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EVALUATION OF THE ANTIFUNGAL PROPERTY OF THE STEM BARK OF *EUGENIA UNIFLORA* L. (MYRTACEAE) FOR CONTROL OF THREE POTENTIAL TOXIGENIC FUNGI

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ABSTRACT

Studies were carried out to determine the potential of using crude aqueous and ethanol extracts of *Eugenia uniflora* L (Myrtaceae) to control three toxigenic fungi, *Aspergillus flavus, Aspergillus niger*, and *Fusarium verticilloides* isolated from vegetables obtained from various markets in Accra. The extracts were serially diluted with autoclaved Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) to obtain the following concentrations, 1:1, 1:2, 1:4, 1:8v/v and undiluted. Radial growth of the test fungi, as well as vegetative growth were assessed in Petri plates and Erlenmeyer flasks (250 ml) respectively. The bark extracts exhibited both growth promotory and inhibitory effects against the test fungi. The aqueous extracts promoted vegetative growth of *A. flavus* and *A. niger*, but depressed both the vegetative and radial growths of *Fusarium verticilloides*. The ethanol extracts depressed both the vegetative and radial growths of the test fungi. Results are discussed in the light of the possible use of *Eugenia uniflora* in integrated control of toxigenic fungi.

Keywords: Toxigenic Fungi, Radial Growth, Vegetative Growth, Growth Promontory

INTRODUCTION

There is a growing interest in plants with antimicrobial properties. Scientists are increasingly becoming involved in the screening of such plants with the aim of establishing their potential antimicrobial effect and identifying the active compounds

[1] for their possible use as biological control agents. The need to search beyond the synthetic chemicals and look at natural alternatives has become imperative due to the environmental and health effects resulting from the continuous use of synthetic compounds. This is partly due to the reluctance on the part of people to consume foods that have been preserved using synthetic chemicals.

Several studies have been conducted with the view of developing eco-friendly antimicrobials. Plant extracts of some Ghanaian weeds *Tridax procumbens* and *Euphorbia heterophylla* have been found to suppress the growth of sclerotia of *Sclerotium rolfsii* **[2]** *Chromolaena odorata* has been shown to be useful in the control of *F. moniliforme* (*F. verticilloides*) **[3]** and *Tapinanthus bangwensis* has potential use in the control of *Sclerotium rolfsii* in the field **[4]**. Extracts of *Allium sativum* and *Zingiber officinale* showed excellent inhibitory effect on seed-borne fungi *Colletotrichum dematium var. truncatum, Macrophomina phaseolina* and *Colletotrichum kikucchii* infecting soybean seed **[5]**.

The success of some of these plant products in laboratories and large scale studies has aroused the interest of several workers in the development of biological products for post harvest application.

Eugenia uniflora L (Myrtaceae) is popularly known as the Surinam cherry or pitanga. It is a native from Surinam, Guyana, and French Guyana to southern Brazil and Uruguay and was introduced into Africa around the year 1911 **[6]**. There are two distinct varieties, the more common ones with bright red fruits and the rarer ones with dark red to crimson fruits which are sweeter and less resinous. The plants are tropical to subtropical, and being deep rooted, can survive a long dry season. They can survive on any kind of soil, even waterlogged soil, for a period, but are intolerant of salt **[6]**.

Some parts of the plant are used traditionally around the world for various purposes. In Brazil the leaf infusion is taken as a stomachic, febrifuge and astringent **[6]**. In Surinam, the leaf decoction is drunk as a cold remedy and, in combination with lemongrass, as a febrifuge **[6]**.

Previously conducted experiments indicate that *E. uniflora* possesses some bacteriological properties. Ethanol-water extract of the plant has been found to present moderate activity against *Staphylococcus aureus* and *Escherichia coli* **[7]**.

Crude extracts (CE), dichloromethane and ethyl acetate extracts of the fruits and leaves of *Eugenia umbelliflora* Berg. (Myrtaceae) have also been found to exhibit excellent

activity against gram positive bacteria **[8]**. The essential oil extract from the dried flower buds of clove, *Eugenia caryophyllata* L Merr. & Perry (Myrtaceae) also exhibited encouraging antifungal, antimicrobial, antioxidant and antiviral activity, and has been investigated on pathogenic bacteria, *Herpes simplex* and Hepatitis C viruses **[9]**.

Aqueous extract of the leaves of *Eugenia uniflora* has been found to inhibit the growth of yeast cells of *Paracoccidioides brasiliensis*, the thermal dimorphic fungus which is the causative agent of Paracoccidioidomycosis (PCM) **[10]**.

The present study was conducted to evaluate the antifungal activity of stem bark of *Eugenia uniflora* against three potentially toxigenic, pathogenic and seed-borne fungi namely, *Aspergillus flavus*, *Aspergillus niger* and *Fusarium verticilloides* (= *F. moniliforme*), isolated from vegetables purchased from markets in Accra.

MATERIALS AND METHODS

Test Fungi

The test fungi, *Aspergillus flavus*, *Aspergillus niger* and *Fusarium verticilloides* were isolated from vegetables obtained from various markets in Accra. These cultures were kept in an incubator at 37°C and sub-cultured on PDA when needed.

Bark of Test Plant

Stem bark of *Eugenia uniflora* was obtained from a plant on the front lawn of the Department of Botany of the University of Ghana. Samples were authenticated at the Ghana Herbarium at the University of Ghana to be that of *Eugenia uniflora*.

Aqueous Extracts

The stem bark of the *Eugenia uniflora* plant was removed from the plant and sun dried for one month to a constant weight. The dried bark was then blended to a powder. Fifty grams of the powdered stem bark was measured and put in a 500 ml beaker. To this, 375 ml of distilled water was added and the mixture stirred. This was then left to stand for 48 hours in the refrigerator with intermittent agitation. After 48 hours, the mixture was decanted and the residue discarded. The supernatant obtained was then centrifuged at 1500 rpm and the residue discarded. The clear liquid obtained was then filtered using a Millipore filter with a 0.45 μm pore size. The supernatant obtained after filtration was distributed into medicinal bottles, autoclaved at a temperature of 121°C and a pressure of 1.1 kg/cm^3 for 15 minutes and refrigerated, awaiting use.

Ethanol Extract

Fifty grams of dried powdered stem bark was measured and put into a 500ml beaker. To this, 375 ml of ethanol was added. The

mixture in the beaker was stirred, covered with aluminium foil and allowed to stand in a refrigerator for 48 hrs with periodic agitation. After 48 hrs, the mixture was decanted and the residue discarded. The supernatant was then concentrated in a rotary evaporator (Model R-210/215) to a mass of 3.84 g. This was dissolved in 250 ml of water. The solution was transferred into a medicinal flask and stored in a refrigerator awaiting use.

Solid Medium Test

The aqueous and ethanol bark extracts of the plant was used in amending Potato Dextrose Agar to obtain solid agar medium of varying extract concentrations as follows: Undiluted, 1:1, 1:2, 1:4: and 1:8v/v. Twenty millilitres of the extract sterilized by heat treatment at 121 ^{o}C for 25 minutes was poured into 9 cm sterile Petri plates. The Petri plates were then inoculated at the centre of the plates with 3mm disc of mycelium taken from the advancing edge of 4-day old cultures, of the test fungi. There were four replicates for each dilution level. The plates were incubated at 30 ^{o}C and the diameter of the plates measured at two day intervals till the mycelia covered the entire 9 cm Petri plate.

Liquid Medium Test

Double strengths of Potato Dextrose Broth was amended in appropriate proportion with either the aqueous or ethanol extract of the stem bark of *E. uniflora* to obtain extract concentrations of 1:1, 1:2, 1:4: and 1:8v/v. PDB without the extracts served as control. Erlenmeyer flasks (250ml) containing 30ml of appropriate dilution of the extract were plugged with non absorbent cotton wool and sterilized at 121 ^{o}C for 25 minutes. The flasks were then inoculated with 3 mm discs of 4-day old culture of the appropriate fungus and incubated at 30 ^{o}C. At pre-determined incubation periods of 2, 4, 6 and 8 days, four flasks were removed and the mycelium harvested using the conventional oven dry weight method with Whatman No. 2 filter papers. Filter papers carrying the harvested mycelia were put in an electrically heated oven (Gallenkamp oven plus series) at 75 ^{o}C for 24 hours. The dry weight of the mycelium was then calculated by the difference in weight.

RESULTS

A. Influence of Heat Sterilized Aqueous Bark Extract of *Eugenia uniflora* on Radial Growth of *Aspergillus flavus, Aspergillus niger* and *Fusarium verticilloides*

The effect of various concentrations of aqueous extracts of stem bark from *Eugenia uniflora* on three toxigenic fungi, *A. flavus*, *A. niger* and *Fusarium verticilloides* on PDA are shown in **Figures 1-3**. Radial growth of mycelium of *A. flavus* and

A. niger was variably improved by the aqueous stem bark extract. **(Figures 1 and 2)**, whereas fungal growth of *Fusarium verticilloides* was variably depressed **(Figure 3)**. Stimulation of radial growth of the mycelium of *A. flavus* and *A. niger* was highest in the undiluted aqueous extract. Further dilution of extract (1:1 – 1:8v/v) gradually removed this stimulatory effect. On the 8th day, radial growth of *A. flavus* was 27 mm (30 %) higher on the undiluted plate than the control **(Figure 1)**. For *A. niger* the mean diameter of the cultures on the undiluted medium was approximately 12 % (11mm) higher than that of cultures on extract free PDA **(Figure 2)**. Compared to the control plate, the undiluted aqueous extract was able to depress growth of *Fusarium verticilloide*s by 26% after 8 days **(Figure 3)**.

B. Influence of Heat Sterilized Ethanol Bark Extract of *Eugenia uniflora* on Radial Growth of *Aspergillus flavus, Aspergillus niger* and *Fusarium verticilloides*

This experiment was a sequel to experiment A in which aqueous extract of the bark of *Eugenia uniflora* was found to be both growth promotory and inhibitory to the test fungi. Radial growth of the three the test fungi was depressed by the highest concentration of the heat sterilized ethanol stem bark extract **(Figures 4-6)**. This depressive effect was commensurate with the concentration that is the higher the concentration the more the depressive effect. However, after 8 days of growth on agar, radial growth of *A. flavus* on agar amended with ethanolic extract approximated that of the control growing on extract-free medium **(Figure 4)**. Growth of *A. niger* and *Fusarium verticilloides* were generally slow and never approximated that of the control after 10 days **(Figure 5 and 6)**.

C. Vegetative Growth of *Aspergillus flavus, Aspergillus niger, Fusarium verticilloides* in Potato Dextrose Broth Amended with Varying Dilutions of Aqueous Stem Bark Extract of *Eugenia uniflora*

The three test fungi showed different behaviour at the end of the incubation period. Dry weight accumulation of *A. flavus* was significantly (p=0.05) increased by the undiluted aqueous extract as compared to the control whereas

Dry matter accumulation of *A. niger* was marginally promoted by the aqueous extract. The accumulation of dry matter in *Fusarium verticilloides* was significantly (p=0.05) depressed by the active principle in the aqueous bark extract **(Figure 7)**.

D. Vegetative Growth of *A. flavus, A. niger and F. verticilloides* in Potato Dextrose Broth Amended with the Ethanol Bark Extract of *E. uniflora*.

Dry weight accumulation of all the three test fungi were significantly depressed by the active principle in the ethanol stem bark extract. **(Figure 8)**.The final dry weight of the highest concentration of the extracts never approximated that of the control.

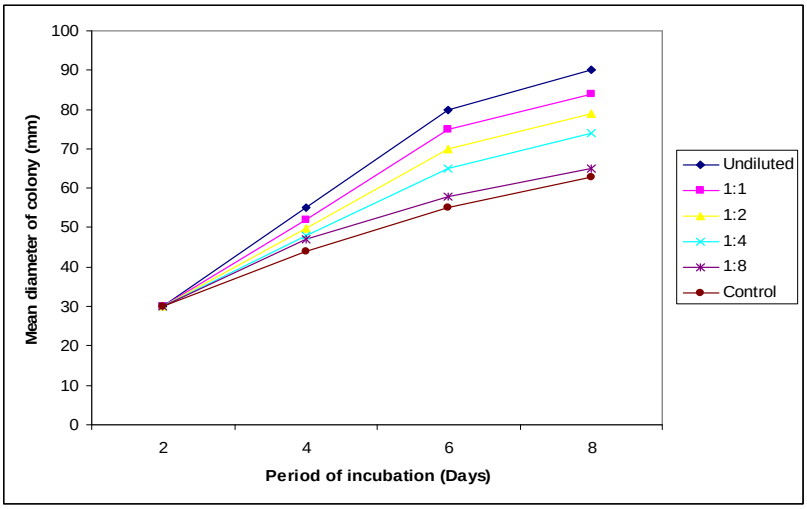

Figure 1: Radial growth of mycelium of *Aspergillus flavus* on PDA amended with varying dilutions of aqueous bark extract of *Eugenia uniflora* at 30 ^{o}C

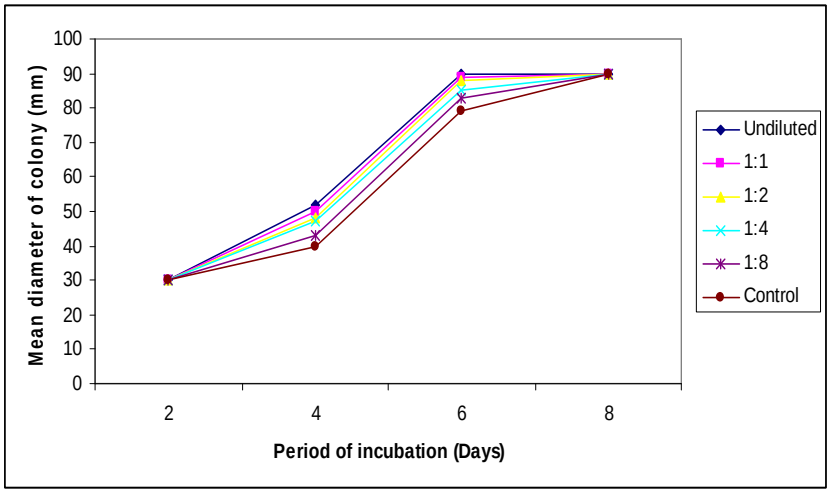

Figure 2: Radial growth of mycelium of *Aspergillus niger* on PDA amended with varying dilutions of aqueous bark extract of *Eugenia uniflora* at 30 °C

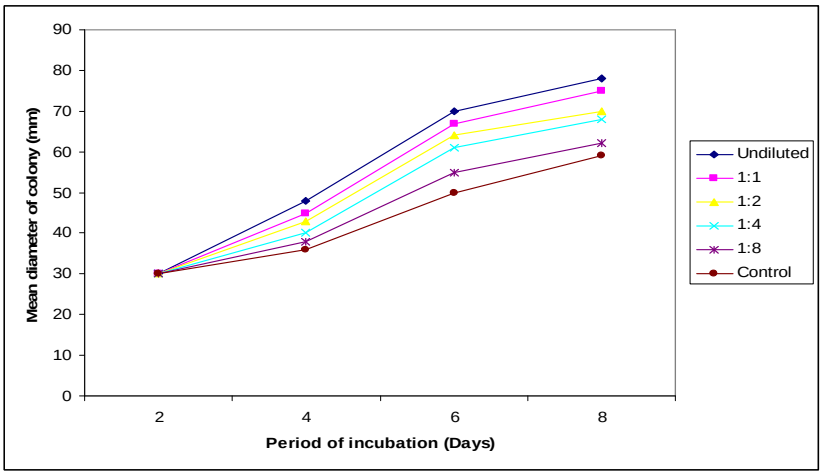

Figure 3: Radial growth of the mycelium of *Fusarium verticilloides* on PDA amended with varying dilutions of aqueous bark extract of *Eugenia uniflora* at 30°C

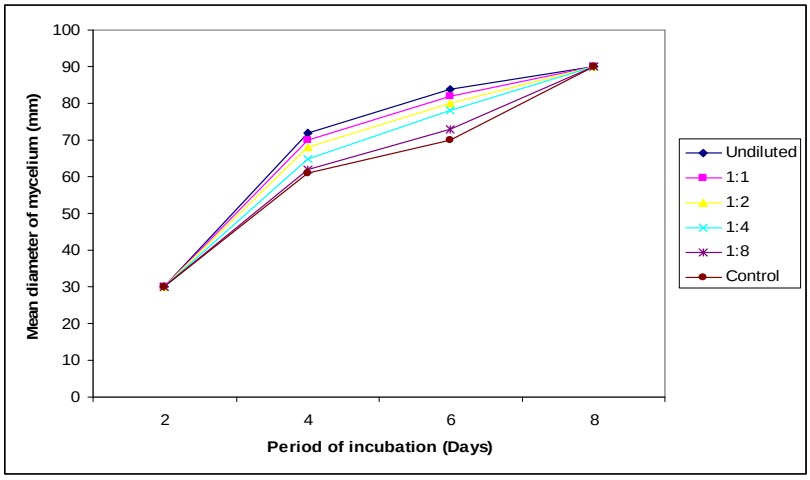

Figure 4: Radial growth of the mycelium of *A. flavus* on PDA amended with varying dilutions of ethanol bark extract of *Eugenia uniflora* at 30°C

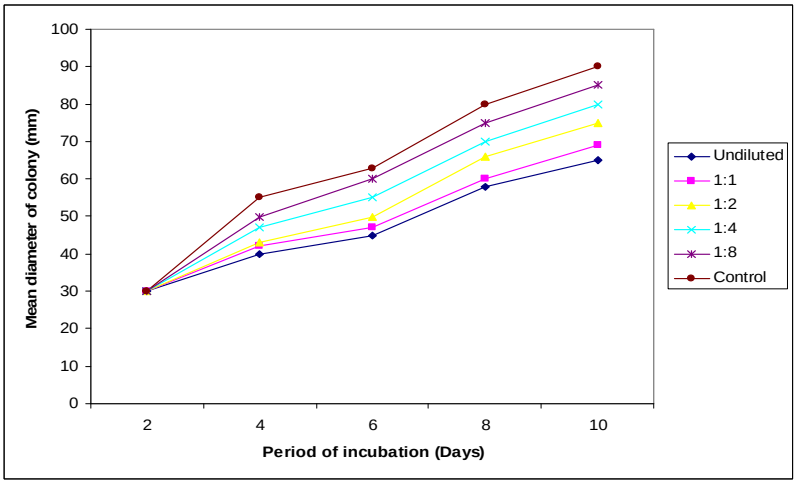

Figure 5: Radial growth of the mycelium of *A. niger* on PDA amended with varying dilutions of ethanol bark extract of *Eugenia uniflora* at 30°C

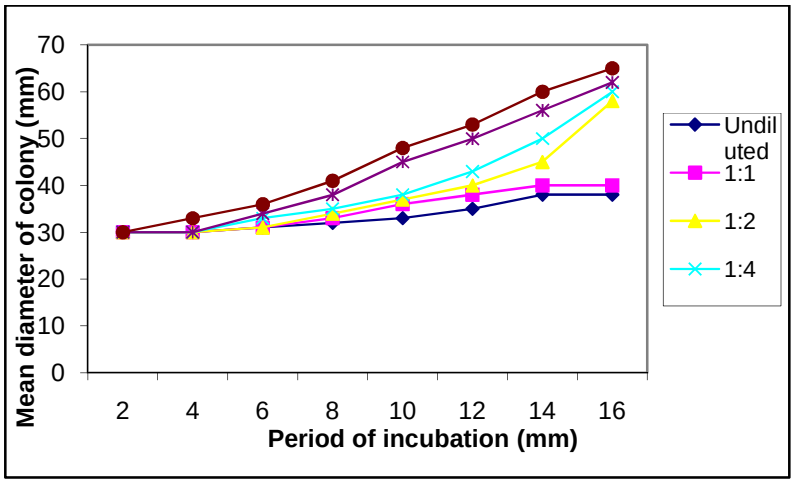

Figure 6: Radial growth of mycelia of *Fusarium verticilloides* on PDA amended with varying dilutions of ethanol bark extract of *Eugenia uniflora* at 30°C

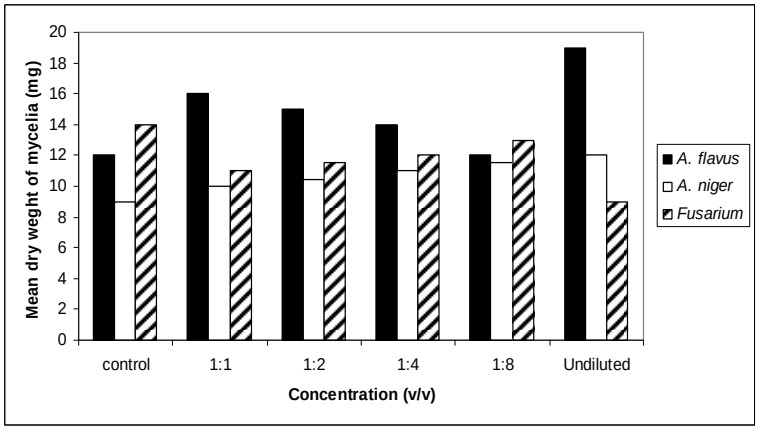

Figure 7: Vegetative growth of *A. flavus*, *A. niger* and *F. verticilloides* in Potato Dextrose Broth amended the indicated varying concentrations of the aqueous stem bark extract of *E. uniflora* for 6 days at 30°C

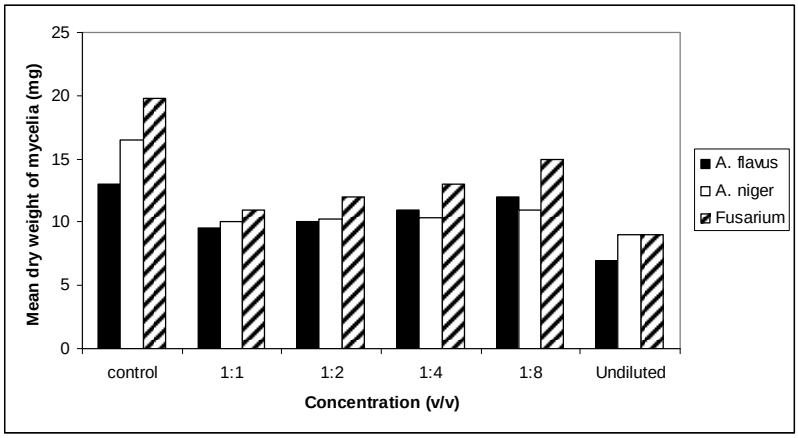

Figure 8: Vegetative growth of *A. flavus*, *A. niger* and *F. verticilloides* in Potato Dextrose Broth amended the indicated varying concentrations of ethanol extract of stem *E. uniflora* for 6 days at 30°C

DISCUSSION

In order to control toxigenic fungi in a more sustainable and environmentally friendly way, research on biofungicides instead of the commonly used fungicides is getting much attention. Plant products and plant extracts provide major sources of naturally occurring biofungicides to realize this aim.

More than 280 plant species have been investigated for their inhibitory effect on toxigenic *Aspergillus spp*., and nearly one hundred of them have some activity on growth or toxic production by these fungi **[11]**. Antifungal and antitoxigenic activity of these plant parts and extracts depend not only on their components but also on the chemical structure of these components **[12]**. The leaves of *E. uniflora* contain compounds such as citrnellel, geranyl-acetate, geraniol, ciberol, terpinene, sesquiterpenes and polyterpenes whereas the bark contains tannins recorded as 28.5% and of use in tanning **[13]**.

The ethanolic extract of *E. uniflora* inhibited the growth of dimorphic fungus *Paracoccidioides brasiliensis* at 750mgml^{-1}. The best minimum inhibitory activity was shown by the ether fraction with minimum inhibitory concentration (MIC) at 187.5mgml^{-1}. **[14]**.

The results of this study showed that aqueous and ethanolic extracts of the bark of *Eugenia uniflora* could be both promotory and inhibitory. The differing effects of the extracts against the test fungi may provide biological confirmation of the variations in chemical composition/active ingredients of the bark extracts. This differing effect, confirms the results of **[15]** who also observed the same effect when they tested for the antimicrobial efficacy of the essential oils of leaves and fruits of *Eugenia uniflora* collected at different times against *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Candida albicans* and *Trichophyton mentagrophytes.*

In the present study, the ethanolic extracts depressed both radial and vegetative growth of the test fungi, whereas the aqueous extract showed differing effects. This also confirms reports that the ethanolic extracts of plants are more inhibitory than aqueous extracts. Presumably, ethanol may be a better extracting solvent than aqueous extracts, the former yielding more potent active ingredients especially for the lower boiling constituents. Whether this is true for *E. uniflora* or not, may be elucidated by further studies.

The bioactivity of the bark extracts diminishes with an increase in extract dilution but high dilutions may even promote growth of the test organism **[16]**. They proposed that the fungal growth promotion may be due to either small

quantities of a very active compound or large quantities of a weakly active compound; but the sensitivity of the fungi to the bark extracts is also likely to be influenced by the nature of the medium. It is also possible that the different test fungi may have common ways of sensing their environments and initial stages of reacting to a toxicant. After this initial control mechanism is "activated", each organism then has its own unique way of reacting to the different active principles in the extracts.

CONCLUSION

In conclusion, this study has demonstrated that bark extracts, especially ethanolic extracts, have the potential of suppressing the growth of some toxigenic fungi. The future of using plant extracts and plant products to control such fungi is promising; moreover, they are less expensive and less hazardous to the environment.

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