Fungicidal Potentials of Leaf and Bark Extracts of *Bryophyllum Pinnatum* on Cowpea (*Vigna Unguiculata* L.) Seed Fungi.

ABSTRACT

In Nigeria, Cowpea (*Vigna unguiculata* [L.] Walp) is an important grain legume which besides being a food crop serves as a major source of income for rural households. The action of pathogens on stored seeds causes huge post-harvest and economic losses to cowpea farmers in Nigeria. This research was aimed at isolating and identifying the spoilage pathogens of cowpea seed and testing the potentiality of ethanol extracts of *Bryophyllum pinnatum* on the isolates. Spoilt cowpea seed samples were purchased from different markets in Anambra State. They were transported to Alpha Research Laboratory, Awka in sterile polythene bags for microbial isolation. The spoilage organisms were isolated from the seeds through microbial culture. The media used for the isolation were Sabouraud Dextrose Agar and Potato Dextrose Agar. Pathogenic fungi were isolated from the spoilt cowpea seeds. The findings indicated that *Aspergillus aculeatus, Fusarium solani, Penicillium citrinum, Rhizopus stolonifer,* and *Aspergillus flavus* were present. The pathogenicity test also showed that the above organisms were responsible for cowpea spoilage. These isolates were tested for their *in-vitro* antifungal activity against the leaf and bark extracts of *Bryophyllum Pinnatum*. The antifungal activity ethanol extracts of *Bryophyllum pinnatum* on the isolated microorganisms were investigated using the disc method. Three different concentrations (25%, 50%, 100%) of extracts were used for the test. *Fusarium solani* showed the highest percentage of inhibition with antifungal medication while *Penicillium citrinum* showed the least percentage of inhibition with antifungal medication. The antifungal potential of *Bryophyllum pinnatum* extract on spoilage pathogens of stored cocoyam corms encourages more research on the active ingredient of the plant for easy use by farmers as alternative to commercial/ synthetic fungicides.

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INTRODUCTION

Cowpea (*Vigna unguiculata* [L.] Walp) is a multi-purpose, under-utilized legume crop mostly grown in dry tropical areas. It is one of the ancient, important and most widely consumed grain legumes in the world. All cultivated cowpeas are grouped under the species *Vigna unguiculata*, which is sub-divided into four cultivar groups: *unguiculata*, *biflora*, *sesquipedalis* and *textilis*. They can be distinguished from one another by different physiological factors such as seed size and colour, taste, yield and maturity time [1] [2][3][4].

Cowpea (*Vigna unguiculata*) is a neglected crop native to Africa, with an outstanding potential to contribute to the major challenges in food and nutrition security, as well as in agricultural sustainability [5]. With the rising interest in orphan crops due to their nutritional potential and ability to thrive in arid and semi-arid lands, the cultivation of cowpea is being promoted in many countries, although this crop still has a limited value chain [6] [3] [5] [7].

The mycotoxins, when ingested during the consumption of infected seed and other food stuffs, can lead to serious health complications in animals as well as humans. It is well documented that various legume seeds are prone to fungal infestation and subsequent mycotoxin contamination [8][9][10]. Over the years, there is a need to identify and isolate the microorganisms associated with spoilage as a way of finding means of controlling it. The plant world is a rich storehouse of natural chemicals that could be exploited for use as biopesticides [11].

Bryophyllum pinnatum (Family: *Crassulaceae*) is an erect, succulent, perennial shrub that grows about 1.5m tall and reproduces through seeds and also vegetatively from leaf bulbils [12]. It is native to Madagascar and Southern Africa and grows mainly in the tropics [13]It is commonly called air plant, life plant, love plant or miracle leaf [14]. Leaves and other parts of *B. pinnatum* have been reported to contain alkaloids, saponins, tannins, flavonoids, anthraquinones, xanthones and bryophyllin A and B [15]. The green callus of the plant contains

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malic acid, quinones and tocopherol [16]. The presence of phenolic compounds in *B. pinnatum* as confirmed by [17] suggests that the plant has antibacterial potential against bacterial pathogens.

The aim of this study is to assess fungicidal potentials of leaf and bark extracts of *Bryophyllum Pinnatum* on Cowpea (*Vigna Unguiculata*) seed fungi.

2. MATERIALS AND METHODS

2.1 Samples Collection

Samples of spoilt cowpea seeds were be collected from Anambra State, aculeatusia, from 6 distinct locations (Nkwo-Amaenyi, Eke-Awka, Ifite, Afor-Nkpor, Nkwo-Nnewi, Nkwo-Umunze) in Anambra State. Laboratory and other facilities used in the practical work were obtained from Alpha research laboratory Awka, Anambra state.

2.2 Phytochemical screening

The extracts were subjected to quantitative and qualitative phytochemical screening for their presence or absence of active phytochemical constituents by the following methods according to [18].

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2.3 Fungal Isolation

2.3.1 Sabouraud dextrose agar media preparation

2.3.1.1 PDA media preparation

About 39g of the medium were suspended in one litre of distilled water, heated over a Bunsen flame while being stirred frequently, and allowed to boil for one minute to thoroughly dissolve the medium/contents. The solution was autoclaved for 15 minutes at a temperature of 121°C and one atmosphere of pressure (15 Psi). Allow to cool for ten minutes after withdrawing from the autoclave. To act as antibiotics, 500 mg of streptomycin sulphate was added to the molten solution.

2.3.1.1 SDA media preparation

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About 65g of the medium were suspended and dissolved in 1 litre of distilled water by heating to boiling and stirring frequently. It was heated for one minute to dissolve the solution, and then sterilised for 15 minutes at 121°C in an autoclave. After that, while the solution was still molten, 500mg of the antibiotic streptomycin was added.

2.3.2 Isolation of fungi

One gram of each sample was aseptically collected and serially diluted in normal saline to the fourth dilution using a ten-fold serial dilution. About 0.1ml aliquot of each dilution was inoculated onto a freshly prepared SDA agar and incubated at room temperature (37°C) for three (3) days.

2.3.3 Sub-culturing techniques

Resulting colonies were then sub-cultured onto Sabouraud Dextrose Agar (SDA), process was repeated whenever more than a single colony of fungi was observed in the petri-dishes, until pure cultures were obtained.

2.2.4 Identification of isolated fungi

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All the various species of fungi isolated were identified, both macroscopic and microscopic features, and their various characteristics studied, (i.e) colour, texture, form of hyphae, form of

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conidia, presence of conidiophores, shape of conidial heads [19].The microscopic identification was aided by appropriate taxonomic keys.

2.3.4 Pathogenicity of isolated fungi

Pathogenicity or decay test was carried out in order to know if the isolated fungi were really responsible for the spoilage of cowpea seed. Healthy seeds were surface sterilized with ethanol. Cylindrical plug tissues were cut out from the fruits using a sterilized 2mm sized cork borer. Agar plate containing a week-old fungal culture were aseptically placed in these holes, then covered and sealed off by means of petroleum jelly. The procedure was repeated separately across each of the fungal isolates. The inoculated samples and the control were placed in sterile polythene bags and incubated in an oven for 5days. The point of inoculation of each type of fungus was examined and recorded. The diameter of the rotten portion of the watermelon fruits was measured. The fungi were later re–isolated from the inoculated fruits and compared with the initial isolates.

2.3.5 In vitro antifungal tests with plant extracts

The 100µl adjusted fungi suspension was pipetted using a micropipette and applied on the surface of sabouraud dextrose agar and swabbed at 60º rotation to uniformly distribute yeast throughout the media surface using a cotton swab. The swabbed sabouraud dextrose agar stood for 15 min to provide time for the attachment of fungi on the media. After that, the sterilised 6 mm diameter cork borer was used to perforate the swabbed media to create a 6 mm diameter of wells. The concentration of extracts for the experiment was determined based on a previous study on the plant. The created wells were filled with the 50 µl extracts at 400, 200 and 100 mg/ml, negative, and positive control. The inoculated Petri dishes were placed in the refrigerator at 4°C for 2 h to facilitate diffusion of extracts or fractions in the media. Next to that, Petri dishes were incubated at 37°C for 24 h in the incubator. The inhibition zone diameter

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after 24 h incubation was measured by a ruler in millimetre and recorded. The experiment was done in triplicate.

2.3.6 Determination of minimum inhibitory concentration for pathogenic fungi

The serial double dilution technique was employed for extracts in broth filled wells commenced from the first to tenth wells. The serial double dilution was performed as 100 µl extracts or fractions were added to the first well and thoroughly mixed five times by rinsing using a micropipette and 100 µl of the mixture was transferred to the second well using a new micropipette tip and thoroughly mixed as above. A 100 µl of the second well mixture was pipetted using a new micropipette tip and transferred to the third well and thoroughly mixed as above. The process was continued until the tenth well and 100 µl mixture of the tenth well was pipetted and discarded to have an equal volume of fluid in the wells (EUCAST, 2003). The twofold serially diluted concentrations of extracts for the experiment were determined from a previous study on the plant. The serial double dilution concentrations used in the experiment were 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0.3906 mg/ml. The 100 µl broth-filled 11th and 20th wells were used as growth and sterility control, respectively. The 10 µl diluted yeast suspension (10% of 100 µl broth volume) was pipetted to wells from the eleventh to first wells to reduce contamination on sterility control and the attained final concentration of yeast suspension (2.5×10^4 CFU/ml) in each well, but 10 µl broth was pipetted to the 12th well. The incubated microtitre plate wells were filled with 0.01% resazurin sodium salt indicator from the 12th to the 1st well and incubated for 2 h at 37°C. The MIC of extracts and fractions were determined as blue or purple resazurin colour changed to pink or colourless (Blazic *et al*., 2019; Ohikhena *et al*., 2017). The experiment was done in triplicate.

2.4 Statistical Analysis

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Percentages and means of fungal colonies were calculated. Data obtained was subjected to Analysis of Variance (ANOVA), and Duncan Multiple Range Test (DMRT) was used to separate the treatment means when significant at 5% level of probability.

3. RESULTS

3.1 Comparative proximate composition of fresh and spoilt cowpea seeds

The nutritional composition of fresh and spoilt cowpea seeds is presented (Table 1).

Table 1: Proximate composition of the fresh and spoilt cowpea seed samples

Proximate (%)	Fresh Cowpea Seed	Spoilt Cowpea Seed
Moisture Content	14.60 ±0.053	22.43 ± 0.030
Ash Content	4.36 ± 0.002	4.87 ± 0.020
Protein Content	19.71 ± 0.002	13.57 ± 0.020
Crude fibre Content	4.11 ± 0.100	2.77 ± 0.060
Fats and Oil	3.86 ± 0.050	3.43 ± 0.005
Carbohydrate	53.36 ± 0.000	52.93 ± 0.000

***Values are mean scores ± Standard deviation of three (3) replicates**
***Data in the same column bearing different superscript differ significantly ($p < 0.05$).**

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3.2 Qualitative phytochemical composition

The result of the qualitative phytochemical composition of the leaf and bark ethanolic extracts of *Bryophyllum pinnatum* leaf and bark (Table 2).

Table 2: Qualitative phytochemical composition of the ethanolic extracts of *Bryophyllum pinnatum* leaf and bark.

Parameters	*Bryophyllum pinnatum*	
	LEAF	**BARK**
SAPONIN	+++	++
FLAVONOID	-	+++
ALKALOID	++	+++
TANNIN	+	+++
STEROIDS	-	+
TERPENIODS	++	+
GLYCOSIDES	+	-
ANTHROCYNIN	+++	-
PHENOL	+++	+
OIL AND RESIN	-	-
REDUCING SUGAR	-	-

Key

+++ = Present in high concentration

++= Present in moderate concentration

+ = Slightly or sparingly present

- = Absent

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3.3 Quantitative phytochemical composition

The result of the quantitative phytochemical study on the *B. pinnatum* leaf and bark are presented in table 3.

Table 3: Quantitative phytochemical composition of the ethanolic extracts of *B. pinnatum, M.pudica and H. crepitans* leaf and bark

Parameters	*Bryophyllum pinnatum*	
	LEAF	**BARK**
ALKALOID	8.82 ± 0.09	26.86 ± 0.50
FLAVONOID	1.67 ± 0.00	3.05 ± 0.10
PHENOL	3.05 ± 0.10	24.36 ± 0.02
SAPONIN	34.16 ± 0.05	26.73 ± 0.41
TANNIN	26.73 ± 0.41	13.33 ± 0.25
STEROID	0.13 ± 0.05	5.40 ± 0.02
TERPENOID	3.30 ± 0.04	2.06 ± 0.05
CARDIAC GLYCOSIDE	2.47 ± 0.00	6.11 ± 0.01

***Values are mean scores ± Standard deviation of three (3) replicates**
***Data in the same column bearing different superscript differ significantly ($p < 0.05$)**

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4.4 Fungi Count of Spoilt Cowpea Seeds

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The fungi isolates that were gotten from the spoilt cowpea seeds were *Aspergillus aculeatus*, *Fusarium solani*, *Penicillium citrinum*, *Rhizopus stolonifer*, and *Aspergillus flavus*. The result from the mean total fungi count ($x10^2$cfu/g) of cowpea samples from 6 different locations is presented (Table 4).

Table 4: Total fungal count of cowpea seed sample

Locations	Mean total fungi count ($x10^2$cfu/g)
Nkwo-Amaenyi	126.00 ± 0.27^b
Eke-Awka	114.00 ± 0.20^d
Ifite Awka	129.00 ± 0.05^a
Afor-Nkpor	123.00 ± 0.00^c
Nkwo-Nnewi	76.00 ± 0.10^f
Nkwo-Umunze	112.00 ± 0.05^e
Mean Fungi count	**113.00 ± 0.00**

***Values are mean scores of three (3) replicates**
*** Values in the column with the same letters are not significantly different ($p<0.05$).**

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3.5 Pathogenicity Test of Isolated Fungi

The pathogenicity test showed that all five test fungi (*Aspergillus aculeatus, Fusarium solani, Penicillium citrinum, Rhizopus stolonifer,* and *Aspergillus flavus*) were pathogenic, hence causing rot in healthy cowpea after twenty-one (21) days of inoculation.

Table 5: Result of the Pathogencity Test

Locations	*Aspergillus aculeatus (mm)*	*Rhizopus stolonifer (mm)*	*Penicillium citrinum (mm)*	*Fusarium solani (mm)*	*Aspergillus flavus (mm)*
Nkwo-Amaenyi	9.80 ± 0.01^{e}	10.50± 0.00^{d}	0.00± 0.00	0.00± 0.00	13.00 ± 0.01^{b}
Eke-Awka	42.10 ± 0.01^{bc}	29.00± 0.00^{b}	8.70± 0.00^{c}	0.00± 0.00	0.00± 0.00
Ifite	9.00 ± 0.12^{d}	27.60 ± 0.01^{c}	56.00± 0.00^{a}	50.00± 0.10^{b}	50.00 ± 0.00^{a}
Afor-Nkpor	50.00 ± 0.10^{a}	55.00 ± 0.01^{a}	9.00 ± 0.02^{b}	0.00± 0.00	8.70± 0.00^{c}
Nkwo-Nnewi	48.00± 0.00^{b}	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
Nkwo-Umunze	11.70± 0.00^{d}	0.00± 0.00	0.00± 0.00	76.00± 0.00^{a}	0.00± 0.00

*** Values in the column with the same letters are not significantly different (p<0.05).**

Key:

+: mildly pathogenic (>10/50mm in diameter).
++: very pathogenic (>/50mm in diameter).
- = not detected

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3.7. In-vitro antifungal activity of plant extracts

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The effect of concentrations of extracts on the test organisms was significant ($P < 0.05$). The colony diameter of the inhibition increased as the concentration of the extract increased as follows (100%> 50%> 25%).

Table 6: Zone of inhibition of the extract against the spoilage fungi

EXTRACTS	*Aspergillus aculeatus*	*Penicillium citrinum*	*Aspergillus flavus*	*Fusarium solani*	*Rhizopus stolonifer*
***B.pinatum* leaf extract @100%**	22.40±1.00^{b}	21.17±0.02^{d}	39.00±2.00^{b}	0.00±0.00	18.90±0.55^{a}
***B.pinatum* leaf extract @ 50%**	11.80±0.01^{e}	20.80± 0.06^{d}	20.50±1.00^{d}	0.00±0.00	17.00±0.10^{b}
***B.pinatum* leaf extract @ 25%**	11.00±0.30^{e}	13.50±0.60^{e}	19.66±0.20^{e}	0.00±0.00	10.00±0.05^{c}
***B.pinatum* bark extract @100%**	25.60±1.00^{a}	36.50±0.10^{a}	21.00±2.00^{c}	0.00±0.00	0.00±0.00
***B.pinatum* bark extract @50%**	20.75±0.01^{c}	27.80±0.22^{b}	12.70±1.00^{f}	0.00±0.00	0.00±0.00
***B.pinatum* bark extract @25%**	18.30±0.39^{d}	24.36± 0.11^{c}	5.06±0.20^{g}	0.00± 0.00	0.00±0.00
Fluconazole 30µg/ml	22.33±0.11^{b}	34.833±0.15^{a}	41.60±1.00^{a}	34.83± 0.3^{a}	19.16±1.00^{a}

***Values are mean scores ± Standard deviation of three (3) replicates**
N/B: **CLSI specification and standard zone of inhibition ranges:**
Less than or equal to 15mm = microbes are resistant to the extract
16 - 20 = Microbes are intermediate to the extract
21 and above = Microbes are susceptible to the extract.

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Plate 1: Post Harvest Cowpea (*Vigna unguiculata*).

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Plate 2: *Bryophyllum pinnatum* leaf

DISCUSSION

The comparative proximate analysis of fresh and spoilt cowpea seeds (Table 1) clearly showed a reduction in the essential nutritional composition as a result of spoilage. Comparing the proximate composition of fresh and spoilt cowpea seeds highlights the impact of spoilage on nutritional quality [20].

Qualitative phytochemical screening of *B. pinnatum* leaf extract showed the absence of flavonoids, steroids, oil and resin, and reducing sugar, while *B. pinnatum* bark extract showed the absence of glycosides, anthrocynin, oil and resin, and reducing sugar (Table 2). This outcome is consistent who also reported the presence of saponin, alkaloids, and phenol from [21]. This study supports the findings of [22], which found that *B. pinnatum* leaf extract included alkaloids, saponins, and terpenoids. The presence of saponin, alkaloids, and phenol, which were also discovered and identified in this study, gives *B. pinnatum* extracts its antimicrobial properties, according to [23].

The result of quantitative phytochemical screening of the *B. pinnatum* leaf and bark showed that all the plant parts contained phytochemicals associated with various medicinal properties (Table 3). This corresponds with the findings of [22], who reported the presence of alkaloid, flavonoid, phenol, tannin, and saponin. Research by Amaral *et al*. [24] also reported that *B. pinnatum* extracts included alkaloid, saponin, flavonoid, phenols, and glycosides, but did not mention that tannin was found in the study. The variation can be brought on by the different locations where the findings were acquired. The antimicrobial potential of the plant extract was likely due to the presence of alkaloids and flavonoids in the plant extract [25].

The literatures revealed that many fungi were reported to be associated with cowpea seeds from many countries [26] [27] [8] and [10]. In the present study, however, only five fungi (*Aspergillus*

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aculeatus, Fusarium solani, Penicillium citrinum, Rhizopus stolonifer, and *Aspergillus flavus*) were associated with spoilage of cowpea seeds obtained from different markets within Anambra State (Table 4). These organisms have been found to significantly degrade cowpea seeds [28] [10] and [29].

The pathogenicity test (Table 5) implicated these spoilage fungi as the main culprits. Olisa *et al*. [30] reported that the warm southern part of the country is always prone to seed pathogen infestation because of the prevailing environmental conditions. This region is associated with high relative humidity and high temperature. These environmental conditions are conducive for the growth of pathogens and disease development. This agreed with the findings of Akintunde *et al.* [31].

Generally, contamination of agricultural produce is a function of many factors, including infestation in the field before harvest, handling during harvesting, and methods of packaging and distribution of produce to the market, as reported by [27]. This agreed with the findings of Khare *et al*. [10], who claimed that *Aspergillus niger*., *Aspergillus flavus.*, and *Rhizopus stolonifer*. were to blame for the spoilage of cowpea seeds.

This study found that the leaf and bark extracts of *B. pinnatum* contained fungicidal compounds (Table 7) that inhibited the growth of fungal isolates. This finding is consistent with earlier reports of numerous studies that focused on different fungi, which reported that plant extracts could be used to protect against decay or spoilage of cowpea seed. The effectiveness of the extracts varied depending on the plant material, concentration, extraction solvent, and individual test fungus [31].

The radial mycelia growth of the fungi studied was significantly inhibited by commercial fungicides (Fluconazole), whereas *Fusarium solani* showed the highest percentage of inhibition

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with antifungal medication while *Penicillium citrinum* showed the least percentage of inhibition with antifungal medication. Regarding concentration, there was a consistent pattern in the fungitoxic activity of all the plant extracts. On all the test species, 100% extract concentration was the most fungitoxic concentration, followed by 50% extract concentration, and 25% extract concentration had the least inhibitory impact. This supports De Lange *et al.* [32] findings that there is a substantial variation in the mycelia growth values reported on the various plant extract concentrations.

CONCLUSION

This study has revealed the potentials of *B. pinnatum* extracts in the control of cowpea seed spoilage, with *B. pinnatum,* leaf and bark extracts exhibiting the most fungitoxic activity, followed by showed the least antifungal effect. This study also depicted that ethanol extracts of these plant extracts could be an alternative or complimentary to synthetic chemicals in controlling spoilage fungi.

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