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

ABSTRACT

Cowpea (*Vigna unguiculata* [L.] Walp) is commonly known as "Akidi" in Igbo language, is a special type of legume. It is relished as a native delicacy and contributes significantly to the meals in the South Eastern part of Nigeria. It is one of the underutilized and oldest legume grain in the world. Fungal attack on crops which over the years have led to pre- and post-harvest loss have been a very significant threat to crop production in the world.This aim of this research is to isolate and identify the spoilage pathogens of cowpea and testing the potentials of ethanol extracts of *Mimosa pudica*leaf and bark on the isolates. Cowpea seeds was purchased from different markets in Anambra State, Nigeria. They were transported to Alpha Research Laboratory, Awka in sterile polythene bags for microbial isolation. The spoilage organisms were isolated from the spoiled seeds through microbial culture. The media used for the isolation were Sabouraud Dextrose Agar and Potato Dextrose Agar. The findings indicated that *Aspergillus aculeatus, Fusarium solani, Penicillium citrinum, Rhizopus stolonifer,* and *Aspergillus flavus* were present. These isolates were tested for their *in-vitro* antifungal activity against the leaf and bark extracts of *Mimosa pudica*.The pathogenicity test also showed that the above organisms were responsible for cowpea seed spoilage. The antifungal potentials of ethanol extracts of *Mimosa pudica* on the isolates were investigated using the disc method. *Penicillium citrinum* showed the highest percentage of inhibition with antifungal medication while *Aspergillus aculeatus showed* showed the least percentage of inhibition with antifungal medication. The antimicrobial potential of *Mimosa pudica*extract on spoilage pathogens of stored Akidi encourages more research on the active ingredient of the plant for easy use by farmers as alternative to commercial/ synthetic fungicides.

Key words: Cowpea; media; spoilage

UNDER PEER REVIEW

INTRODUCTION

Cowpeas (*Vigna unguiculata* [L.] Walp) commonly known as "Akidi" in Igbo language are one of the staple foods in the diets of most Africans and can serve as an important source of proteins, calories and income. Its high protein content makes it an important ingredient in the diet of population groups of many tropical and sub-tropical countries(Afoakwa*et al*., 2010).Cowpea varieties namely Akidi, among others are common in Nigeria. Akidi are mostly consumed by people from eastern Nigeria [1]. It 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 [2].

Cowpea has gained more attention recently from consumers and researchers worldwide due to its exerted health beneficial properties including anti-diabetic, anti-cancer, anti-hyperlipidemic, anti-inflammatory and anti-hypertensive properties. It is also a cash crop for most west and central African farmers. It serves as a significant dietary compliment in developing countries of Africa, Latin America and Asia [3] [4].

Fungal infestation of agricultural commodities was becoming alarming. This was attributed partly to lack of good packaging materials for transporting foodstuffs from the farms and partly to the storage facilities that had provided environmental conditions conducive for effective growth of moulds[5]. 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 [6]. The plant world is a rich storehouse of natural chemicals that could be exploited for use as biopesticides [1]

Mimosa pudica L. belongs to the family of Fabaceae. A creeping annual or perennial herb often grown for its curiosity value as the leaves fold inward and droop when touched and again reopens within minutes. It is also called a "humble plant", "shame plant" and "touch

UNDER PEER REVIEW

me not plant”. This plant is said to have a bitter and astringent taste and has a history of use for the treatment of various ailments. The most commonly used plant part for this purpose is the leaves, but flowers, bark and fruits are also important in folklore medicine [8]. The plant, having various polyphenolics and flavonoid derivatives, has been traditionally used for its antidiarrheal, antihyperglycemic, anticonvulsant and cytotoxic properties [9]. It has been evaluated for a wide range of activities like antibacterial[10], anti-inflammatory [11], antifungal [12], wound healing [13] and anti-ulcer activity [14].

The aim of this study is to assess fungicidal potentials of leaf and bark extracts of *Mimosa Pudica* and *HuraCrepitans*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[15].

UNDER PEER REVIEW

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

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.

UNDER PEER REVIEW

2.2.4 Identification of isolated fungi

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 conidia, presence of conidiophores, shape of conidial heads [16].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 60° 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

UNDER PEER REVIEW

media. Next to that, Petri dishes were incubated at 37°C for 24 h in the incubator. The inhibition zone diameter 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.

UNDER PEER REVIEW

2.4 Statistical Analysis

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). Spoilage significantly affected the proximate composition ($p>0.005$) as moisture content and ash content were increased by spoilage while protein, fats and oil, crude fibre, and carbohydrate were decreased.

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 ($p < 0.05$).**

UNDER PEER REVIEW

3.2 Qualitative phytochemical composition

The result of the qualitative phytochemical composition of the leaf and bark ethanolic extracts of, *Mimosa pudica,* leaf and bark (Table 2). The phytochemical studies revealed the presence of nine (9) out of the eleven (11) phytochemicals determined.

UNDER PEER REVIEW

Table 2: Qualitative phytochemical composition of the ethanolic extracts of *Bryophyllumpinnatum, M.pudica and H. crepitans* leaf and bark

Parameters	*Mimosa pudica*	
	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

3.3 Quantitative phytochemicalcomposition

The result of the quantitative phytochemical study on the *M.pudica*leaf and bark extracts are presented in table 3.

UNDER PEER REVIEW

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

Parameters	*Mimosa pudica*	
	LEAF	**BARK**
ALKALOID	3.75 ± 0.10	11.10 ± 0.15
FLAVONOID	16.75 ± 0.11	12.70 ± 0.04
PHENOL	1.80± 0.02	1.30 ± 0.13
SAPONIN	6.86 ± 0.07	5.33± 0.11
TANNIN	9.76 ± 0.17	13.77± 0.10
STEROID	0.85± 0.17	0.43± 0.01
TERPENOID	1.68± 0.24	1.53± 0.10
CARDIAC GLYCOSIDE	7.75± 0.10	8.62± 0.13

***Values are mean scores ± Standard deviation of three (3) replicates**

***Data in the same column bearing different superscript differ significantly ($p < 0.05$).**

UNDER PEER REVIEW

3.4 Fungi Count of Spoilt Cowpea Seeds

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
IfiteAwka	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).**

UNDER PEER REVIEW

3.5Pathogenicity 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).

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 ± 0.0	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

UNDER PEER REVIEW

3.6 In-vitro antifungal activity of plant extracts

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*
***M pudica*leaf extract @100%**	18.40±1.0b	0.00±0.00	18.13±2.00^{b}	17.00±3.01^{b}	9.000±0.55^{b}
***M pudica* leaf extract @ 50%**	13.67±0.01^{c}	0.00±0.00	12.20±1.00^{c}	7.50±1.110^{c}	0.000±0.00
***M pudica* leaf ethanol 25%**	12.00±0.30^{d}	0.00±0.00	9.86±0.20^{d}	5.00± 2.00^{d}	0.000±0.00
***M pudica*bark extract @100%**	18.40±1.00^{b}	0.00±0.00	18.13±2.00^{b}	17.00±3.01^{b}	0.000±0.00
***M pudica*bark extract @50%**	13.67±0.01^{c}	0.00±0.00	12.20±1.00^{d}	7.50±1.110^{c}	0.000±0.00
***M pudica* bark extract @25%**	12.00±0.30^{d}	0.00±0.00	9.86±0.20^{g}	5.00± 2.00^{d}	0.000±0.00
Fluconazole 30µg/ml	22.33±0.11^{a}	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.

UNDER PEER REVIEW

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

UNDER PEER REVIEW

Plate 2: *Mimosa pudica* 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. Spoilage typically leads to a decrease in protein content and an increase in moisture content due to microbial activity. Additionally, the presence of spoilage microorganisms can lead to the breakdown of complex carbohydrates and the production of undesirable compounds, which can affect the overall nutritional quality and safety of the seeds. Comparing the proximate composition of fresh and spoilt cowpea seeds highlights the impact of spoilage on nutritional quality[17].

The result of the qualitative phytochemical screening of *M. pudica*leafextract showed the absence of flavonoids, steroids, anthocyanin, and reducing sugar, while *M.pudica* bark extract showed the absence of flavonoids, alkaloids, oil and steroid, terpenoids, anthocyanin, phenol, and reducing sugar (Table 2). This outcome is consistent who also reported the presence of saponin, glycosides, and tannin from [18]. This study supports the findings of [19], which found that *M. pudica*leafextract included alkaloids, saponins, and glycosides. According to [20], the presence of saponin, glycosides, and terpenoids, which were also discovered and identified in this study, gives *M. pudica*extracts its antifungal properties.

Quantitative phytochemical screening of *M. pudica*leaf and bark showed the following phytochemicals (Table 3), corresponds with the findings of [18], who reported the presence of alkaloid, flavonoid, phenol, tannin, and saponin. Reports by [20] and [19], opined that *M. pudica* extracts have shown antimicrobial activity against *Aspergillus flavus*, *Trycophytonruburum*, Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and Gram- negative bacteria (*Escherichia coli* and *Klebsiella pneumonia*).

UNDER PEER REVIEW

The literatures revealed that many fungi were reported to be associated with cowpea seeds from many countries [21] [22] [23] and [24].In the present study, however, only five fungi (*Aspergillus 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 [25] [24] and [26].

The pathogenicity test (Table 5) implicated these spoilage fungi as the main culprits. The warm southern part of the country is always prone to seed pathogen infestation because of the prevailing environmental conditions as reported by [27]. 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 [28].[29] and [30] in West Africa and southwestern Asia, respectively, studied cowpea genotypes and revealed that many cowpea seeds are prone to fungal infestation. Also, [24] isolated eight fungal species belonging to five genera, which include *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus*, and *Cylindrocarpon,* from the seeds of three cultivars of cowpea. This finding is consistent with the reports of [31] and [32].

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 [5]. Most often, natural openings and wounds caused by harvesting, shipping, handling, and marketing allow fungi to enter cowpea seeds [33] and [33]. This agreed with the findings of[35], who claimed that *Aspergillus aculeatus.*, *Aspergillus flavus.*, and *Rhizopus stolonifer.* were to blame for the spoilage of cowpea seeds.

UNDER PEER REVIEW

This study found that the leaf and bark extracts of *M. pudica*contained fungicidal compounds (Table 6) 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 [28].

The radial mycelia growth of the fungi studied was significantly inhibited by commercial fungicides (Fluconazole), whereas *Penicillium citrinum* showed the highest 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 isolates, 100% extract concentration was the most fungitoxic concentration, followed by 50% extract concentration, and 25% extract concentration had the least inhibitory impact. This supports [36] findings that there is a substantial variation in the mycelia growth values reported on the various plant extract concentrations. The antifungal properties of the plant extracts are therefore likely due to the presence of phytochemicals, which are anti-microbial agents [37], that are inhibitory to the growth of these pathogens [38].

CONCLUSION

This study has revealed the potentials of *M.pudica*leaf and bark extracts in the control of cowpea seed spoilage. This study also depicted that ethanol extracts of this plant extracts could be an alternative or complimentary to synthetic chemicals in controlling spoilage fungi.

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