

Evaluation of Antifungal Activity of *Amaranthus viridis* L. (Amaranthaceae) on Fusariosis by *Piper nigrum* L. and on Anthracnose by *Musa* sp.

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Abstract: The plants have investigated in the search for new substances against microorganisms resistant to current pesticides and antibiotics. The aim of this research was to evaluate the existence of antiphytopathogenic properties of organic extracts from the leaves of amaranth (*Amaranthus viridis* L.), Amaranthaceae, popularly known as caruru. The hexanic, dichloromethanic, ethylic acetate and ethanolic extracts were obtained, respectively with yields of 2.2, 2.4, 3.2 and 3.6% (m/m). These extracts were used to determine the Minimum Inhibitory Concentration (MIC), through dilution technique using 96 well microplate. After reading the CIM in wells where no fungal growth was observed, the Minimum Fungicidal Concentration (MFC) was determined on plates containing dextrose Sabouraud agar. The experiments with the extracts of *Amaranthus* indicates activity against the fungi *Colletotrichum musae* (Berk. and Curt.) Arx, causing anthracnose of banana and against *Fusarium solani* f. sp. *piperis* responsible for fusariosis in black pepper. In relation to *Colletotrichum musae* extracts obtained with dichloromethane, ethyl acetate and ethanol, the MIC ranged from 15.6-250.0 $\mu\text{g mL}^{-1}$. The hexanic, ethylic acetate and ethanolic extracts showed activity against *Fusarium solani* with MIC ranging from 31.2-250.0 $\mu\text{g mL}^{-1}$. Through this research was showed the presence of antifungal constituents in extracts of *Amaranthus viridis* L., revealing its potential antimicrobial effect against these two phytopathogenic strains tested.

Key words: *Amaranthus viridis*, *Colletotrichum musae*, *Fusarium solani*, *Piper nigrum*, *Musa* sp., Brazil

INTRODUCTION

From the Bordeaux mixture, introduced in 1882 by Pierre-Marie-Alexis Millardet as a fungicide for the control of agricultural pests, researchers have been looking for new substances, obtained both synthetically and by means of extracts from plants or microorganisms, for overcome acquired resistance to fungal products used in agriculture. The plants through their secondary metabolism, producing substances related to your defense mechanism in response to attacks by predators such as viruses, bacteria, fungi, parasites, insects, molluscs or higher animals (Barbosa, 2004; Okunlola *et al.*, 2008; Antwi-Boasiako and Abubakari, 2011). In agriculture, the rot anthracnose or post-harvest banana (*Musa* sp.) is caused by *Colletotrichum musae* (Berk. and Curt.) Arx (Zambolim *et al.*, 2002) and *Fusarium solani* f. sp. *piperis* is linked to Fusarium wilt of black pepper. The Brazilian participation in the international banana market is still relatively small, although it is the second largest producer. This is caused by several factors including the volume of post-harvest losses which in

Brazil is estimated at 1.98 million ton per year that corresponds to about 30% of national production (Benato, 1999). The main post-harvest losses are due to numerous physical, physiological and microbiological. The main form of control of most diseases in post-harvest that affects many fruits is accomplished through the use of thiabendazole, methyl thiophanate, prochloraz and other fungicides. However, the application form and the emergence of resistant pathogens have been limited the use of agrochemicals on the market today. Due to this resistance, several methods for controlling diseases such as physical, biological and alternative in which essential oils are used and/or plant extracts have been studied in order to replace or reduce the use of fungicides (Cia *et al.*, 2007; Bastos and Albuquerque, 2004).

The black pepper was introduced into the North of the Espirito Santo state and South of Bahia state as an alternative to improve the income of small and medium agricultural producers. Associate with cassava, beans, conilon coffee and banana, these cultures provides greater assurance of fixation on field and survival of many farmers. The black pepper (*Piper nigrum* L.) is a climbing

plant belonging to the family Piperaceae. It is originally from Southeast Asia, specifically India and it is the most common and important of the spices (Costa *et al.*, 2011). It is a specie that presents a short period of immaturity which makes commercial production from the second year of cultivation. However, the black pepper is very fragile against an infestation by the fungus *Fusarium solani* f. sp. *piperis*.

Although in recent decades, industries have developed a significant number of new chemical entities, microbial resistance to these substances has increased exponentially. Genetically, the microorganisms acquire resistance to fungicides and transmit to new generations. The development of new agro-chemicals is slow, requiring nearly a decade to be available commercially. In parallel, microbial resistance is rapidly and continuously and induces the adoption of important measures to tackle the problem through, for example control of pesticide use, the continuity of research to improve understanding of genetic mechanisms of microbial resistance and get new natural or synthetic pesticides (Sibtain *et al.*, 2002; Abera *et al.*, 2011).

Among the angiosperms, the Amaranthaceae family is composed by 160 genera and about 2,400 species, most of which is herb or shrublet and there are few trees. It is a cosmopolitan family whose species are found on all continents. The *Amaranthus* genus has several species with medicinal and nutritional value such as *A. blitum*, *A. spinosus* and *A. tricolor* or *A. mangostanus*, originating from Asia. The species *A. caudatus* is originally from South America, mainly in the Andes regions. The *A. cruentus* or *A. paniculatus* is native of Guatemala and Central America, the species *A. leucocarpus* or *A. flavus* are found in Mexico and *A. retroflexus* in North America. The species *A. dubius*, *A. deflexus* and *A. hybridus* are natural in South America and *A. viridis* or *A. gracilis* in Africa (Teutonico and Knorr, 2011; Lorenzi and Matos, 2008).

In studies with *Amaranthus spinosus*, it was verified the presence of 7-p-coumaroylapigenin-4-O- β -D-glucopyranoside, spinoside, xylofuranosyluracil, β -D-ribofuranosyladenine, hydroxycinamates, kaempferol and quercetin glycosides, betalains, betaxanthin, betacyanin, amaranthine and isoamaranthine, gomphrenin, betanin, β -sitosterol, β -sitosterol glycoside, stigmasterol, linoleic acid, rutin and β -carotene (Suryavanshi *et al.*, 2007; Odhava *et al.*, 2007; Barminas *et al.*, 1998). The species *Amaranthus viridis* L. (or *A. gracilis*), popularly known as caruru, caruru-de-mancha, amarantu verde e caruru verdadeiro is an annual herb, erect with height of 40-100 cm, little branched, slightly pigmented with thick stems and rather fleshy and it only multiplies by seeds. It

is a plant native from Africa that is fully adapted to soil conditions of the Brazilian territory where he is currently regarded as a weed (Lorenzi and Matos, 2008; Teutonico and Knorr, 2011).

Due to the need to develop environmentally friendly agrochemicals against microorganisms resistant to current fungicides, this study evaluated the antifungal activity of hexanic, dichloromethanic, ethylic acetate and ethanolic extracts, made from leaves of *Amaranthus viridis* L. (Amaranthaceae), to an alternative to replace synthetic fungicides used to control pathogenic fungi responsible for anthracnose of banana (*Musa* sp.) and fusariosis of black pepper (*Piper nigrum* L.).

MATERIALS AND METHODS

Plant material: *Amaranthus viridis* L. (Amaranthaceae) was collected on the campus of the Universidade Federal of Espirito Santo, Sao Mateus, Espirito Santo. The leaves were washed, dried at $40\pm 5^{\circ}\text{C}$ and then rasured and stored in tightly sealed glass container.

The extraction and phytochemical screening: To obtain the organic extracts, powder *Amaranthus viridis* (50 g) was ground to obtain extracts sequentially with hexane, dichloromethane, ethyl acetate and ethanol by turboextraction in the proportion of 1:10 solvent for 30 min with intervals of 5 min. The following solvent of greater polarity, only was added to plant material after removal of all organic solvent used previously. For each solvent, the resulting solution was filtered through a fine mesh fabric (voil) then filter paper and again on filter paper inserted into the Buchner funnel attached to kitassato and under the reduced pressure. The extracts were concentrated in a rotary evaporator under refrigeration and then subjected to lyophilization for 72 h, yielding, respectively 2.2, 2.4, 3.2 and 3.6% (m/m) of Hexane (EHAS), Dichloromethane (EDAS), Ethyl acetate (EAEAS) and Ethanol (EEAS). The phytochemical screening of extracts for the chemical classes: saponins, organic acids, sugars, polysaccharides, phenols and tannins, flavonoids, alkaloids, cardiac glycosides, steroids, terpenoids and carotenoids was performed according to literature (Barbosa, 2004; Nalubega *et al.*, 2011).

Antifungal assay: The biological tests were performed by microdilution with dextrose Sabouraud broth according to standard methodology for fungi. The inoculum was prepared with recent strains of *Colletotrichum musae* (Berk. and Curt.) Arx and *Fusarium solani* f. sp. *piperis*. Dextrose Sabouraud broth maintained at $28\pm 2^{\circ}\text{C}$ for 48 h.

The inoculum was prepared in saline solution whose suspension was standardized by the tube at 0.5 McFarland scale to obtain the suspension with 1.0×10^6 – 5.0×10^6 spores mL^{-1} .

The tests were performed in 96 well microplate based on the concentration of 1000.0–3.9 $\mu\text{g mL}^{-1}$ of four organic extracts for determination of Minimum Inhibitory Concentration (MIC). To ensure no contamination or the inefficacy of the environment for fungal growth, the last three wells were used for the control of the medium, the corresponding statement extract/cetoconazole and fungus. The experiment was performed in quintuplicate and incubated at $28 \pm 2^\circ\text{C}$ for 60 h to MIC determination. The Minimum Fungicidal Concentration (MFC) was carried out on plates after reading the MIC wells where no fungal growth was observed. The positive control was conducted with ketoconazole at concentrations of $50.0 \mu\text{g mL}^{-1}$. Statistical analysis was performed using analysis of variance test, considering significant values for $p < 0.05$.

RESULTS AND DISCUSSION

Medicinal plants again are the focus of consumer and research in recent decades, both in traditional medicine and for the development of new chemical entities in control of microorganisms resistant to current antimicrobial agents (Hassan *et al.*, 2007; Musyimi *et al.*, 2008; Sarwar *et al.*, 2011). In search of new actives chemical entities, phytochemical screening of the extract from leaves of *Amaranthus viridis* L. (Amaranthaceae) indicated the presence of biologically active constituents: saponins, tannins and phenols, flavonoids, alkaloids, cardiac glycosides, steroids and triterpenoids, showing resemblance to the research done by Maiyo *et al.* (2010) in which there phytochemical constituents and antimicrobial activity of the extracts with hexane, dichloromethane, ethyl acetate and methanol from leaves of *Amaranthus hybridus* species, *Amaranthus caudatus* and *Amaranthus spinosus*. Compounds of the classes of flavonoids, terpenoids and tannins have shown antimicrobial activity (Neogi *et al.*, 2008; Louis *et al.*, 2011). The results obtained from tests on organic extracts from *Amaranthus viridis* was found inhibitory activity ($p < 0.05$) growth of *Colletotrichum musae* (Berk. and Curt.) Arx with extracts obtained with dichloromethane, ethyl acetate and ethanol with MICs between 15.6–250.0 mg mL^{-1} . The extracts with hexane, ethyl acetate and ethanol showed activity for the fungus *Fusarium solani* sp. with MICs between 31.2–250.0 mg mL^{-1} (Table 1). At concentrations up to 1000.0 mg mL^{-1} and the EDAs EHAs showed no activity, respectively

Table 1: Results of evaluation tests by the method of microdilution plates of the activity of organic extracts of *Amaranthus viridis* L. (Amaranthaceae) against phytopathogenic fungi

Substance ($\mu\text{g mL}^{-1}$)	<i>Colletotrichum musae</i> (Berk. and Curt.) Arx		<i>Fusarium solani</i> f. sp. <i>piperis</i>	
	MIC	MFC	MIC	MFC
Control ¹	-	-	-	-
Control ²	+	+	+	+
Control ³	-	-	-	-
EHAS ⁴	-	-	250.0	250.0
EDAS ⁴	125.0	250.0	-	-
EAEAS ⁴	15.6	15.6	31.2	62.5
EEAS ⁴	31.2	125.0	62.5	62.5
Cetoconazole ⁵	-	-	-	-

¹Extract/drug control; ²Microorganism control; ³Dextrose Sabouraud broth control; ⁴Organic extracts: Hexane (EHAS), Dichloromethane (EDAS), Ethyl Acetate (EAEAS) and Ethanol (EEAS) concentrations 1000.0–3.9 $\mu\text{g mL}^{-1}$; ⁵Antifungal control 50 $\mu\text{g mL}^{-1}$; (-) not detected microbial growth, (+) microbial growth

against *Fusarium solani* and *Colletotrichum musae*. For the analysis of Table 1, extracts of *Amaranthus viridis* L. (Amaranthaceae), the ethylic acetate extract was the most power to *Colletotrichum musae* (Berk. and Curt.) Arx because it has fungicidal activity in small concentration, i.e., concentration 15.6 mg mL^{-1} and both strains fungi did not grow in the positive control chosen, i.e., in the middle with the antifungal ketoconazole. According to Rios and Recio (2005), plant extracts with MIC $< 100 \text{ mg mL}^{-1}$ are very promising. For *Colletotrichum musae* (Berk. and Curt.) Arx, all extracts with polar solvents dichloromethane, ethyl acetate and ethanol which extract polar substances with chemical groups: hydroxyl, carbonyl, carboxyl, amino, nitro, halide, nitro and cyan had fungicidal activity, changing only the minimum inhibitory and fungicides concentrations which suggests and serves as an indication for fractionation and identification of substances that could serve as prototypes for the development of new antifungal agents. In the study conducted by Pinho *et al.* (2010) with several varieties of banana notes that all are susceptible to anthracnose caused by *Colletotrichum musae* which requires applications of fungicides throughout the production process. Fact observed with fusariosis of black pepper kingdom whose fungi eventually acquiring resistance (Miranda *et al.*, 2007).

Ali *et al.* (2010) evaluated the antimicrobial activity of extracts obtained with hexane, chloroform and methanol of dried barks of *Cinnamomum impressicostatum* and *Cinnamomum pubescens*. The hexane and chloroform extracts of the plants strongly suppressed the growth of the four fungi: *Saccharomyces cerevisiae*, *Candida lipolytica*, *Candida albicans* and *Microsporium canis* but range from moderate to weak towards bacteria: *Staphylococcus aureus*, *Streptococcus epidermidis*, *Bacillus cereus*, *Pseudomonas aeruginosa* and

Burkholderia cepacia. The methanol extracts only displayed weak activity on some of these organisms. Regasini *et al.* (2010) in research on plant extracts *Pterogyne nitens* Tul. observed that the fractions extracted with butanol showed antifungal activity with MIC from 15.6 mg mL⁻¹ for fungi *C. albicans*, *C. krusei*, *C. parapsilosis* and *C. neoformans*.

Costa *et al.* (2011) investigated the antifungal activity of essential oil of *Syzygium aromaticum* (L.) Merr. and L. M. Perry on the *in vitro* mycelial growth of pathogenic fungi *Rhizoctonia solani*, *Fusarium solani*, *Fusarium oxysporum* and *Macrophomina phaseolina*. The essential oil of clove showed fungicidal activity at a concentration of 0.15% on the growth of *R. solani*, *F. oxysporum* and *F. solani* but showed no such activity on *M. phaseolina*. The analysis by gas chromatography coupled with mass spectrometry enabled the identification of eugenol (83.6%), eugenil acetate (11.6%) and caryophyllene (4.2%). In organic agriculture has been widely used plant extracts to control agricultural pests in order to have an ecologically correct production. Among these, we mention the extracts of Neem (*Azadirachta indica* A. Juss), Nettle (*Nettle dioica* L.) and garlic (*Allium sativum* L.) used in agricultural pest control (Solomon *et al.*, 2008; Deffune, 2001; Santos *et al.*, 2010).

This research supports the need to continue searching for antifungal substances in the flora, however it should be noted that environmental conditions such as soil, moisture and temperature can influence the antimicrobial activity of extracts and essential oils so that for the same plant collected from different environments, due to the variation of phytochemicals present due to change of the stress to which the plant is submitted (Celiktas *et al.*, 2007).

CONCLUSION

In the extraction of the leaves of *Amaranthus viridis* L. (Amaranthaceae) by turboextraction with solvent hexane, dichloromethane, ethyl acetate and ethanol were obtained with respectively 2.2, 2.4, 3.2 and 3.6% (m/m) extracts. The phytochemical screening showed the presence of biologically active constituents belonging to the group of saponins, tannins and phenols, flavonoids, alkaloids, cardiac glycosides, steroids and triterpenoids in the extracts of *Amaranthus viridis* L. In the biological tests was identified antifungal activity of extracts against *Colletotrichum musae* (Berk. and Curt.) Arx and *Fusarium solani* f. sp. *piperis*.

To *Colletotrichum musae* (Berk. and Curt.) Arx with extracts obtained with dichloromethane, ethyl acetate and ethanol, the MIC ranged from 15.6-250.0 µg mL⁻¹. Hexanic,

ethyl acetate and ethanolic extracts showed activity against *Fusarium solani* sp. with MIC between 31.2-250.0 µg mL⁻¹. Therefore, this extract is a good candidate for isolation and identification of substance with potential fungicide activity.

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