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In Vitro Activity of Xylopia Aethiopica Extracts Against Monacrosporium Bembicodes Isolated from Powdered Soyabean Samples

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Abstract
Several incidences of antimycotic resistant have been reported and traced from the consumption of contaminated foods and their products. This study was carried out to determine the effects of *Xylopia aethiopica* on *Monocrosporium bembicoides* isolated from powdered Soyabean samples. A total of 120 powderd Soya bean sample were collected randomly from Uli community, Ihiala Local Government Area of Anambra State and screened for the presence of *Monocrosporium bembicoides* using spread plate technique. The isolates were subcultured and characterized using their macroscopic and microscopic characteristics. The seeds of *Xylopia aethiopica* was collected and screened for phytochemical constituents using spectrophotometer and gravimetric methods. The antifungal activity of the extract was carried out using agar well diffution technique. Tube dilution method was used to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Xylopia aethiopica* seed extract. A total of 24 powdered Soyabean samples out of 120 samples were positive to *Monocrosporium bembicoides*, of which the organism was seen most in samples collected from location C (Eke Agbagba market). The phytochemical analysis revealed the presence of alkaloids, flavonoids, phenolics, cardiac glycosides, tannins, steroids and saponins. The extracts showed pronounced activity against the tested *Monocrosporium bembicoides* of which ethanolic extract significantly ($p< 0.05$) inhibited the organisms more than the aqueous extracts. The ethanolic extract and ketoconazole had similar MIC and MFC. This study has shown the occurrence of *Monocrosporium bembicoides* in the studied powdered Soyabean samples, and *Xylopia aethiopica* seed extracts showed pronounced activity against the organism, of which ethanolic extract was more pronounced.
Keywords: *Monocrosporium bembicoides, Xylopia aethiopica,* Soyabean,Phytochemical, Antifungal Activity

INTRODUCTION

Soyabean, known as *Glycine max* is a worldwide economic crop and the most important cultivated legume with hundreds of food, feed and industrial uses. It is an introduced crop in Bangladesh. The crop can be grown in tropical, sub- tropical as well as the temperate regions. It is a primary source of vegetable oil and protein concentrates. Soyabean is an excellent source of major nutrients, about 40% of dry matter is protein and 20% fat. As soybean acreage has expanded throughout the world, diseases have increased in number and severity (Newell and Hymowitz, 2003).

All parts of the soyabean plant are susceptible to a number of pathogens which reduce the quality and/or quantity of seed yield. Soyabean suffers from as many as 150 different diseases. Generally one or more diseases can be found in one field wherever soyabean are grown. A specific one may be very destructive in one season and difficult or impossible to find in the next season. Among the serious diseases of soyabean, most of them are seed-transmitted (Newell and Hymowitz, 2003*)*.

Monacrosporium bembicoides, a type of nematophagous fungi have been found in all regions of the world, from the tropics to Antarctica. It appears to be common in most soils, especially in tropical areas, occuring abundantly and ubiquitously. They have been reported from agricultural, garden and forest soils, and are especially abundant in soils rich in organic material. In agricultural soils in temperate regions the nematode trapping fungi follow a seasonal variation, with highest densities and number of species in late summer and autumn, possibly due to the higher soil temperature and increased input of organic debris. The fungi are most frequent in the upper 20 cm of the soil and appear to be almost absent below 40 cm (Persmark *et al*., 2001). Mostly, plant-parasitic nematodes attack plant roots and, therefore, the ability of the nematophagous fungi to grow in the rhizosphere is of great importance for their capacity to control these nematodes. Many nematode trapping fungi especially the specie of *Monacrosporium* have been found to occur more frequently in the rhizospheres of several plants, especially leguminous plants, e.g. soybean and pea, than in root-free soil. This effect could possibly be due to increased or changed root exudation in these plants. To evaluate whether trapping structures and consequently trapping of nematodes are actually more abundant in rhizosphere soil, new techniques have to be developed to examine the activity of nematophagous fungi in situ (Jaffee *et al*., 2002).

Medicinal plants such as *Xylopia aethiopica* and many others have continued to play central roles in the

healthcare of large proportion of the world's population. This is particularly true in the developing countries, where herbal medicine has a long and uninterrupted history of use. Recognition and development of medicinal and economic benefits of these plants are on the increase in both developing and industrialized nations (Bordallo *et al*., 2002). Among the use of herbal therapy in the treatment of infective diseases affecting man all over the world today, the results presently arising from the use of available chemotherapeutic agents are even encouraging factors to use of herbs. (Davise *et al*., 2011). The medicinal values of these plants lie in bioactive phytochemical constituents that produce definite physiological action on the human body (Bordallo *et al*., 2002). These natural compounds formed the foundations of modern prescription of drugs as we know today (Farrell *et al*., 2006).

Anambra State is one of the few States in Nigeria with large consumption of Soya bean, majorly in powdered form. Previous studies focused on microorganisms associated with Soya bean (Ahma *et al.,* 2002). Hence there is no known information on effect of some natural antimicrobial substances against these nematophagous fungi. This study was carried out to evaluate the effect of *Xylopia aethiopica* seed extracts on *Monacrosporium bembicoides.*

MATERIALS AND METHODS

Study Area: Ihiala L.G.A. is one of the 21 L.G.A in Anambra State of Nigeria. It lays at the most southerly part of Anambra State and is located at the southern end of the south senatorial zone of the states. Ihiala L.G.A occupies a total land area of approximately 204 square kilometers and lies in a tropical rain-forest belt of Nigeria. However due to human activities, particularly in the form of deforestation for purposes of farming and construction works, the forest vegetation has largely appeared giving way to derived savannah vegetation of shrub land and bushes. The climate of the city is typically an equatorial rain forest type characterized by two main seasons; the rainy season, which lasts between April and October and the dry season which lasts between November and March. In most part of Ihiala L.G.A, the temperature is usually high all the year around with average minimum temperature at about 32 $^{\circ}C$ and 25 $^{\circ}C$ respectively.

Sample Collection: A total of 120 samples of soyabean powder were collected from different shops and open markets; Amanputu market, Nkwo ogbe market, Eke agbagba market, and Ubahudara market, all within Ihiala Local Government Area, using sterile polyethene bags, and kept in disinfected cooler. Sampling was performed manually from different bags such that the product was collected from different parts of the bags. The sample was pooled and mixed properly and formed one cup of the Soyabean sample. The samples were brought to the laboratory in a cooler maintaining low temperature (< 4^{o}C) using ice blocks. The collected samples were processed within 2 h of its collection. The samples were collected randomly and each collected sample was marked with identification code with respect to the data and time of collection. Sample and the collection criteria were not limited to any specific part.

Isolation of Fungal Organism: This was carried out using the method of Pitt and Hocking (2002). Exactly 1 g of each powdered Soyabean sample was added into 100 ml beaker, mixed thoroughly with 4 ml sterile distilled water using sterile spatula and then made up to10 ml with sterile distilled water. The supernatant was decanted and 0.1 ml of the mixture was inoculated into labeled plate containing Sabouraud Dextrose Agar (SDA) that contained 0.05% chloramphenicol and incubated at room temperature (30±2 $^{\circ}C$) for 3-5days. Thereafter, plates were examined macroscopically for characteristic colonies.

Identification of Fungal Isolates: The fungal isolates were identified to the genus/species level based on macroscopic and microscopic characteristics of the isolates obtained from pure cultures. The characterized isolates were ascertained with the aid of fungal atlas (Watanabe, 2002).

Macroscopy: The colonies were carefully examined for fungal characteristics. The rapidity of growth, color, and nature of the reverse side of the plate, shape, texture, consistency of the growth and other peculiar features of the colonies were observed according to the method of Watanabe, (2002).

Microscopy: This was carried out using Needle mount technique. A drop of lactophenol cotton blue solution was placed on the center of a clean grease-free slide. A fragment of the colony was placed in the drop using sterile wire loop and was covered with a cover-slip, avoiding bubbles. Excess fluid from the outside of the cover slip was wiped with cotton wool and slide was passed through the flame to warm so as to remove the remaining air bubbles and facilitate staining of the fungal element. The slide was then examined under the microscope, using low-power objective of ×10 magnifications, followed by high-power objective of ×40 magnifications to reveal the nature of the hyphae, shape, size, texture and arrangement of the conidia. The pictorial nature of the fungal organisms was confirmed using the fungal atlas (Watanabe, 2002).

Preparations of plant materials: The seeds of *Xylopia aethiopica* were collected from Uli market, Ihiala L.G.A of Anambra State, Nigeria. The sample was authenticated appropriately. The seeds were ground to powder form using sterile electric grinder. Five killogram of the ground seeds was macerated in 50 ml of distilled water and ethanol respectively for 72 h. The mixture was filtered using what man No.1 filter paper. The extracts were

concentrated by evaporating to dryness at room temperature in a steady air current (Iheukwumere and Umedum, 2013).

Phytochemical analysis of the plant extracts: The phytochemical components (alkaloids, glycosides, flavonoids, phenolics, tannins, steroids and saponins) of the seed extracts were determined quantitatively using the methods described by Iheukwumere and Umedum (2013).

Alkaloids: Five milliliters of the sample was mixed with 96% ethanol and 20% tetraoxosulphate (VI) acid (1:1). One milliliter of the filtrate from the mixture was added to 5 ml of 60% tertraoxosulphate (VI) acid and allowed to stand for 5 minutes. Then 5 ml of 0.5% formaldehyde was added and allowed to stand for 3 h. The reading was taken at absorbance of 550nm.

Glycosides: This was carried out using Buljet's reagent. One gram of the fine powder of the sample was soaked in 10 ml of 70% alcohol for 2 h and then filtered with Whatman No. 1 filter paper. The extract was then purified using lead acetate solution and disodium hydrogen tetraoxosulphate (VI) solution before the addition of freshly prepared Buljet's reagent. The absorbance was taken at of 550nm.

Flavonoids: Five millilitres of the extract was mixed with 5 ml of dilute hydrochloric acid and boiled for 30 minutes. The boiled extract was allowed to cool and then filtered with Whatman No. 1 filter paper. One millilitre of the filtrate was added to 5 ml of ethyl acetate and 5 ml of 1% ammonia solution. The absorbance was taken at 420nm.

Phenolics: Ten millilitres of the sample was boiled with 50 ml acetone for 15 minutes. Five millilitres of the solution was pipette into 50 ml flask. The 10 ml of distilled water was added. This was followed by addition of 2 M ammonium hydroxide solution and 5 ml of concentrated amyl alcohol solution. The mixture was left for 30 minutes and absorbance was taken at 550nm.

Tannins: Ten millilitres was pipette into 50 ml plastic containing 50 ml of distilled water. This was mixed for 1 h on a sterile mechanical shaker. The solution was filtered with Whatman No. 1 filter paper, and 5 ml of the filtrate was mixed with 2 ml of iron (III) chloride solution in 0.1 N hydrochloric acid. The absorbance was taken at 550nm.

Steroids: The extract was eluted with normal ammonium hydroxide solution. Two millilitres of eluate was mixed with 2 ml of chloroform in a test tube. Three millilitres of ice cold acetic anhydride was added to the mixture and allowed to cool. The absorbance was taken at 420nm.

Saponins: Five millilitres of the sample was dissolved in aqueous methanol. The 0.25 ml of aliquot was taken for spectrophotometric determination for total saponins at 544nm.

Determination of extract value of the plant materials: The concentration of the extract was determined by evaporating 1.0 g of the extract in an evaporating dish of known weight in an oven to dryness and weighed. The dish containing the residue was allowed to cool and weighed. The weight of the residue was obtained by subtracting the weight of the empty dish from the weight of the dish and residue. The above method was done in triplicate (Iheukwumere *et al*., 2012).

Preparation of the test samples of the plant extracts for *in vitro* antifungal susceptibility tests: In this study the concentration of 500 mg/ml of the extract was used to screen for the antimicrobial activity. This was done by using the modified method of Iheukwumere *et al*. (2012). Here, 2.5 g of the extract was dissolved in 5.0 ml of peptone water.

***In vitro* antifungal susceptibility testing of the plant extracts using agar well diffusion method:** This was carried out by the modified method of Iheukwumere and Umedum (2013). Each labeled plate was uniformly inoculated with the test organism using pour plate method with SDA. A sterile cork borer of 5mm diameter was used to make wells on the medium. One tenth millilitre of various concentrations of the extracts was dropped into each labeled well and then incubated at room temperature (30±2 $^{\circ}C$) for 5 days. Antifungal activity was determined by measuring the diameter of the zones of inhibition (mm) produced after incubation.

Determination of minimum inhibitory concentration (MIC): This was carried out by serial-diluting the stock (500 mg/ml) solution to get various concentrations of the stock; 500, 250 and 125mg/ml. One millilitre (1 ml) of each dilution was inoculated with 1ml of test organism, another test tube containing the dilution without the test organism were used as control. The test tubes were incubated at room temperature (30±2 $^{\circ}C$) for 72 h. The lowest concentration that show no visible growth was recorded as the minimum inhibitory concentration (MIC) for the organisms (Iheukwumere *et al.,* 2012)

Statistical Analysis : The result of the data generated in this study were expressed as mean ± Standard deviation ($X \pm SD$), The statistical analysis of the data generated from this pathogenic study was carried out using student "t" test at 95% confidence limit (Iheukwumere and Umedum, 2013).

RESULTS

The powdered Soyabean samples that were positive to *Monacrosporium bembicodes* are shown in Table 1. A

total of 120 powdered Soyabean samples were collected from different markets of Amanputu, Nkwo ogbe, Eke agbagba, and Ubahudara. Out of the 120 samples, 24(20.00 %) samples were positive to the test isolate whereas 96 (80.00%) samples were negative. It was observed that *Monacrosporium bembicodes* was significantly ($P<0.05$) seen most in location C compared to other locations.

The characteristics and identity of the test organism isolated from powdered Soyabean samples are shown in Table 2. The result revealed the presence of *monacrosporium bembicodes*. The isolate was characterized and identified by its macroscopic characteristics and microscopic characteristics. The macroscopic characteristics were based on the colour, pigmentation, growth rate, texture and consistency of their colonies. The microscopic characteristics were based on the nature of hyphae, phialides, vesicles, conidiophores and arrangement of conidia (figure 1).

The phytochemical constituents of *Xylopia aethiopica* seed extract revealed the presence of alkaloids, flavonoids, saponins, phenolics, tannins, steroids and cardiac glycosides (Table 3). These phytochemicals may responsible for the antifungal activies of the extracts. The diameter Zones of inhibition of the extracts against *Monacrosporium bembecoides* using 5mm cork borer is shown in Table 4. The ethanolic extract significantly ($p< 0.05$) inhibited the test organisms more than the aqueous extract. No inhibition was observed from absolute ethanol (0.1ml) and distilled water (0.1ml) used as negative control. The activities of the extracts were significant ($p<0.05$) when compared to Ketoconazole (positive control). The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) studied revealed the pronounced activity of the extracts against *Monacrosporium bembecoides*(Table 5The activity of the ethanolic extract was similar to that of ketoconazole (positive control).

Table 1: Soyabean (*Glycine max*) samples that were positive to the test isolate

Source	Positive samples (%)	N= 10 Negative samples (%)	Total (%)
A	4 (13.33%)	26(86.67%)	30(25.00%)
B	6(20.00%)	24(80.00%)	30(25.00%)
C	8(26.67%)	22(73.33%)	30(25.00%)
D	6(20.00%)	24(80.00%)	30(25.00%)
Total (%)	24(20.00%)	96(80.00%)	120(100.00%)

A = Amanputu Market, B = Nkwo Ogbe Market, C = Eke Agbagba Market, D = Ubahudara Market

Table 2: Macroscopic and microscopic characteristics of the isolate from powdered Soyabean (*Glycine max*) samples

Isolate	Macroscopic Characteristic	Microscopic characteristic
Monacrosporium bembicodes	The characteristic colour of the colony is greenish-brown, while the reverse side on a Petri dish is pale to brownish colour. Surface of the colony was coarsely rough. The conidiophores appeared as a powdery mass. The shape of the organism was cylindrical or bottle shaped or elongated obovoid. The colonies grew rapidly (45- 55 mm in 7 days). The optimum temperature for their growth is 25^0C	The spores are two celled the distal cell usually 1.5 to 2 times longer than the proximal cells. The size of the spores varies between 18- 30 µm long and 9-14 µm wide on SDA medium. Produces constricting rings when viewed under microscope

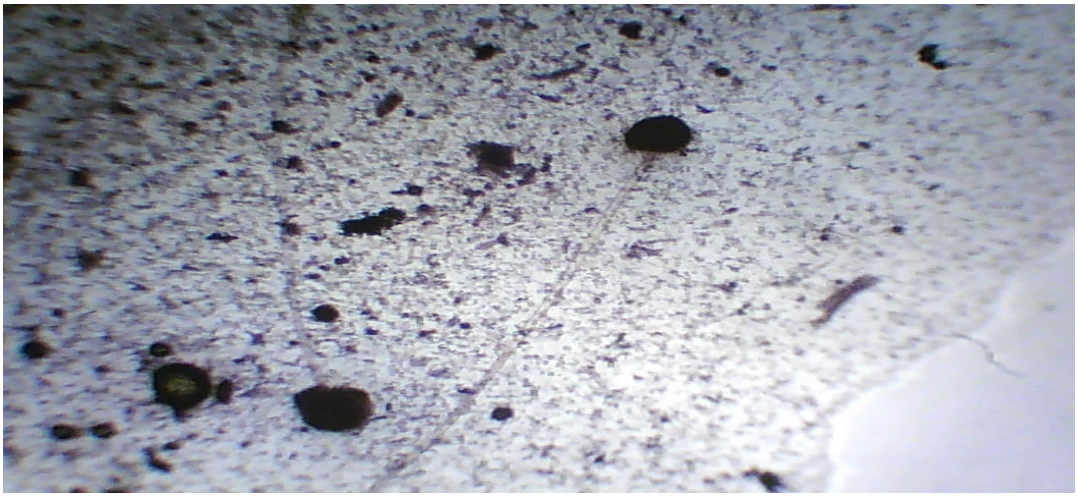

Plate 1: The microscopic characteristic of *Monacrosporium bembicodes*

Table 3: Phytochemical constituents of the *Xylopia aethiopica* extract

Phytochemical	Value (Mg/ 100g)
Alkaloids	1.92
Tannins	0.62
Saponins	0.22
Flavonoids	0.44
Phenolics	1.51
Steroids	0.11
Cardiac glcosides	0.35

Table 4: Diameter (mm) zones of inhibition of the extract against the *Monocrosporium bembecoides* using 5 mm cork bore

Extract (800 mg/ml)	Mean Diameter ± SD (mm)
Ethanolic Extract	18.50± 0.50
Aqueous Extract	12.87 ± 0.47
Ketoconazole	19.00 ± 0.00
Absolute Ethanol (0.1 ml)	0.00 ± 0.00
Distilled water (0.1 ml)	0.00 ± 1.00

SD = Standard Deviation

Table 5: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MBC) of the extracts

Extract	MIC (mg/ml)	MFC (mg/ml)
Ethanolic Extract	400	800
Aqueous Extract	800	0
Ketoconazole	400	800

DISCUSSION

Contamination of staple foods such as Soyabean with *Monacrosporium bembicodes* affects the well being of man as well as livestocks when they consume crops infected with the organism which is a type of nematophagous organism. In many developing countries, it imposes subsequential burden on the health care

systems, increases economic loss and reduces agricultural sector productivity and output (Rosen *et al*., 2006).

The presence of *Monacrosporium bembicodes* in studied soyabean powder (*Glycine max*) samples can be traced from certain conditions such as high moisture during harvesting, inadequate drying and poor ventilation during storage of crops, poor agricultural practices. Similar observations were reported by many other researchers (Bewaji and Baabunmi, 2000; Jaffee, 2002; Shapira *et al*., 2010). Truckesses and Scott (2002) stated that different biotic factors like temperature, moisture, and relative humidity are also the main causes of fungal contamination on soyabean seeds. This shows that planting, pre and post harvesting practices and storage methods require microbiological safety regulation to escape microbiological contamination of soyabean seeds. Similar deduction was drawn by (Persmark *et al*., 2001).

In this study, the phytochemical constituents present in the *Xylopia aethiopica* extract could be responsible for the antifungicidal activity of the various sample extracts. Similar findings were made by different researchers (Parekh *et al*., 2005; Iheukwumere *et al*., 2012). The antifungal activity shown by *Xylopia aethiopica* extract in the study agrees with the findings of Yu *et al*. (2009). The antifungal properties of *Xylopia aethiopica* extract is traced to its chemical compound. The active phytochemical constituents of the extracts had more ability to dissolve in ethanol (organic solvent) than in water (inorganic solvent). Similar conclusion was drawn by different researchers (Iheukwumere et al., 2012; Iheukwumere and Umedum, 2013). This study further highlighted that ethanol was able to extract more of the phytochemical constituents because ethanol is an organic and polar solvent. And most of the phytochemical constituents are organic in nature. This observation suggested that the organic solvent extraction is suitable to verify the antifungal properties of medicinal plants (Iheukwumere *et al*., 2012).

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the extracts of *Xylopia aethiopica* showed that ethanolic extracts exhibited similar activity in their MICs when compared to ketoconazole (control) which had more pronounced activity. This means that infections caused by *Monacrosporium bembicodes* could be managed effectively using the single dose of this plant extract. Also, further research involving *in vivo* assays will be needed to establish the relationship between MICs and MFCs obtained in this study and the effective dosage that should be administered in ethnomedical practice

CONCLUSION

This study revealed the presence of *Monacrosporium bembicodes* in the powdered Soyabean (*Glycine max*) samples randomly collected from four different markets in Ihiala Local Government Area of Anambra State. The *Xylopia aethiopica* seed extracts showed pronounced against the isolate. The ethanolic extract and ketoconazole had similar MICs and MFCs, of which the activity of ethanol was more pronounced than that of aqueous extract.

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