19, 20, 21, 22]. Similarly, [23, 24] reported the antifungal efficacy of common bioactive compounds in botanical fungicides as may suppress the growth of phytopathogenic fungi with their actions being poisonous to the cell membranes, organelles, and walls of fungi preventing the germination of spores, the growth of mycelia and hypha. They further inhibit the production of critical enzymes, DNA, proteins and as well prevent fungi *Aspergillus* spp. and *Fusarium* spp. from producing toxic compounds such as aflatoxin and fumonisin, respectively, without negative side effects [14], Honey only, in combination with ethanolic leaf extracts of *P. amarus* and combination with ethanolic leaf extract of *T. portulacastrum* was found to be most effective inhibitor of the radial growth of the isolated phytopathogenic fungi of sunflower seeds. This result also further reaffirms the report by [25], that *in vitro* and *in-vivo* tests of the application of honey only at high concentrations resulted in inhibition in the growth of many fungi species. This is possibly due to the components and a few numbers of enzymes such as diastase, invertase, glucose oxidase, catalase, and phosphatase, prominently active in a honey. Thus, using honey in combination with many other plant products could be a panacea for treating phytogenic fungal diseases.

Table 1: Occurrence and percentage of occurrence of isolates of sunflower seeds

Fungi spp.	Number of Occurrence	Percentage (%)
Fusarium oxysporum	68c	56.2
Aspergillus flavus	9a	7.4
Penicillium spp.	5a	4.1
Aspergillus niger	39b	32.2
Total	121	100

Means followed by the same letter(s) are statistically not significant at 0.05 level of probability using N-DMRT

Table 2: The radial growth inhibition of fungi of sunflower seeds against ethanolic extracts and honey at 5 DAI

Treatment	*A. niger*	*Penicillium* spp.	*F. oxysporum*	*A. flavus*
	Inhibition (mm) (%)			
Control	0.00^{a} (0)	0.00^{a} (0)	0.00^{a} (0)	0.00^{a}(0)
Phyllanthus amarus	1.74^{b} (3.8)	3.06^{d} (6.8)	1.52^{b} (3.3)	1.20^{b}(2.6)
Phyllanthus amarus + Honey	2.00^{c} (4.4)	2.72^{c} (6.0)	2.86^{cd} (6.3)	1.80^{b}(4.0)
T. portulacastrum	3.06^{d} (6.8)	3.20^{d} (7.1)	2.94^{cd} (6.5)	1.20^{b}(2.6)
T. portulacastrum + Honey	2.54^{c} (5.6)	3.20^{d} (7.1)	2.86^{cd} (6.3)	3.14^{d}(6.9)
Honey	3.66^{d} (8.0)	4.20^{e} (9.3)	3.00^{d} (6.6)	3.14^{d}(6.9)

Means followed by the same letter(s) are statistically not significant at 0.05 level of probability using N-DMRT

5. Conclusion

The potential of honey and ethanolic leaf extract of *T. portulacastrum* leaves could be explored for proactive management of seed-borne fungi *Penicillium* spp, *Aspergillus niger*, *Aspergillus flavus* and *Fusarium oxysporum* in sunflower production. However, the efficacy of these potential fungicides should be investigated on the field.

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7. Declaration of any conflict of interest

No conflict of interest.

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