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Full Length Research Paper

In-ovo biological activities of *Phyllanthus amarus* leaf extracts against Newcastle disease virus

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Newcastle disease virus is a paramyxovirus which causes Newcastle disease in birds. Investigation was done on the effect of leaf extract of *Phyllanthus amarus* against Newcastle disease virus (NDV) using an *in-ovo* assay. Nine to eleven day-old viable embryonated chicken eggs (ECE) were used for the assay, these were divided into six groups of six eggs each. Methanol, aqueous and n-hexane extracts of the plant leaves were administered to the various groups at concentrations varying from 50 to 5 mg/ml. Embryonated eggs were incubated and embryo survival was monitored daily. Negative control and diluents control groups received phosphate buffer saline (PBS) and dimethly suphoxide (DMSO), respectively. The other group was uninoculated while a virus control group received 100 EID_{50}/0.1 ml NDV alone. Bacteria free allantoic fluid from the embryonated eggs in different treatment groups were harvested and collected for spot hemagglutination (HA) test and HA assay to detect the presence of NDV viral particles and the viral titre, respectively. Leaf extracts were assayed for presence of phytochemicals and antioxidant potentials. It was observed from the results that the extract was toxic to the embryo at a concentration above 50 mg/ml and further results showed that the HA viral titre reduction was directly proportional to increasing extract concentration. The phytochemical assays of leaf extract revealed the presence of phytochemicals including alkaloids, tannins, saponins, flavonoids, phenols, steroids, glycosides. The current findings have demonstrated that leaf extract from *P. amarus* has potentials of medicinal value as well as antiviral activity against NDV *in-ovo*. Further experimental assays using live animal models are recommended to validate the use of *P. amarus* plant extract in therapeutic measure in chickens.

Key words: Antiviral, *Phyllanthus amarus*, Newcastle disease virus (NDV), embryonated chicken eggs (ECE).

INTRODUCTION

For the past years, viruses remains a threat to human and animal lives, the prevention and alleviation of these viruses and its infections is necessary and cannot be overemphasized (Fajimi and Taiwo, 2005). However,

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vaccines have been developed for some of these viral diseases but there is need for pursuit in discovery of therapeutic agents for cases of unvaccinated individuals or animals exposed to these infections. Most of the antiviral agents that are currently being used are limited in therapeutic potencies and may possess several other problems in the clinical practices such as tolerability, contraindications, toxicity amongst others not to mention the availability and cost of the drugs, hence the need for an alternative approach or therapeutic measures (Asres et al., 2005; Verma et al., 2014).

Plants on the other hand are a rich source of essential nutrients (Abad et al., 2000), chemicals and other active component (Babich et al., 2003). Some of the existing therapeutic agents or drugs were developed based on chemical compounds isolated from plants (Babich et al., 2003). These plants exist in different parts of the world such as Asia, Europe and Africa. However, these plants and many more may have not been exhaustively explored which brings us to the ideas of possibilities of creating or discovering new products or antiviral agents from plants as the use of medicinal plants in treatment of animal and human diseases has become more prominent. In Nigeria and many other African countries, rural and urban communities have continued to use or consult local herbs/medicine-men for remedies to a variety of diseases. Such plants include *Phyllanthus amarus* which is one of the species belonging to a large family, Euphorbiaceae. It is found in the tropical and sub-tropical region worldwide (Joseph and Raj, 2011). It is commonly called “iyin olobisowo” in yoruba and said to possess claims of medicinal values which include hepatoprotective, anti-diabetic, anti-hypertensive, analgesic, anti-inflammatory and anti-microbial properties (Adeneye et al., 2006; Okiki et al., 2015). Chemical compounds including alkaloids, flavonoids, lignans and phenols have been reportedly isolated from this plant (Adeneye et al., 2006). However, the antiviral potentials of this plant have not been exhaustively explored.

Newcastle disease (ND) is a highly infectious disease of domestic and wild birds which is caused by Newcastle disease virus (NDV) and is widely regarded as one of the most important avian diseases belonging to the family paramyxoviridae (Young et al., 2002). It is a threat to most avian species but chickens are the most susceptible with clinical disease (Young et al., 2002). Newcastle disease virus can be a causative agent for conjunctivitis in humans usually in persons exposed to large quantities of the virus.

Newcastle disease currently can be prevented or managed by due and proper vaccination but has no treatment. Beyond this, the cost, challenges faced in availability and distribution of vaccine and lack of treatment is a major setback in alleviation of this disease. In view of aforementioned, this study evaluated the antiviral potentials of plant extracts from *P. amarus* against NDV which will provide information that may serve as basis for further researches and also may lead to development of cheaper, more available and effective alternative approach to control and or novel drugs for therapeutic purposes.

MATERIALS AND METHODS

Collection and identification of the plant material

Leaf parts of the study plant were collected from their natural habitats in Ado-Ekiti, Nigeria through the months of April and June, 2016. It was identified at the Department of Biological Sciences, Afe Babalola University, Ado-Ekiti using standard keys and description.

Preparation of plant extract

Leaves of study plant were air dried under shade and ground into powdery form prior to extraction process. The extraction was carried out according to Oladunmoye (2012). The resulting weight of the powdered form was 500 g which was exhaustively extracted at a ratio of 1:4 (powder weight to solvent volume) with multiple solvents namely n-hexane, water and methanol. The leaf extract was concentrated *in vacuo* using a rotary evaporator at 40°C, while the un-evaporated solvent remaining in the extract was left to air-dry which gave a residue weighing 10.50 g. The concentrated extract was reconstituted in 10.5 ml of dimethyl sulphoxide (DMSO) to give a stock of a concentration of 1000 mg/ml, which was used for further testing at varying concentrations.

Antiviral assay

Acute toxicity assay of leaf extract

This was done to evaluate the effect of extract toxicity on the embryo of the embryonated chicken egg (ECE). Egg toxicity assay was used to estimate the minimum toxic concentration of the extract in nine days old ECE using 0.2 ml of each concentration to inoculate five viable ECE and the control group received 0.2 ml DMSO. Inoculated eggs were incubated for 4 days at 37°C and monitored twice daily for mortality by the process of candling. Extract toxicity was determined by examination of embryo for lesions and hemorrhages and by the percentage mortality of embryos.

Source of virus and 9-day old embryonated chicken eggs (ECE)

Velogenic strain of NDV was obtained from Viral Research Department, NVRI, Vom and embryonated chicken eggs (ECE) aged nine days were obtained from poultry division, NVRI, Vom, Nigeria.

Determination of EID_{50} of the virus

Fifty percent embryo infectious dose (EID_{50}) of the virus was determined according to the method of Young et al. (2002). From this, 100 EID_{50}/0.1 ml of the virus stock was made for the experiment.

Preparation of leaf extract/inoculum

This experiment was carried out in three groups, extracts were

treated with antibiotics (Penicillin, Streptomycin, Gentamycin, Amphotericin B (5x PSGA). The embryonated chicken eggs were properly labeled according to the extract concentrations and groups used. Sets of plastic egg crates/trays were thoroughly disinfected with Virkon and the embryonated chicken eggs were adequately swabbed with 70% alcohol before transferring into the disinfected trays to avoid contamination. Under a properly sterilized bio-safety cabinet, nine-day-old embryonated chicken eggs were divided into groups of six, the eggs were punched and inoculated with the extract and virus according to the grouping through the allantoic route. The predetermined concentrations studied were 50, 25, 10 and 5 mg/ml. In the first group designed to test the prophylactic effect, each predetermined concentration of extract at 0.2 ml was inoculated into the ECE and incubated for 90 min before the inoculation of 100 EID_{50}/0.1 ml of the virus. In the second group, the therapeutic effect was tested by inoculating 100 EID_{50}/0.1 ml of virus and incubated for 90 min before inoculation of the extracts after the 90 min at the different concentrations studied. The third group, a mixture containing 100 EID_{50}/0.1 ml of virus and 0.2 ml of the extracts at varying concentration was incubated at 4°C for 90 min, after which the mixture was inoculated at the same time into the ECE to determine the inhibitory potentials. A group inoculated with standard NDV 100 EID_{50}/0.1 ml was used as virus control, another group inoculated with 0.2 ml DMSO served as diluent control and another group of eggs also was not inoculated nor punched and this serves as un-inoculated control. The ECE were sealed with nail polish and incubated at 37°C in a humidified incubator. The ECE were candled daily and embryo survival observed. The experiment was terminated by chilling at +4°C after 96 h when the virus control group have all died (after 48 h). Bacteria free allantoic fluid from the different test groups was harvested for spot test and haemagglutination assay to detect the presence of NDV in the ECE.

Harvesting and spot haemagglutination test

Harvesting was done after chilling the eggs overnight in a 4°C refrigerator. Embryos that had been chilled were brought out of the refrigerator and kept at room temperature for about 30 min to thaw. The eggs were swabbed and put in the bio-safety cabinet. The shell of each egg was cracked opened at the air space and the allantoic fluid was siphoned with a syringe while using an harvesting spoon to prevent obstruction of the fluid, this was dispensed into a sterile universal bottle (which was labelled accordingly). Sterility of allantoic fluid was checked by culturing on blood agar and nutrient agar to confirm absence of bacteria. To spot test, a pipette was used to dispense a drop of 1% washed chicken red blood cells on a clean white tile while clean and sterile rubber wire loops was used to pick a drop of the allantoic fluid which was mixed with the drop of blood. The tile was gently rocked and observed for visible agglutination to indicate viral presence (OIE, 2012).

Haemagglutination assay

Haemagglutination assay was used to determine the presence and quantity of virus/viral particle in the allantoic fluid of the eggs. The haemagglutination assay was performed in V bottom shaped microtitre plates using 25 µl each of phosphate buffered saline (PBS), allantoic fluid and 1% chicken red bloods cells (OIE, 2012). This was performed in replicates and the mean titre value was recorded.

Qualitative phytochemical analysis of plant extracts

This was carried out in order to determine the presence of glycosides, tannin, saponin, phenol, alkaloid and flavonoid in the plant extracts (Hadi and Bremner, 2001; Wadood et al. (2013; Desphande and Kadam, 2013).

Determination of total phenol content and antioxidant capacity of plant sample

Total phenols were determined using Folin Ciocalteu reagent (McDonald et al., 2001) and antioxidant capacity assayed according to the method of Serpen et al. (2012) using inhibition of 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radicals expressed in trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) of extract expressed in gallic acid equivalents (GAE).

Data analysis

Data were analysed using SPSS version 21(IBM, USA). Values were reported in mean ± SEM. The mean differences were analysed using one way analysis of variance (ANOVA) for comparison between different extract types and significance reported at $P<0.05$. Further comparison within concentrations was done using a post hoc test (Turkey HSD).

RESULTS AND DISCUSSION

Table 1 shows the maximum toxic concentration of the methanol, aqueous and n-hexane leaf extracts against the embryonated chicken egg. It was observed that the maximum tolerable concentration of extract as measured by the embryonic death and morphology of the embryo in comparison with the controls was 50 mg/ml, therefore test were subjected to a concentration of 50 mg/ml and below. The diluent was safe for the embryo as no mortality was recorded. The antiviral assay revealed decreasing HA viral titre with increase in concentration of the extracts. It was observed that at a concentration of 50 mg/ml, the n-hexane extracts reduced the viral HA titre to 2, while the methanol and aqueous extracts reduced the HA titre to 56 and 128, respectively, however at a low concentration of 5 mg/ml, the leaf extracts exhibited insignificant reduction of viral titre in comparison with the viral control (Figure 1). Figure 2 shows the therapeutic activities of the leaf extracts of *P. amarus* against NDV, it was observed that n-hexane extracts reduced the viral HA titre from 1024 to 4 at a concentration of 50 mg/ml, while at 25 mg/ml, the viral HA titre was reduced to 32 and 128 at the lowest concentration of 5 mg/ml, however methanol and aqueous extracts did not show reduction in viral titre below 64 at the highest concentration studied. *P. amarus* n-hexane leaf extracts exhibited some inhibitory antiviral potentials by reducing the viral HA titre to 4 at 50 mg/ml and 128 at 5 mg/ml, while methanol and aqueous leaf extracts reduced the viral HA titre to 128 and 64 at a concentration of 50 mg/ml respectively. The methanol and aqueous extracts showed little to insignificant reduction in the viral titre value at concentration of 5 mg/ml (Figure 3). The phytochemicals screening revealed the qualitative presence of bioactive

Table 1. Acute toxicity assay of leaf extract of test plant.

Extract	Concentration (mg/ml)	No of eggs	No. of embryonic death/hrs				
			24 h	48 h	72 h	96 h	Total
***P. amarus* Aq**	250	5	0/5	0/5	1/5	2/5	3/5
	100	5	0/5	0/5	0/5	1/5	1/5
	50	5	0/5	0/5	0/5	0/5	0/5
	25	5	0/5	0/5	0/5	0/5	0/5
	10	5	0/5	0/5	0/5	0/5	0/5
***P. amarus* Hex**	250	5	0/5	0/5	1/5	2/5	3/5
	100	5	0/5	0/5	0/5	1/5	1/5
	50	5	0/5	0/5	0/5	0/5	0/5
	25	5	0/5	0/5	0/5	0/5	0/5
	10	5	0/5	0/5	0/5	0/5	0/5
***P. amarus* meth**	250	5	0/5	1/5	4/5	0/0	5/5
	100	5	0/5	1/5	2/5	1/2	4/5
	50	5	0/5	0/5	0/5	0/0	0/5
	25	5	0/5	0/5	0/5	0/0	0/5
	10	5	0/5	0/5	0/5	0/0	0/5
Control	PBS	5	0/5	0/5	0/5	0/5	0/5
	DMSO	5	0/5	0/5	0/5	0/5	0/5
	Uc	5	0/5	0/5	0/5	0/5	0/5

B- *Phyllanthus amarus,* Vc- virus control, Uc- uninoculated control, Dc- diluent control, Meth- methanol, Hex- hexane, Aq- aqueous.

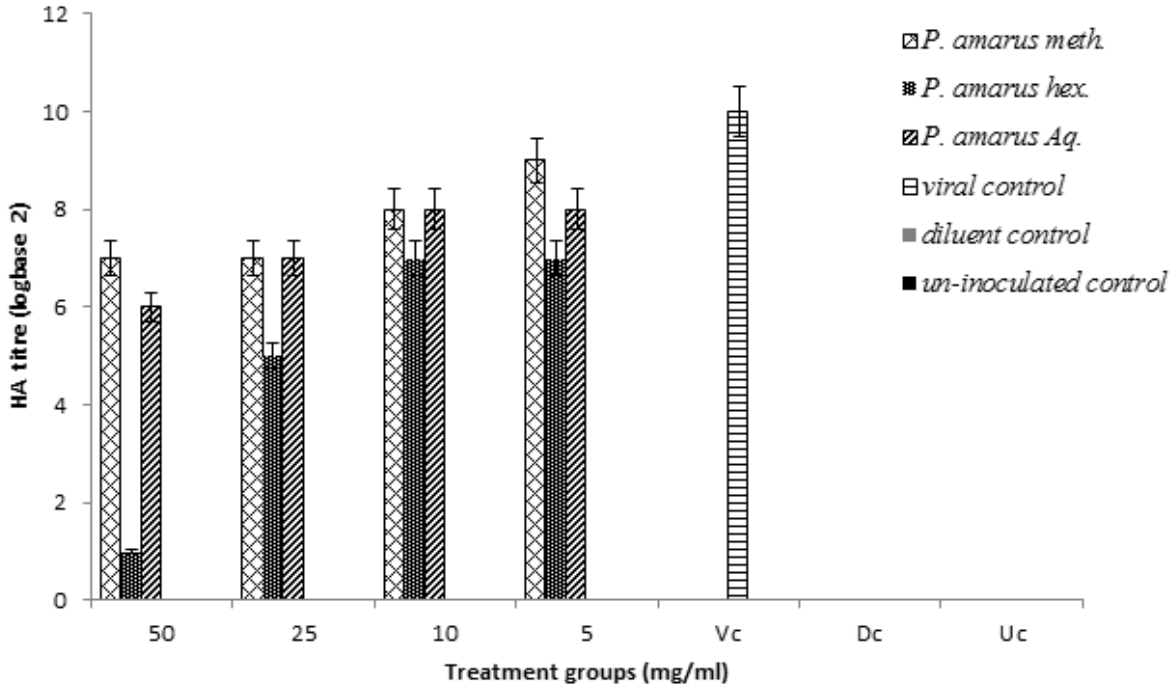

Figure 1. Prophylactic potentials of different extracts of test plant.

Table 2. Qualitative phytochemical properties of plant extracts.

Metabolites	B_{aq}	B_{Hex}	B_{Meth}
Alkaloids	+	+	+
Tannins	++	++	++
Phenol	++	++	++
Saponin	++	++	++
Terpenoids	+	+	+
Steroids	+	+	+
Cardiac glycosides	-	-	-
Glycosides	+	+	+
Flavonoid	+	+	+
Phlobatannin	+	-	-

B- *Phyllanthus amarus,* Meth- methanol, Hex- hexane, Aq- aqueous, - not detected, + slightly present, ++moderately present, +++ strongly present.

Table 3. Quantitative pytochemical values of plant leaf extracts.

Extracts	Alkaloids (mg/ml)	Phenols (mg/ml)	Saponin (mg/ml)	Tanins (mg/ml)	Flavonoids (mg/ml)
B(Aq)	0.248± 0.18	1.044± 0.02	0.252± 0.14	0.599± 0.10	0.696± 0.15
B(Hex)	0.290± 0.14	1.127± 0.19	0.288± 0.14	0.621± 0.01	0.590± 0.11
B(Meth)	0.263± 0.19	1.010± 0.17	0.264± 0.15	0.615± 0.11	0.593± 0.11

Results reported in mean of replicates ± SEM, n=3. B- *P. amarus,* Meth- methanol, Hex- hexane, Aq- aqueous.

ingredients in varying degrees which may be attributed to the bioactivity seen in the antiviral assay (Table 2). Table 3 revealed the quantitative presentation of the important active ingredients in the extracts. The study also revealed that the extract possesses antioxidant capacity as indicated by the value 38.943 ± 0.11 (mmol TEAC/100 g of sample) obtained from its percentage DPPH free-radical scavenging activity as well as the percentage ferric reducing antioxidant power of 165.076± 0.24 (mgGAE/100 g sample).

This antiviral investigation of *P. amarus* is suggestive of potentials present in the plant leaf extracts. It was observed that the leaf extracts exhibited no acute toxicity against the embryo at the concentration of 50 mg/ml and below, as no death was recorded and embryo appears normal which prompted further investigation on the extract's antiviral potencies (Table 1). The extracts were safe to the embryo at the concentration studied. However, it was revealed that the different extracts possess antiviral activities against NDV at different degrees. The extract demonstrated reduction in viral titre with increase in extract concentration. The treatment group exhibited a significant decrease ($F(4,10)=33.0$, $p=0.001$) in HA viral titre (Figures 1 to 3). This reduction led to the survival of some of the embryo of the ECE in the treatment group beyond the viral control group that has a titre of 1024 which all died 48 h post viral inoculation.

Further analysis showed that there was no significant mean difference between the viral titre at concentrations of 5 and 10 mg/ml ($P>0.05$), however at the concentration of 50 mg/ml, there was significant reduction ($P < 0.05$) in titre to a value of 2. It was observed that the prophylactic test group showed most significant reduction ($P< 0.05$) in viral titre than the other groups which could suggest that this plant extract when introduced into ECE before NDV infection could alienate the virus and serve as a prophylaxis. In the therapeutic and inhibitory groups, the leaf extracts also showed antiviral activities measured by reduction in the HA titre in comparison with the virus control group. The hexane extract showed significant reduction in the HA titre at all groups studied at the concentration of 50 mg/ml, indicating more potency when compared with the methanol and aqueous extracts (Figures 1 to 3). The diluents and un-inoculated control groups showed no reaction/agglutination as well as zero HA titre as no viral particle nor extract were inoculated into it. This biological properties exhibited by *P. amarus* could be attributed to the phytochemicals revealed in the plant extracts (Sofowora, 1993), however the varying degrees of the presence of this phytochemicals could be due to the ability of the solvents to extract some of the active ingredient or substances from the plant leaf based on its polarity (Table 3) (Simon et al., 2015). The free-radical scavenging activity of the leaf extracts may also possibly contribute to the antiviral effect exerted by the

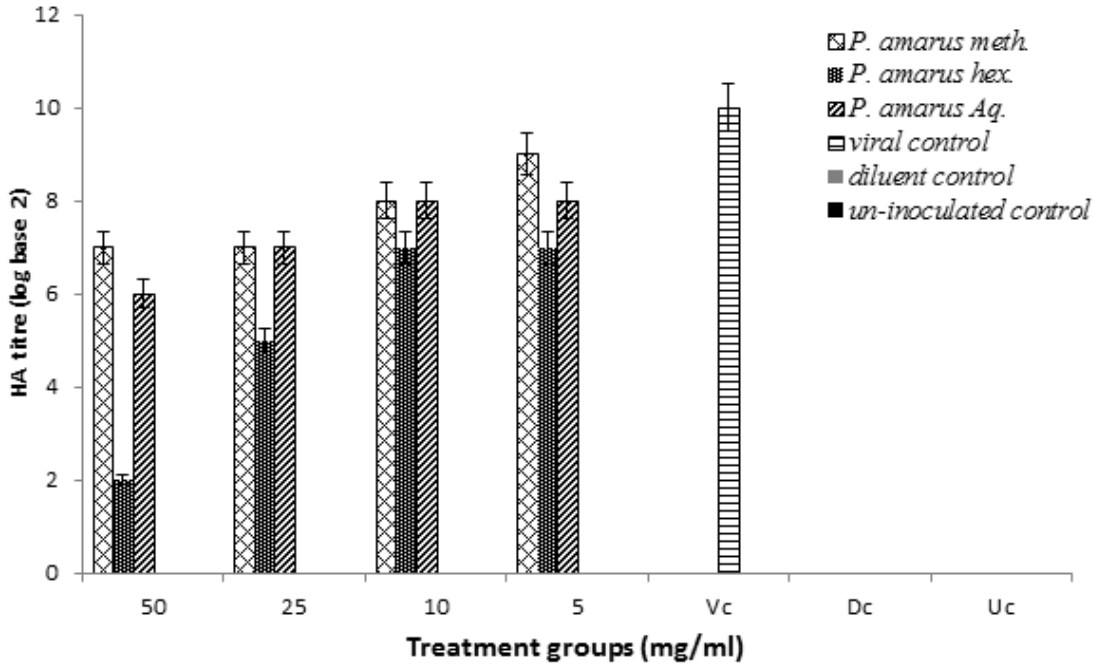

Figure 2. Therapeutic potentials of different extracts of test plant.

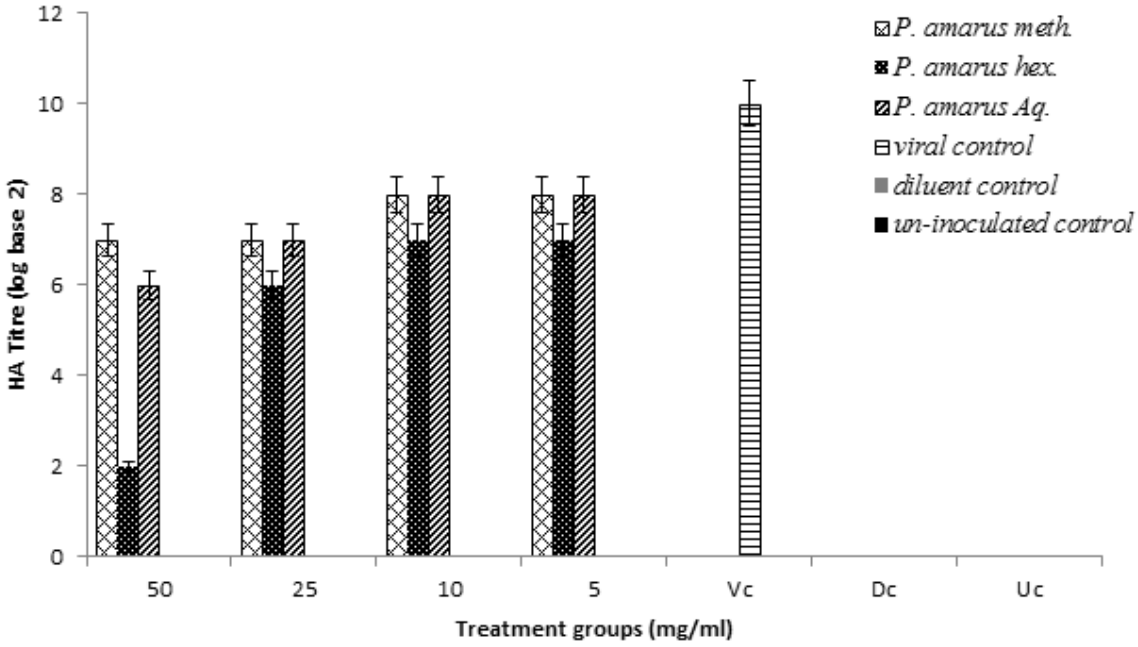

Figure 3. Inhibitory potentials of different extracts of test plant.

bioactive compounds present in the extract (Ayo et al., 2009), however this require validation by further investigation.

This research shows that the study plant may emanate as an alternative approach to prophylactic and therapeutic effect of viral infection which is in agreement with studies conducted by Chollom et al. (2012) and Bakari et al. (2013) that showed the anti-NDV potentials

Moringa olifera and C*ommiphora swynnertonii*, respectively.

Based on the result obtained from this study, it can be said that *P. amarus* extracts possess antiviral activity against NDV which can be a baseline for larger and further researches into tapping the antiviral potentials of this plant. It is recommended that a larger study and an *in vivo* study using live chickens that could possibly accommodate higher and varying concentrations be conducted in order to further validate the efficacy of the plant extract on anti-NDV and antioxidant activities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of essential oils for preventive or curative management of melon gummy stem blight and plant toxicity

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Melon cultivation is frequently heavily reliant on synthetic fungicides, including products used to control gummy stem blight caused by *Stagonosporopsis cucurbitacearum*. The essential oils used in controlling plant pathogens may offer an alternative to chemical pesticides. This study evaluated the effectiveness of essential oils to control the gummy stem blight in melon plants. *In vitro* tests were carried out using essential oils obtained from ripe noni fruit (*Morinda citrifolia*) and dehydrated leaves of the following plants: lemon grass (*Cymbopogon citratus*), citronella grass (*Cymbopogon nardus*), basil (*Ocimum basilicum*) and Mexican tea (*Chenopodium ambrosioides*) at different concentrations. A synthetic fungicide was used as control treatment. Results showed that the essential oils from noni and lemongrass had the highest effect on mycelial growth inhibition in *S. cucurbitacearum*. When applied on melon plants as a preventive measure, the essential oils from noni and lemongrass controlled gummy stem blight at the following concentrations: 0.03, 0.05, 0.1 and 0.3%. These results highlight the potential of essential oils to manage melon fungal diseases, which may result in reduction of pesticide application.

Key words: *Cucumis melo*, *Stagonosporopsis cucurbitacearum*. *Didymella bryoniae*, alternative control, plant disease.

INTRODUCTION

Gummy stem blight (GSB) is a major disease that strikes several cucurbit species and is caused by *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley, also known as *Didymella bryoniae* (Auersw.) Rehm (Steward et al., 2015). The fungus causes seedling damping-off, foliar lesions, as well as, stalk and stems cankers. It can be universally found in every continent and attacks at least 12 genera and 23

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species of cucurbit plants (Keinath, 2011), including some of the most popular crops grown in Brazil, such as watermelon, *Citrullus lanatus*, (Thunb.) Matsum. & Nakai and melon (*Cucumis melo* L).

Growers have considered synthetic fungicides to control cucurbit diseases. However, in addition to environmental pollution and contamination of consumable products, several cases of resistance to the main active fungicides on the market have been reported for GSB (Thomas et al., 2012; Keinath, 2013). Thus, there is a need for alternative control measures, such as dry season cultivation, thus avoiding environmental conditions favorable to the disease. According to Santos et al. (2011), leaf wetness, high relative humidity and temperatures between 20 and 30°C favor the development of the pathogen. Other methods of reducing the damage caused by GSB, include the use of partial genetic resistance (Santos et al., 2009) and biocontrol (Zhao et al., 2012).

Plant essential oils have been in use around the world mainly as insect repellents (Isman, 2000). More recently, their effect on the control of plant pathogens has aroused the interest of a number of researchers. For example, Guimarães et al. (2011) confirmed the inhibition of *Alternaria alternata in vitro* by the essential oil of lemon grass (*Cymbopogon citratus* (DC.) Stapf) and Aguiar et al. (2014) demonstrated the fungitoxic potential of eucalyptus essential oils (*Corymbia citriodora* (Hook.) K.D. Hill & L.A.S. Johnson) and citronella grass (*Cymbopogon nardus* (L.) Rendle) in the *in vitro* control of *Pyricularia grisea*, *Aspergillus* spp. and *Colletotrichum musae*. In another study, Hussain et al. (2008) described how basil oil (*Ocimum basilicum* L.) showed fungitoxic activity on *Aspergillus niger, Mucor mucedo, Fusarium solani, Botryodiplodia theobromae* and *Rhizopus solani.*

This study describes the effectiveness of the application of essential oils of noni fruit (*Morinda citrifolia* L.), lemon grass, citronella, basil and mastruz (*Chenopodium ambrosioides* L.) in the control of *S. cucurbitacearum* and of the gummy stem blight disease in melon plants.

MATERIALS AND METHODS

The study was carried out at the Phytopathology Laboratory of the Federal University of Tocantins, Gurupi campus as follows: one isolate of *S. cucurbitacearum* was taken from naturally infected melon plants in the municipality of Gurupi, TO. The identity of the isolate was confirmed by using the primers D7S (Koch and Utkhede, 2002) and UNLO28S22 (Bakkeren et al. 2000), which amplified 535 bp fragments of *S. cucurbitacearum*, in accordance with Gasparotto et al. (2011).

Securing the essential oils

The essential oils obtained from ripe noni fruit and the dehydrated leaves of lemon grass, citronella grass, basil and mastruz were extracted by the hydro distillation method, which consists of depositing 0.02 kg of material for extraction in 500 ml of distilled water for a period of two hours in a *Clevenger apparatus* (1928). At the end of the extraction period, the essential oils were collected in the form of supernatant, stored in amber flasks, identified, and finally kept at 4°C until the bioassay was implanted (Seixas et al., 2012, adapted).

Identification and quantification of the chemical constituents of essential oils

Qualitative analyses of the essential oils were carried out by gas chromatography coupled to GC-MS mass spectrometry. The constituents were identified by comparing their spectral profiles with those in the databases of the 229 Nist and Wiley libraries. Compounds had their identities confirmed by comparing their calculated retention indices with those found in Nist (2015) and in the literature (Adams, 2007). The retention index of a component is a number, obtained by interpolation, which links the retention time of the component under study to the retention time of two standards (usually hydrocarbons) eluted before and after the peak of the compound examined (Inczedy et al., 1998).

The quantification of compound contents, expressed as a percentage based on the normalization of areas, was obtained by gas chromatography and Shimadzu GC-2010 flame ionization detector (DIC).

In vitro mycelial growth inhibition tests

In vitro bioassays were carried out on Petri dishes (70 mm diameter) by testing different concentrations of essential oils (0.0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0%) and were compared with the synthetic fungicide Methyl Thiophanate + Chlorothalonil at 2000 ppm (Cerconil®), which served as a control treatment. A completely randomized experimental design was used in a factorial scheme, with four replications over five evaluation periods (2, 4, 6, 8 and 10 days of incubation). Each plate with an oil combination of X concentration was considered an experimental unit.

The different essential oil concentrations were diluted in a sterile water solution added to Tween 80 (1.0%). Subsequently, 100 µl of the different concentrations were scattered on the surface of the BDA culture medium with the aid of a Drigalsky loop. Next, in the center of each plate, a disk (4 mm) containing mycelium of *S. cucurbitacearum* was placed. The plates were sealed, identified and kept in an incubation chamber at 25°C for ten days and a 12 h photoperiod.

The evaluation consisted of measuring the mean diameter of the colonies in two diametrically opposed directions, at regular intervals of 48 h. From the values obtained of the mean diameter of the fungus, the mycelial growth rate index (MGVI) was calculated, using the formula described by Maia et al. (2011). Regression equations of mycelial growth in cm vs. time period in days were also calculated.

Phytotoxicity tests in melon plants

Phytotoxicity tests were performed applying the essential oils of noni fruit and lemon grass to melon plant at five concentration levels (0.1, 0.5, 1.0, 1.5 and 2.0% v/v). Two controls were used: one with the application of distilled water only and the other with a distilled water solution mixed with Tween 80.

Aliquots of essential oils were added to a stock solution of Tween 80 (1.0%), to obtain the different concentration levels, duly homogenized in a laboratory stirrer. Melon plants cv. Eldorado 300® were sprayed with 10 mL of essential oil solutions using a hand

Table 1. Regression equations plotting of mycelial growth in cm vs. time in days and mycelial growth velocity index (MGVI) for *Stagnosporopsis cucurbitacearum,* subjected to increasing concentrations of essential oils of noni fruit (*Morinda citrifolia*), lemon grass (*Cymbopogon citratus*), and fungicide, evaluated up to the tenth day of incubation. Gurupi, UFT, 2015.

Concentration levels	Noni fruit oil			Lemon grass oil **		
	Regression equation	R^2	MGVI*	Regression index	R^2	MGVI*
Fungicide[a]	y=8.8x+0.9	1.00	10.5[c]	y=8.7x+0.9	1.0	10.5[f]
0.0%	y=8.7x+21.1	0.8	18.8[a]	y=11.7x+21.9	0.9	22.0[a]
0.1%	y=12.1x+17	0.8	20.1[a]	y=13.1x+17.7	0.8	21.2[a]
0.5%	y=12.1x+8.5	0.78	16.2[b]	y=14.3x+10	0.9	19.3[b]
1.0%	-	-	-	y=16.1x+2.8	0.9	17.5[c]
1.5%	-	-	-	y=19.4x-13.2	0.9	13.2d
2.0%	-	-	-	y=19.5x-14.8	0.9	12.3[de]
2.5%	-	-	-	y=20.6x-22.0	0.9	10.9[ef]
3.0%	-	-	-	y=18.8x-21	1.0	10.1[f]

[a]Methyl Thiophanate + Chlorothalonil at 2000 ppm. *Means and days followed by the same letter in the column by each oil type, do not differ from each other according to Tukey test at 5% probability. **Mycelial growth completely inhibited starting at the 3.5% concentration level.

spray. The plants were then kept in the laboratory at 25°C for 24 h. Phytotoxicity was estimated based on scales adapted from Dequech et al. (2008), Freitas et al. (2009) and Cogliatti et al. (2011): 0% = absence of phytotoxicity; 1 - 25% = mild leaf necrosis or mild chlorosis of the plant; 26 - 50% = moderate leaf necrosis or moderate chlorosis of the plant; 51 - 75% = high leaf necrosis or high chlorosis of the plant; 76 - 100% = wilt and dryness of the plant.

Preventive and curative control tests on melon plant

Following the results of the phytotoxicity tests, the concentrations of essential oils of noni fruit and lemongrass were suitably adjusted for testing the controllability of GSB in melon plants cv. Eldorado 300®, in preventive and curative applications. Six different concentrations at graduated levels (0.03, 0.05, 0.1, 0.3, 0.5 and 0.75% v/v) were prepared in the same manner as described above, in addition to treatment with the fungicide Methyl Thiophanate + Chlorothalonil at 2000 ppm (Cerconil®) and a control with distilled water only.

A conidial solution of *S. cucurbitacearum* was prepared by adding 20 mL of distilled water to fungal colonies growing in PDA medium. Conidia were then removed using a soft-bristle brush. The solution was filtered through gauze and the conidia quantified in a Neubauer chamber, adjusting the concentration to 10^6 conidia mL^{-1} before inoculation to the melon plants.

The evaluation of the effect of preventive application of the different concentrations of essential oils was carried out by manually spraying plants at the growth stage of two well-defined fully expanded leaves (20 days after planting). Once the essential oil solution had dried (*c*. 2 h after the application), the pathogen was inoculated to the leaves and the plants were kept in a humid and dark chamber for 48 h. Subsequently, the melon plants were transferred to a natural environment with temperatures ranging from 26 to 34°C for four more days. The severity of the disease was assessed four days after inoculation, using the scale adopted by Santos et al. (2005) where 0 = healthy plant; 1 = less than 1% of diseased leaf area; 3 = 1 to 5% of diseased leaf area; 5 = 6 to 25% of diseased leaf area; 7 = 26 to 50% of diseased leaf area; 9 = more than 50% of diseased leaf area.

To verify the curative effect of the essential oils, plants of the same age described in the previous experiment were first inoculated with 10 mL of conidia solution at a concentration of 10^6 conídia mL^{-1} and kept in a dark, humid chamber for 48 h. After five days, the presence of lesions characteristic of GSB in the leaves was confirmed, after the application of different concentrations of essential oils. Disease severity assessments were made following the methodology prescribed for preventive control.

The *in vitro* data of mycelial growth as a function of essential oil dosage, phytotoxicity and *in vivo* effect to the melon plants were evaluated by analysis of variance, and divergence of means by Tukey test, as well as regression analysis. The quality of fit of the regression equations was evaluated to determine whether coefficients had 1 or 5% probability.

RESULTS

In vitro mycelial growth inhibition tests

The results of the mycelial growth tests of *S. cucurbitacearum* at different essential oils concentrations are shown separately for noni fruit and lemon grass (Table 1) and citronella grass, basil and mastruz (Table 2).

Starting at the 0.5% dosage level, there was inhibition of mycelial growth by noni fruit essential oil, rising to total inhibition of fungus growth at concentrations of 1% and above (Table 1). The reading of the mycelial growth index (MGVI) was also significantly reduced at the 0.5% level (Table 1). There is little information on noni fruit essential oil in terms of antifungal activities.

Lemon grass presented an inhibitory effect on mycelial growth in *S. cucurbitacearum* starting at the 3.5% concentration level, where total inhibition of fungal development was observed (data not shown). From the 2.5% concentration level upwards, the inhibition measured by the MGVI was equivalent to the inhibition produced by the synthetic fungicide (Table 1). On the tenth day of incubation, the fungus almost reached

Table 2. Regression equations plotting of mycelial growth in cm vs. time in days and mycelial growth velocity index (MGVI) for *Stagnosporopsis cucurbitacearum* subjected to 12 concentration levels of the essential oils of citronela grass (*Cymbopogon nardus*), basil (*Ocimum basilicum*) mastruz (*Chenopodium ambrosioides*), and fungicide, evaluated up to the tenth day of incubation. Gurupi, UFT, 2015.

Concentration Levels	Citronella grass oil			Basil leaf oil			Mastruz leaf oil		
	Regression Equation	R^2	MGVI*	Regression Equation	R^2	MGVI*	Regression Equation	R^2	MGVI*
Fungicide[a]	y=8.8x+0.9	1.1	10.5[c]	y=8.8x+0.9	1.0	10.5g	y=8.8x+0.9	1.0	10.5[c]
0.0%	y=12.2x+22	0.8	22.2[a]	y=11.3x+26.2	0.7	23.2a	y=11.9x+16.7	0.9	20.0[a]
0.1%	y=11.6x+21.6	0.8	21.7[a]	y=11.0x+26.5	0.8	23.4a	y=11.7x+17.8	0.9	20.3[a]
0.5%	y=12.7x+15.1	0.9	20.3[a]	y=12.2x+20.2	0.9	21.9ab	y=12.0x+17.1	0.9	20.4[a]
1.0%	y=16.1x-1.0	1.0	16.7[b]	y=11.7x+18.2	0.9	20.3b	y=12.1x+17.9	0.6	20.7[a]
1.5%	y=15.2x-1.1	1.0	16.2[b]	y=13.9x+11	0.9	19.9bc	y=11.9x+17.0	0.9	20.3[a]
2.0%	y=15.9x-6.1	1.0	15.0[b]	y=11.9x+10.0	0.9	17.1cd	y=12.9x+14.1	0.9	20.2[a]
2.5%	y=15.7x-6.8	1.0	14.4[b]	y=15.0x-1.2	1.0	15.8de	y=13.8x+12.3	0.9	19.8[ab]
3.0%	y=15.6x-19.5	1.0	9.1[c]	y=14.5x+1.0	1.0	16.0de	y=12.9x+12.9	0.9	17.3[ab]
3.5%	y=15.4x-20.4	0.9	8.9[c]	y=15.2x-3.4	1.0	15.0def	y=14.3x+7.8	0.9	18.6[ab]
4.0%	y=12.9x-21.2	0.9	6.3[d]	y=14.1x-3.4	1.0	14.4def	y=14.5x+5.4	0.9	17.5[ab]
4.5%	y=10.4x-16.5	0.9	5.0[de]	y=14.9x-10.0	1.0	12.5fg	y=14.9x+3.6	1.0	17.6[ab]
5.0%	y=6.5x-9.6	1.0	3.1[e]	y=15.8x-9.4	1.0	13.5ef	y=14.3x+2.1	1.0	16.2[b]

*Means followed by the same letter in the column by each oil type do not differ from each other according to Tukey test at 5% probability.
[a]Methyl Thiophanate + Chlorotalonil at 2000 ppm.

maximum mycelial growth (73 mm) irrespective of concentration level, except for the concentration of 3.0% lemon grass with a diameter of 67.2 mm. Only the synthetic fungicide was able to significantly reduce the development of fungus, with mycelial growth of 45.2 mm by the tenth day.

The essential oils effects of citronella grass, basil and mastruz on the mycelial growth of *S. cucurbitacearum* are presented in Table 2. On the tenth day of the evaluation, Citronella grass oil caused the lowest mycelial growth (23.0 mm) and MGVI (3.1) at a concentration level of 5.0%. Basil and mastruz did not control fungus growth satisfactorily.

Chemical constituents of essential oils

The qualitative and quantitative analyzes of the components present in noni fruit and lemongrass oils are presented in Table 3. The analyses showed the presence of octanoic acid, also called caprylic acid, as the major component of noni essential oil (82.24%), followed by hexanoic acid (caproic acid, 8.26%). Liu et al. (2008), reported high inhibitive capacity of mycelial growth for both caprylic and caproic acids on *Alternaria solani, Colletotrichum lagenarium, Fusarium oxysporum f. sp. cucumerinum* and *Fusarium oxysporum f. sp. lycopersici*, as well as the inhibition of spore germination.

The major components of lemongrass essential oil were geranial (41.46%) and neral (32.43%) which were also detected in the essential oil of *Lippia rehmannii* by Linde et al. (2010), which, in similar concentrations, showed inhibition of the growth of hyphae of *Fusarium oxysporum* and *Rhizoctonia solani*, important plant pathogens. Thus, the origin of the geranial and neral compounds from botanical species of different families did not alter their fungistatic effect.

Phytotoxicity of essential oils to the melon plant

Phytotoxicity tests were performed with the essential oils of noni and lemon grass, since they presented the best results in the *in vitro* tests (Figure 1). Noni fruit essential oil caused phytotoxicity right from the initial concentration level of 0.1%. However, at this concentration level, the damage was minimal, with 1 to 25% of the leaves presenting mild chlorosis and slight necrosis. Lemon grass essential oil caused phytotoxicity at a concentration level of 0.5% to the melon plant. However, at concentration levels in excess of 1.0%, there was increasing phytotoxic symptoms (chlorosis and necrosis).

Evaluation of preventive and curative controls in melon plant

Both essential oils of noni fruit and lemongrass were efficient in preventive applications only, giving rise to major reductions in the severity of GSB (Table 4), and the concentration levels of 0.03, 0.05, 0.1 and 0.3% for the two essential oils were efficient in controlling the disease.

Table 3. Relative percentages (% area), obtained by Gas Chromatography, coupled to a Mass Spectrometry Detector, of the constituents of essential oils. Gurupi-TO, 2015.

Constituents	Retention Índex**	(%)
	Noni	
3-Methyl-3-butenyl-1-acetate	888	-*
2-Heptanone	897	-
Methyl Hexanoate	922	-
Hexanoic Acid	987	8.26
Ethyl Hetanoate	999	2.48
Methyl octanoate	1123	-
Octanoic Acid	1177	82.24
Ethyl octanoate	1196	-
Isopentyl Hexanoate	1259	1.60
3-Methyl-2-butenyl Hexanoate	1292	-
3-Methylbutyl octanoate	1457	4.25
3-Methylbutyl-2-enyl octanoate	1489	-
Cymbopogon citratus		
Mircene	986	9,73
(Z)-β-Oxime	1020	0,32
(E)-β-Oxygen	1029	0,16
Linalol	1074	1,64
Neral	1209	32,43
Geraniol	1220	4,52
Geranial	1239	41,46
2-Undecanone	1359	0,35
Geranyl acetate	1443	0,42
E-Caryofylene	1641	0,17
Others	-	8.8

*Not quantified (values < 0,05). **The retention index is a component of a number, obtained by interpolation, relating the retention time of a component under study to the retention time of two patterns (generally hydrocarbonates) eluted before and after the peak in interest (Inczedy et al., 1998).

Table 4. Severity of gummy stem blight in melon plant as a function of preventive and curative application of different concentrations of the essential oils of noni fruit (*Morinda citrifolia*) and lemon grass (*Cymbopogon citratus*). Gurupi-TO, 2015.

Treatment	Concentration/Severity (%age Diseased Foliar Area)				
	0%	**0.03%**	**0.05%**	**0.1%**	**0.3%**
Noni preventive	75.5^{a}*	4.88^{b}	8.63^{b}	4.88^{b}	6.13^{b}
Noni curative	75.5^{a}	75.5^{a}	75.5^{a}	75.5^{a}	66.1^{a}
Lemon grass preventive	75.5^{a}	5.50^{b}	1.75^{b}	3.00^{b}	1.75^{b}
Lemon grass curative	75.5^{a}	75.5^{a}	75.5^{a}	75.5^{a}	75.5^{a}

*Means followed by the same letter in the column do not differ from each other according to Tukey test at 5% probability.

Concentrations of 0.5 and 0.75% were also tested; however, these levels were found to provoke a high degree of phytotoxicity in plants.

The curative application was not efficient in disease control and, therefore, is not recommended for GSB management.

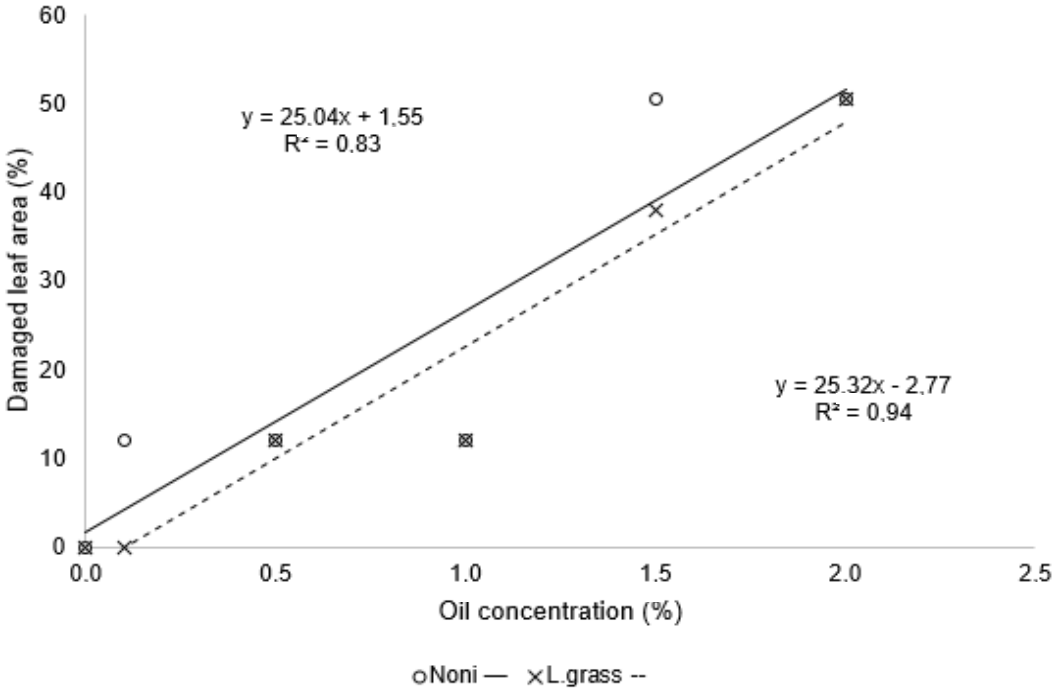

Figure 1. Percentage of foliar area damaged (phytotoxicity) as a function of different concentration levels of two essential oils of noni fruit (*Morinda citrifolia*) and lemon grass (*Cymbopogon citratus*) in melon plant.

DISCUSSION

In vitro essays

There are reports relative to the use of juice from noni fruits to treat diabetes, diarrhea, pain, hypertension, arthritis, stress and cancer. More than 160 chemical constituents present in noni fruit juice have been identified, including asperuloside, scopolamine and antraquinones, as well as vitamins and several amino acids (Franchi et al., 2008). However, so far, the effect of noni extract on the causative agent of GSB is yet to be investigated.

Sarmento-Brum et al. (2014) found mycelial growth of *D. bryoniae* (= *S. cucurbitacearum*) of 22.4 mm at ten days of incubation at a concentration of 0.15% of citronella grass essential oil. Seixas et al. (2012) also reported that citronella grass essential oil showed higher inhibition "*in vitro*" of the causative agent of GSB than the other essential oils. However, these results were not confirmed by the present study. The essential oils from basil and mastruz had no fungitoxic potential at ten days of incubation, though there was some delay on the growth rate of hyphae, as measured by MGVI (mainly basil essential oil).

Phytotoxicity

The phytotoxicity test determines the dosage that is effective for controlling the disease, whist sparing the plant host of damage. Thus, an essential oil efficient in controlling such a disease may be inadequate for use, due to the intolerance of plants to the chemical constituents present in the oil, even in low concentrations. Silva et al. (2012) did not observed lemon grass phytotoxicity to lettuce (*Lactuca sativa*) after application at concentration <1.0%. The evaluation of phytotoxicity is an important both at field level as well as in post-harvest too. Oliveira Junior et al. (2013) used essential oil of redfish, *Schinus terebinthifolius*, at a concentration level of 0.5% (v/v) in papaya fruit, to test protection against *Colletotrichum gloeosporioides*. Despite the promising "*in vitro"* response, the oil cannot be recommended due to the high levels of phytotoxicity to the fruit which make it unfit for marketing.

In vivo essays

Perini et al. (2011) have also observed similar results with those obtained in the present study, evidencing the superiority of preventive over curative control in rice blast control. Another result that proves the efficiency of the essential oils effect against pathogenic fungi was developed by Tomazoni et al. (2017), which obtained control of Alternaria in tomato plants, both in preventive and curative treatment. These results demonstrate that the efficiency of the application type varies according to

the essential oil content and fungus species.

Conclusions

Noni fruit essential oil reduced GSB disease levels between 60 and 70.6%, depending on the concentration used. As for the lemongrass essential oil, the decrease in disease severity at the same concentration was even higher, between 70 and 73.8%, similar to that observed with the application of fungicide that showed a reduction in 75% of the affected leaf area. In all, the essential oils of lemongrass and noni fruit showed that control is effective when applied preventively against gummy growth in melon plant. This result demonstrates the potential of essential oils as an additional tool in the management of plant diseases, which can, thus, contribute to reducing the use of pesticides in the control of GSB in the melon crop.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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