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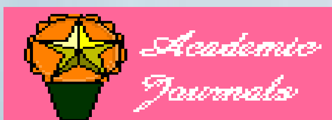

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Full Length Research Paper

Modulatory effects of *Hilleria latifolia* and *Laportea ovalifolia* on activity of selected antibiotics

Susana Oteng Dapaah, Christian Agyare*, Yaw Duah Boakye and Theresa Appiah

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Microorganisms are becoming resistant to almost all existing and newly discovered antimicrobial agents. This has led to ineffective treatment of infectious diseases with increased risk of complications. Few medicinal plants have been found to exhibit the ability of reversing the resistance mechanisms of microbes to antibiotics. The study investigates the influence of leaf methanol extracts of *Hilleria latifolia* and *Laportea ovalifolia* on some commonly used antibiotics. Micro-dilution technique was used to determine the antimicrobial activity and minimum inhibitory concentration (MIC) of the leaf extracts and the selected antibiotics. MICs of the antibiotics in presence of sub-inhibitory concentration of the extracts were determined. MIC of *H. latifolia* and *L. ovalifolia* extracts ranged from 50 to 100 mg/ml. In the presence of sub-inhibitory concentration (5 mg/ml) of the extracts, the activity of the antibiotics was modified with enhanced or reduced activity. The activity of amoxicillin was potentiated by 8-folds, 4-folds, 2-folds, 8-folds, and 2-folds against *Escherichia coli, Bacillus subtilis, Salmonella typhi, Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively in the presence of leaf extract of *H. latifolia*. Activity of ampicillin was potentiated by 2- and 4-folds against *E. coli* and *S. typhi*, respectively, as well as tetracycline, 4-folds, against *Klebsiella pneumonia* in the presence of leaf extract of *H. latifolia*. Sub-inhibitory concentrations of *H. latifolia* and *L. ovalifolia* extracts reduced the activities of erythromycin and ciprofloxacin against all the test organisms. Sub-inhibitory concentrations of *H. latifolia* and *L. ovalifolia* extracts modified the activities of the selected antibiotics.

Key words: Minimum inhibitory concentration, antibiotics, microbial resistance, antibiotic-resistance modifying agents.

INTRODUCTION

Pathogenic organisms which were previously known to have been killed or inhibited by antibiotics are now resistant to these same antimicrobial agents (Levy and Marshall, 2004). This has forced a number of pharmaceutical companies to leave the field of antibiotic discovery and production to rather produce more profitable medications for treating other diseases especially non-communicable diseases (Projan, 2003). The problem of resistance is now posing great threat on public health more than ever before, due to increasing

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multi-drug resistance in a single organism drastically limiting therapeutic options (Levy, 2005).

Naturally, bacteria have the ability to genetically develop resistance to antibacterial agents (Nascimento et al., 2000). They use different ways and strategies to acquire or develop resistance to antibiotics, which include, active efflux of drugs, alteration of target sites and inactivation of antibiotics by producing enzymes that degrade them (Sibanda and Okoh, 2007). Also, inappropriate diagnosis, drug counterfeit, use of antibiotics in food production and animal rearing, non-compliance and under dosing and sometimes uncontrolled use of antimicrobial agents are factors that contribute greatly to the overwhelming increase in the microbial resistant menace in this generation (Adu et al., 2014).

Microbial resistance to antimicrobial agents has limited the use of known cheap but effective antibiotics (Ranjan et al., 2012). This has necessitated the search for new potent antimicrobial agents to combat the threat posed by resistant microbes. In search of antimicrobial agents, various sources such as the synthetic compounds as well as bioactive agents from natural products (aquatic microorganisms and medicinal plants) are taken into consideration (Agyare et al., 2012). Medicinal plants are great sources of antimicrobial agents and the idea that plants have been used as effective drugs for treatment of infectious diseases was well accepted even before the discovery of microbes (Rios and Recio, 2005).

Coates et al. (2002) reported the occurrence of cross resistance to newly identified antibiotics and other antimicrobial agents suggesting that newly discovered antimicrobial agents may be rendered ineffective in the near future. Even though the appropriate use of these antimicrobial agents can reduce the rate of resistance development, it cannot eliminate the emergence of resistant strains (Sibanda and Okoh, 2007). There is therefore the need to discover and develop new compounds that will target and block resistance mechanisms to help treat infections from these resistant strains (Oluwatuyi et al., 2004).

Medicinal plants have been found to contain compounds with or without antimicrobial property that can cause resistant microorganisms to be susceptible to a previously impotent antibiotic (Aiyegoro et al., 2009). In most developing countries, where majority of the people rely on medicinal plants and natural products, they combine their orthodox medications with these plants in the treatment of various diseases (Adu et al., 2014; Agyare et al., 2009). This study, therefore, investigates the influence of leaf methanol extracts of *Hilleria latifolia* and *Laportea ovalifolia* on some commonly used antibiotics. *H. latifolia* (Lam.) H. Walt belongs to the family Phytolaccaceae and is locally known by the Ewes as '*avegboma*' and '*anafranaku*' by the Asantes in Ghana. It is a perennial herb of 30 to 120 cm high, with ovate-elliptic leaves and numerous short hair-like structures on lower surface. The leaves are used in Ghana for the management of rheumatism, boils and wounds (Agyare et al., 2009). The leaves are used to treat general oedema, asthma and some skin diseases (Mshana, 2000; Dokosi, 1998). It is also used to treat cough with blood (Schmelzer and Gurib-Fakim, 2008).

L. ovalifolia (Schumach.) Chew belongs to the family Urticaceae. It is known by the Asantes in Ghana as 'akyekyenwonsa', 'abrewa nom taa' or 'Kumasi otuo'. It is a herbaceous weed more often creeping than erect and densely covered with stinging hairs (Chew, 1969). *L. ovalifolia* is of two varieties, that is, male and female (Essiett et al., 2011). Leaves are used to treat wounds (Agyare et al., 2009) and the fruits are used as a poison antidote (Bouch, 2004). The root extract is used to prevent or reduce excessive menstrual bleeding (Sofowora, 1996)..

MATERIALS AND METHODS

Plant collection

Leaves of *H. latifolia* and *L. ovalifolia* were collected from Aburi in the Eastern region of Ghana on February, 2014. The plants were authenticated by Dr. Alex Asase of the Department of Botany, University of Ghana, and voucher specimen AA 63 and AA 71, respectively deposited in the Ghana Herbarium, Department of Botany, University of Ghana, Legon, Accra, Ghana.

Plant extraction

The fresh leaves collected were washed thoroughly under running tap-water and dried under shade between 25 and 28°C for two weeks, after which they were pulverized into coarse powder using the laboratory milling machine (Christy and Norris, Chelmsford, England). 800 g each of the powdered plant materials were soaked in 2.5 L of 70% v/v methanol. They were extracted with the aid of ultra-turrax (T 25 Janke and Kunkel, Labortenik, Germany) under ice-cooling at a speed of 24000 rpm for 3 to 5 min, and then filtered using a laboratory sieve (Retsch, Haan, Germany) of mesh number 200 with aperture of 75 µm and Whatmann filter paper No.1. The filtrates were concentrated with the rotary evaporator (Rotavapor BÜCHI R-200 with heating bath B-490, Büchi, Konstanz) at 40°C under reduced pressure and lyophilized and then stored in air tight containers at 4 to 8°C. The yields of the extracts of *H. latifolia* and *L. ovalifolia* were 17.4 and 11.29% w/w, related to the dried material, respectively.

Preliminary phytochemical screening

Methanol leaf extracts of *H. latifolia* (HLLE) and *L. ovalifolia* (LOLE) and their respective powdered dried plant materials were subjected to qualitative phytochemical analysis to identify various secondary metabolites such as tannins, glycosides, saponins, alkaloids, flavonoids, steroids and terpenoids present using standard methods described by Usman et al. (2014), Trease and Evans (2002) and Sofowora (1993).

High performance liquid chromatography (HPLC) profile of extracts

HPLC analysis was performed to identify the profile or finger-prints

Table 1. Phytochemical screening of methanol leaf extracts of *H. latifolia* and *L. ovalifolia*, and their dried powdered plant materials.

Secondary metabolites	***H. latifolia* leaf**		***L. ovalifolia* leaf**	
	HLLE	**Powdered plant material**	**LOLE**	**Powdered plant material**
Tannins	+	+	+	+
Flavonoids	+	+	-	-
Glycosides	+	+	+	+
Saponin	+	+	-	+
Alkaloids	+	+	-	-
Sterols	+	+	+	+
Terpenoids	+	+	+	+

(+) = Presence of secondary metabolites; (-) = absence of secondary metabolites. HLLE: Methanol leaf extract of *H. latifolia*; LOLE: methanol leaf extract of *L. ovalifolia*.

of the crude extracts with a UV-detector set at a wavelength of 254 nm. The running conditions included injection volume of 10 µl, mobile phase of methanol:water (20:80 v/v, isocratic condition), flow rate of 1 ml/min and pressure of 15 MPa. The chromatographic data were determined using Chrom Quest® software.

Test organisms

Test organisms were obtained from the microbiology laboratory of the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. They include *Pseudomonas aeruginosa* ATCC 4853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NCTC 10073, *Enterococcus faecalis* ATCC 29212 and clinical strains of *Streptococcus pyogenes*, *Salmonella typhi* and *Klebsiella pneumonia*. They were stored in 30% glycerol broth at -4°C in a frost free freezer (Mistral, UK) until needed, whereby 100 µl of the stock suspension was transferred into 10 ml nutrient broth (Oxoid Limited, United Kingdom) and incubated at 37°C for 24 h (sub-cultured) before use.

Determination of antibacterial activity and minimum inhibitory concentration (MIC) of extracts

Micro-dilution method described by Eloff (1998) and modified by Agyare et al. (2012) was used to determine the antibacterial activity and the MIC of the extracts. Each well of micro-titre plate (96 wells) was filled with 100 µl of double strength nutrient broth, 20 µl of 10^6 cfu/ml of the test organisms and 80 µl of different concentrations of HLLE and LOLE ranging from 1.56 to 100 mg/ml. Ciprofloxacin (Sigma-Aldrich, Michigan, USA) at concentration range of 1.0 to 128 µg/ml was used as reference antibiotic drug. Control wells were filled with broth only and broth and test organisms only. After 24 h of incubation at 37°C, 20 µl of 1.25 mg/ml of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Taufkirchen, Germany) was added to each well and observed for a purple colouration after incubation at 37°C for 30 min which indicated microbial growth. The minimum concentrations of HLLE, LOLE and reference drug that did not show any colour change in the wells were recorded as the MIC. The method was replicated three times to validate the results.

Microbial resistance modifying activity of the extracts

This study was done to determine the effect of a sub-inhibitory concentration of the extracts on the activity of some selected antibiotics including amoxicillin, erythromycin, ciprofloxacin, tetracycline and ampicillin (Sigma-Aldrich, Michigan, USA). The micro-dilution technique with some modifications as described by Adu et al. (2014) was employed. The MICs of the antibiotics were first determined using concentrations ranging from 1 to 1024 µg/ml. Each of the 96 wells of the micro-titre plate was filled with 100 µl of double strength nutrient broth, appropriate volume of different concentrations of the antibiotics and 20 µl of 10^6 cfu/ml of the test organisms. The plate was incubated for 24 h at 37°C, after which 20 µl MTT was added to the wells and MIC determined as the lowest concentration at which no growth was observed (that is, no colour change from yellow to purple). MICs of the antibiotics were re-determined in the presence of sub-inhibitory concentration (5 mg/ml) of the extracts (HLLE and LOLE).

RESULTS

Various phytochemical tests were performed on HLLE and LOLE and their dried powdered plant materials to identify their phytochemical composition. Phytochemical screening of HLLE and dried powdered leaf material of *H. latifolia* revealed the presence of tannins, flavonoids, glycosides, saponins, alkaloids, sterols and terpenoids. LOLE and the pulverized leaf material of *L. ovalifolia* also showed the presence of tannins, glycosides, sterols and terpenoids. However, saponins was found in the powdered leaf material of *L. ovalifolia* but was absent in its extract (Table 1).

HPLC profiles of extracts

Chemical profiles of the extracts indicate the present of metabolites/compounds at the wavelength used and these will serve as a guide in identifying the plants (Figures 1 and 2).

Antibacterial activity and MIC of extracts

MICs of HLLE and LOLE against typed and clinical strains of organisms, consisting of Gram-positive bacteria

Table 2. MIC of methanol leaf extracts of *H. latifolia* and *L. ovalifolia* against test organisms.

Test organisms	MIC of extracts and reference drug		
	HLLE (mg/ml)	LOLE (mg/ml)	Cip (µg/ml)
B. subtilis	50.0	50.0	2.0
S. aureus	50.0	100.0	4.0
E. feacalis	50.0	100.0	4.0
S. pyogenes	50.0	100.0	4.0
E. coli	50.0	100.0	2.0
S. typhi	50.0	50.0	4.0
K. pneumoniae	50.0	50.0	4.0
P. aeruginosa	50.0	50.0	4.0

HLLE: Methanol leaf extract of *H. latifolia*; LOLE: methanol leaf extract of *L. ovalifolia;* Cip: Ciprofloxacin.

(*B. subtilis*, *S. aureus*, *E. feacalis*, *S. pyogenes*) and Gram-negative bacteria (*E. coli*, *S. typhi*, *K. pneumonia*, *P. aeruginosa*) were between 50 and 100 mg/ml (Table 2).

Antibiotic modulatory activity of methanol leaf extracts of *H. latifolia* (HLLE) and *L. ovalifolia* (LOLE)

Activities of the selected antibiotics (amoxicillin, erythromycin, ciprofloxacin, tetracycline and ampicillin) against the test microorganisms were modified in the presence of sub-inhibitory concentration (5 mg/ml) of the extracts, either by enhanced or reduced activity. For instance, the activity of amoxicillin against *E. coli, B. subtilis, S. typhi, S. aureus* and *P. aeruginosa* potentiated by 8-, 4-, 2-, 8- and 2-folds, respectively, in the presence of the sub-inhibitory concentration of HLLE. Sub-inhibitory concentration of HLLE again enhanced the activity of ampicillin 2- and 4-folds against *E. coli* and *S. typhi*, respectively, as well as tetracycline, 4 folds, against *K. pneumonia*. The activity of amoxicillin against S. aureus and E. coli was enhanced (2-folds) in the presence of LOLE. However, both HLLE and LOLE sub-inhibitory concentrations reduced the activities of erythromycin and ciprofloxacin against all test organisms (Table 3).

DISCUSSION

Secondary metabolites such as tannins, flavonoids, glycosides, alkaloids, terpenoids and steroids present in the plants may be responsible for their pharmacological and biological properties (Maganha et al., 2010; Barbosa-Filho et al., 2006; Mbagwu et al., 2007; Sofowora, 1993). These secondary metabolites act alone or in synergy leading to the healing potentials of plants (Jenke-Kodama et al., 2008; Gurib-Fakim, 2006). Phytochemical screening of plant materials revealed the presence of tannins, glycosides, saponins, flavonoids, alkaloids, sterols and terpenoids in the leaves of *H. latifolia*, which is similar to reports on the same plant by Abotsi et al. (2012) and Schmelzer and Gurib-Fakim (2008). Tannins, glycosides, sterols and terpenoids were also present in the leaves of *L. ovalifolia*, however, flavonoids and alkaloids were absent (Table 1). Essiett et al. (2011) reported the presence of phytochemicals such as tannins, glycosides, saponins, flavonoids and alkaloids in the leaves of *L. ovalifolia*. The absence of flavonoids and alkaloids in the leaves of *L. ovalifolia* as observed in this study may be due to the different geographical location of the plant, the season and time of collection which are all contributing factors leading to variations in the phytochemical constituents of plants of the same species (Stackpole et al., 2011; González-Martínez et al., 2006).

In addition to the phytochemical screening, HLPC profile of the 70% methanol extracts (HLLE, and LOLE) were developed for identification purposes. HPLC profiling is more specific and helps in easy identification and confirmation of plant on the basis of specific phytochemicals present. The HPLC profiles of the extracts (Figures 1 and 2) showed that the peaks representing compounds present in the extracts appeared in the early part (early elution) of the chromatogram. This observation may be due to the polar solvent (70% methanol) used for the extraction. The profiles indicate the complex chemical composition of the extracts and provide identification parameters to figure out alterations in crude extracts (Tistaert et al., 2012).

HLLE and LOLE exhibited a broad spectrum antibacterial activity against *B. subtilis*, *S. aureus*, *E. feacalis*, *S. pyogenes*, *E. coli*, *S. typhi*, *K. pneumonia* and *P. aeruginosa* with MIC ranging from 50 to 100 mg/ml (Table 2). The antibacterial activity observed may be attributed to the phytochemical constituents present in the extracts, since these phyto-constituents have been reported to exhibit antimicrobial properties (Edeoga et al., 2005; Nweze et al., 2004).

The low antibacterial activity of HLLE and LOLE may be as a result of low amount of the active constituents

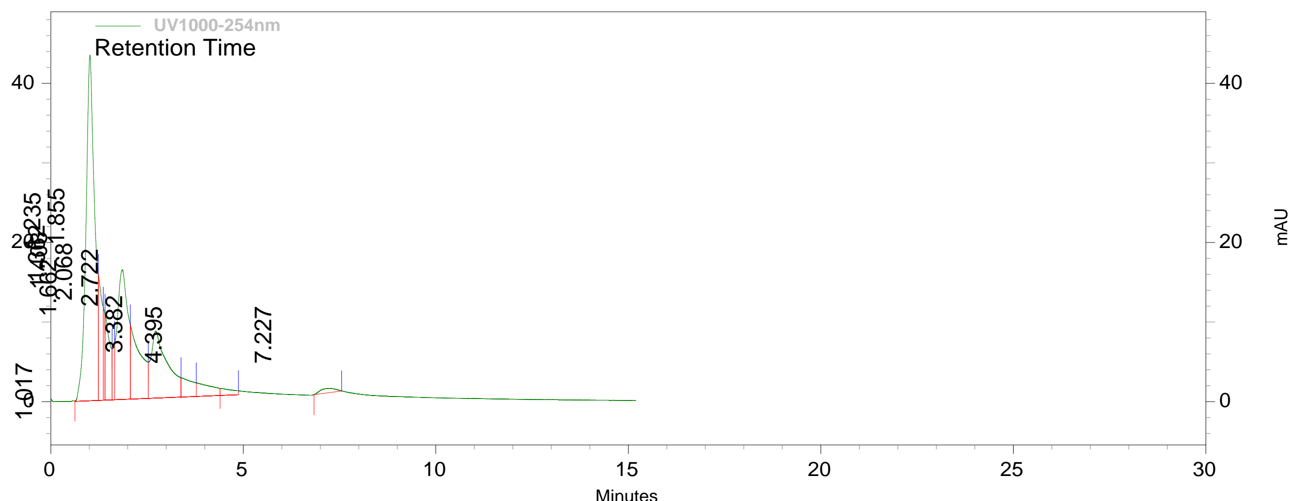

Figure 1. HPLC profile of methanol leaf extract of *H. latifolia* (HLLE) at λ 254 nm.

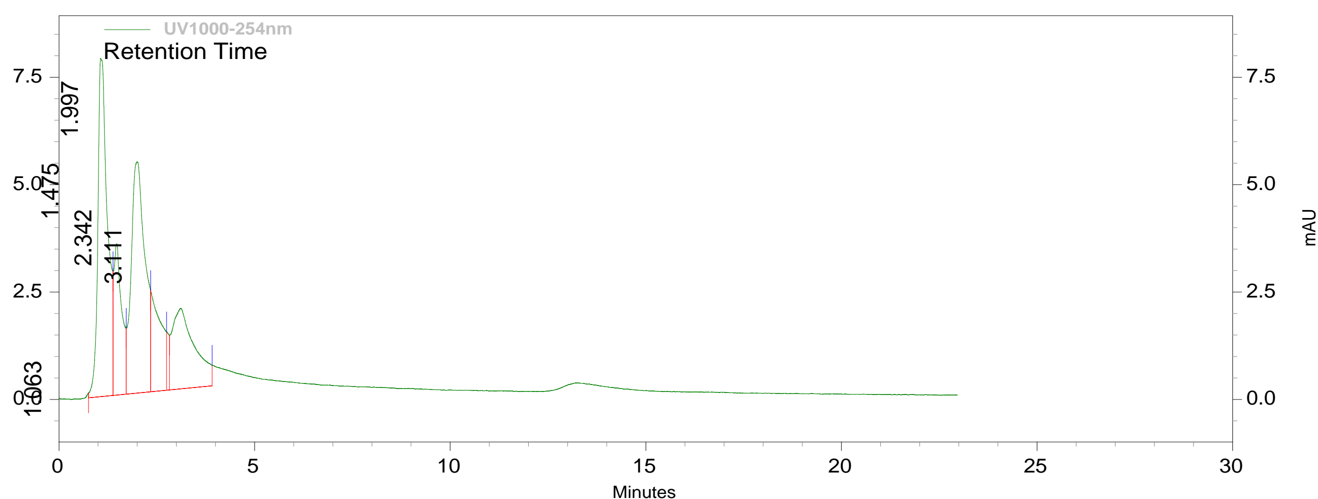

Figure 2. HPLC profile of methanol leaf extract of *L. ovalifolia* (LOLE) at λ 254 nm.

in the extracts. Similar to this observation, Okwulehie and Akanwa (2013) reported a low antimicrobial activity of *L. ovalifolia*. At MIC of 50 mg/ml, *L. ovalifolia* did not inhibit the growth of the test organisms. Also, Assob et al. (2011) reported on the antimicrobial activity of *H. latifolia* with MIC of 0.6 to 2.5 mg/ml. The high MIC (50 to 100 mg/ml) observed for *H. latifolia* in this study may be as a result of different extraction procedures used and different locality of the plant materials used which may lead to different composition in terms of primary and secondary metabolites.

Even though, HLLE and LOLE may not be potential source of antimicrobial agents as reported by Navarro and Delgado (1999) and Fabry et al. (1998) because of their relatively high MICs, it is also well noted that plant extracts with low antimicrobial activity may have some phyto-constituents that can modify the antimicrobial activity of some existing antimicrobial agents, especially against resistant bacteria (Adu et al., 2009). Sub-inhibitory concentrations (5 mg/ml) of HLLE and LOLE modified the activity of amoxicillin, erythromycin, ciprofloxacin, tetracycline and ampicillin by either potentiating or reducing their activity against the test organisms.

The increased activity of these antibiotics in the presence of the sub-inhibitory concentration of the extracts may be attributed to the phytochemicals present in the extracts. For instance, flavonoids have been reported to have the ability to reverse the resistance of *S. aureus* to some antibiotics (Aiyegoro et al., 2009). The

Table 3. Effect of sub-inhibitory concentration (5mg/mL) of HLLE and LOLE on activity of selected antibiotics against test organisms.

Antibiotic only/Antibiotic + HLLE/ LOLE		Test organisms							
		BS	SA	EF	SP	EC	ST	KP	PA
		Number of folds increase in activity							
Amx	HLLE	4	8	1	>0.25	8	2	1	2
	LOLE	1	2	1	>0.25	2	0.5	0.5	1
Amp	HLLE	1	1	1	>0.0625	2	4	1	1
	LOLE	0.25	1	1	>0.0625	1	0.25	0.5	>0.0625
Tet	HLLE	1	1	1	1	0.5	1	4	2
	LOLE	0.125	0.25	0.25	0.25	0.125	0.25	0.5	0.5
Ery	HLLE	0.25	0.0625	0.125	0.125	0.0625	0.0625	0.125	0.25
	LOLE	0.5	0.125	0.25	0.0625	0.0625	0.125	0.125	0.125
Cip	HLLE	0.125	0.5	0.5	0.5	0.125	0.25	0.5	0.25
	LOLE	0.0625	0.125	0.25	0.25	0.125	0.125	0.125	0.125

EC: *E. coli,* BS: *B. subtilis,* ST: *S. typhi,* KP*: K. pneumonia,* SA: *S. aureus,* EF: *E. feacalis,* SP: *S. pyogenes,* PA: *P. aeruginosa.* Amx: amoxicillin, Amp: ampicillin, Tet: tetracycline, Ery: erythromycin, Cip: ciprofloxacin, HLLE: methanol leaf extract of *Hilleria latifolia*, LOLE: methanol leaf extract of *Laportea ovalifolia.*

activity of the test antibiotics was enhanced mainly by HLLE as compared to LOLE. This may be as a result of the presence of flavonoids in HLLE and its absence in LOLE Antimicrobials from plants, at sub-inhibitory concen-trations, have been reported to be efficient in synergism with antibiotics by enhancing their antimicrobial actions (Kamatou et al., 2006). The phytochemicals act by reversing the resistance mechanisms of some microorganisms, thereby rendering them susceptible to previously inactive antibiotics (Tenover, 2006). Plants have also been known to produce multi-drug resistance inhibitors (MDRIs) to enhance the antimicrobial activities of compounds (Stermitz et al., 2000).

The reduced or nullified activity of the antibiotics may be as a result of interactions between the phytochemicals in the extract and the antibiotics or the microorganisms. The phyto-constituents may react chemically with the antibiotics leading to loss of activity (Adu et al., 2009, 2014). It has been established that certain substances including plant constituents can shield microorganisms from the lethal effects of some antimicrobial agents (Keweloh et al., 1989). For example, some phytochemicals can bind to the surface structures of microorganisms thereby reducing their permeability to antibiotics (Adu et al., 2014). Furthermore, some of the phyto-contituents may act as protein activators or co-enzymes which bind to and activate enzymes responsible for resistance in an organism, making them resistant to a previously potent antibiotic (Lambert, 2002). There is need to isolate the bioactive agents or compounds responsible for the antibiotic resistance modifying properties especially those that potentiated the activity of the extracts against resistant bacterial strains.

Conclusion

The sub-inhibitory concentrations of the methanol leaf extracts of *H. latifolia* and *L. ovalifolia* modified the activity of some antibiotics by either potentiating or reducing their antibacterial activities.

Conflict of interests

The authors have declare no conflict of interests.

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Evaluation of new protocols to *Curcuma longa* micropropagation: A medicinal and ornamental specie

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Turmeric (*Curcuma longa*) is an Asian native species used for ornamental, medicinal and food purposes. However, its conventional propagation method is costly and inefficient. Therefore, this study aimed to verify appropriate asepsis methods for shoots from rhizomes, evaluate concentrations of activated charcoal and sucrose, and determine appropriate salt concentrations in Murashige and Skoog (MS) medium for turmeric growth. Three independent assays were performed. First, asepsis methods were verified for explants; second was for concentrations of sucrose and activated charcoal and in the final assay salt concentrations was evaluated. In all assays, MS medium supplemented with 30 g/L of sucrose, 6.5 g/L of agar and pH adjusted to 5.8 was used. Two growth regulators were added to the culture medium: 4.44 µM of 6-benzyl-amino-purine (BAP) and 1.08 µM of α-naphthalene acetic acid (NAA). Data obtained were subjected to analysis of variance (ANOVA) at $p \leq 0.05$ probability. The averages were compared by Tukey's test and polynomial regression at $p \leq 0.05$. All the asepsis methods evaluated showed similar effects. The highest concentration was obtained by utilizing 60 mg/L sucrose for the most part of the evaluated characteristics; however, it is recommended that activated charcoal be used at a concentration of 4.5 g/L. For salt concentrations, averages were compared by polynomial regression at ($p \leq 0.05$) probability in 50 to 60% MS medium, and resulted in longer root systems, greater numbers of shoots and more leaves. The usual dose of 3% sucrose resulted in lower development of *in vitro* seedlings. Salt concentrations higher than 60% were toxic to turmeric tissues and consequently compromised the root system and the aerial part of developing seedlings.

Key words: Turmeric, *Curcuma longa*, Zingiberaceae, culture medium, micropropagation.

INTRODUCTION

Turmeric (*Curcuma longa*), popularly known as turmeric, is a perennial herb from the Asian native Zingiberaceae family (Chakraborty et al., 2011). It is broadly used as a medicine because it is rich in compounds like curcumin, sodium curcuminate and methyl-curcumin (Li et al., 2011; Ramadan et al., 2011) that have been studied due to their anti- inflammatory, antioxidant and antitumor potential (Chainani, 2003; Kainsa et al., 2012; Green and

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Mitchell, 2014). It also has an antimicrobial effect against *Aspergillus* sp. fungi and bacteria from *Pseudomonas, Staphylococcus* and *Salmonella* genera (Rao and Mittal, 2014). In the cosmetics industry, it is used to produce body lotions and moisturizers (Gonçalves et al., 2014), whereas in the food industry, rhizomes are used to manufacture colorings that are usually used in pasta, in the preparation of sauces and breads (Shaw and Panda, 2015). It is also commonly used in landscaping projects for gardens and parks, and in flower commerce for floral arrangements and bouquets (Velayudhan et al., 2012).

In Brazil, turmeric has adapted very well to cultivation conditions. Nevertheless producers still lack information, mainly regarding to the cultivation methods and to varieties which may promote curcuminoids increment and starch production, once this specie has expressive amount of this type of carbohydrate (76 and 86, 62%) (Santana and Meireles, 2014) end in particular regarding the initial phase of plant propagation (Berni et al., 2014).

Currently, the rhizome propagation method is conventionally used (Berni et al., 2014). Rhizomes are usually collected and re-planted in October in most of the country due to their dormancy and the beginning of the rainfall season. This technique presents some limiting factors for seedling production, such as cost, production time, labor, and utilization of the commercialized part the rhizomes (Miachir et al., 2004). The contamination of the propagation material with soil fungi, bacteria and nematodes present in soil (Faridah et al., 2011) is another obstacle that results in loss of the propagation material. Moreover, a rhizome has low yield because it propagates, at most, four seedlings.

One way to relieve these limiting factors for turmeric establishment may be through the culture medium in vegetal tissues. This technique obtains desirable agronomic characteristics, such as pathogen-free plants, abundant and fast seedling production throughout the year (Sidhu, 2010; Ahmadian et al., 2013). For example, one single explant can result in up to 300 seedlings, with large-scale production of excellent quality material (Yildiz, 2012). *Etlingera elatior* seedlings were capable of forming at the end of 30 days from 8 to 10 shoots in MS medium (Colombo et al., 2010). In addition, homogenous seedlings usually reach physiological maturity faster in the field, with shorter plant juvenility and an earlier production start (Souza et al., 2011).

However, for the micropropagation of vegetable material, it is necessary to develop the appropriate culture medium for each species (Sridhar and Aswath, 2014). This culture medium should consist of ideally balanced compounds in order to favor morphogenesis and produce vigorous plants (Yildiz, 2012; Ahmadian et al., 2013). Several aspects must be standardized in the culture medium such as asepsis, salt concentration, carbon sources, antioxidants, pH and growth regulators. The latter are mostly verified in assays involving tissue culture. However, aspects like asepsis, salt concentration, and carbon and antioxidant content should not be neglected. Therefore they should be investigated to standardize protocols because inappropriate nutrition can cause difficulties in seedling growth (Sridhar and Aswath, 2014).

Nevertheless there is lack of information about culture medium constituents for turmeric, and research results have often been inconclusive. According to Babaei et al. (2013), asepsis is the most limiting phase of the micro-propagation process, mainly due to the bacteria from *Bacillus*, *Pseudomonas*, *Staphylococcus* and *Lactobacillus* genera and fungi from *Aspergillus* sp. *Penicillium* spp. and *Fusarium* spp. Genera, which are among the most commonly found contaminants that cause explant death and serious economic, propagating material and time losses over the micropropagation process (Sidhu, 2010; Yildiz, 2012). The use of disinfecting products in the sterilization process of several species Zingiberaceae is essential because explant sources in assays are axillary buds obtained for example from rhizomes or shoots of philippine wax flower and ginger (Colombo et al., 2010; Abdelmageed et al., 2011; Faridah et al., 2011). Rhizomes, which are underground materials, are exposed to a great variety of pathogens, such as fungi and bacteria (Rescarolli and Zaffari, 2009), making explant establishing in the initial cultivation phase difficult. As is the case with rhizome and root rotting caused by (*Rhizoctonia solani* and *Fusarium oxysporum* f. sp. cubense) at *E. elatior* and *Heliconia chartacea* cv. Sex Pink. and nematodes from Meloidogyne, Radopholus and Helicotylenchus species and genus (Lins and Coelho, 2004).

Salt concentration is another important factor as media with usual concentration fare (100%) is not always ideal in a micropropagation protocol. Excessive salt can cause the opposite effect, limiting the development of roots and aerial parts. Moreover, some species such as *Ocimum basilicum* are frequently intolerant to high salt concentrations in the range of (70 to 100%) (Tarchoune et al., 2012). Species with concentration lower than 70% have been more favorable to the development and with regard to *Solanum tuberosum* where 50% of the MS medium salts caused vigorous and health seedlings (Bandinelli et al., 2013). As reported by Islam et al. (2004), a 75% salt concentration was more important for induction of microrhizomes in turmeric than was treatment with 100% salt concentration.

Activated charcoal is the most common antioxidant used to absorb unwanted compounds from the medium (Thomas, 2008). However, it also absorbs growth regulators, vitamins, copper and zinc. Therefore the previous standardization of activated charcoal is recommended (Thomas, 2008; Ahmadian et al., 2013). Its beneficial effect was verified in *Strelitzia reginae*, which phenolics were reduced at 53% in the medium, when compared with media supplemented with ascorbic acid (North et al., 2012). Sucrose is generally used as the

Table 1. Asepsis methods used for *Curcuma longa* shoots kept under *in vitro* conditions.

Treatments (t)	Description
1	Immersion in sodium hypochlorite solution at 2% (v v^{-1}) for 20 min, followed by washing with deionized water and autoclaved for three times
2	Similarly as T1–time increased to 40 min in sodium hypochlorite solution at 2% (v v^{-1}), followed by triple washing
3	Immersion in fungicide: 100 µl/100 ml of Rovral® water[1] 100 µl/100 ml of Locker® water[2] 100 µl/100 ml of Priori xtra®water[3]. Shoots were left in fungicide for 30 minutes and another 20 min in sodium hypochlorite at 2% (v v^{-1}), followed by triple washing
4	As for T3, but with time increased to 60 min in fungicide and another 20 min in sodium hypochlorite at 2% (v v^{-1}),followed by triple washing.
5	Immersion in fungicide: 300 µl/100 ml of Rovral® water[1] 300 µl/100 ml of Locker® water[2] 300 µl/100 ml of Priori xtra®water[3], left for 30 min in fungicide and another 20 min in sodium hypochlorite at 2% (v v^{-1}),followed by triple washing
6	As for T5, but with time increased to 60 min in fungicide and another 20 min in sodium hypochlorite at 2% (v v^{-1}),followed by triple washing

[1]Rovral®-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxo imidazoline-1-carboxamide (IPRODIONE). [2]Locker® - Carbendazim 200 g/L + Tebuconazole 100 g/L + Kresoxim-methyl 125 g/L. [3]Priori xtra® - Azoxystrobin 200g/L + Cyproconazol 80 g/L.

carbon source at 3% concentration in MS medium (Yassen et al., 2013), but higher concentrations provided greater growth of aerial parts and roots, which is beneficial over the micropropagation process once seedling may develop faster over a short period in the laboratory. Some studies have shown that species of the Zingiberaceae family respond favorably to increases in sucrose concentration from 3 to 6% (Islam et al., 2004; Kusumastuti et al., 2014). This has been observed for *Curcuma aromatica*, which showed 70% more rooting at 6% concentration when at the usual 3% concentration (Nayak, 2000). Other Zingiberaceae species enhanced due the higher concentrations of sucrose from were *C. Longa* with 6% (Jala 2012), *Curcuma xanthorrhiza* with 9% (Kusumastuti et al., 2014) and *Zingiber officinale* 8% of sucrose (Singh et al., 2014).

Therefore, this study aimed to verify appropriate asepsis methods for shoots from rhizomes, evaluate concentrations of activated charcoal and sucrose, and verify salt concentrations in Murashige and Skoog (MS) medium in the growth of turmeric.

MATERIALS AND METHODS

Turmeric rhizomes were obtained from *Cooperativa dos Produtores de Açafrão* Mara Rosa - Goiás, Laboratory of Molecular Biology and Culture of Vegetal Tissues from Paranaense University (UNIPAR, Umuarama PR, Brazil). Cracked, withered rhizomes with disease symptoms were discarded and only the most vigorous rhizomes were selected. These were kept in laboratory ambient conditions without temperature and moisture control until the start of the experiments. Shoots from the rhizomes were used for assay installation and were removed with a scalpel. The shoot length was standardized to approximately 1.5 cm ± 0.3.

Shoot asepsis

To evaluate asepsis methods, shoots previously obtained were submitted to six different procedures as described in Table 1.The procedures were carried out in a laminar flow hood. Shoots from the asepsis step were inoculated in 350 ml clear glass flasks containing MS culture medium (Murashige and Skoog, 1962), supplemented with 30 g/L of sucrose and 6.5 g/L of agar. Two growth regulators of 4.44 µM of 6-benzyl-amino-purine (BAP) and 1.08 µM of α-naphthalene acetic acid (NAA) were added to the culture medium (Prakash et al., 2008). The pH was adjusted to 5.8 and then sterilized by autoclaving at 121°C for 20 min. Inoculation was carried out in an aseptic chamber. The shoots were individually displayed in flasks containing 50 ml of culture medium, closed with transparent plastic lids, and sealed with polyvinyl chloride (PVC).

The material was then kept in a growth room for 90 days at a temperature of 25 ± 2°C, and submitted to light intensity of 60 µmoles/m^2/s and 24-h light photo periods. The evaluated parameters were: the contamination total fungal and bacterial (expressed as percentage), and explant oxidation (expressed as percentage; number of leaves; length of aerial part and root (mm);

Table 2. Percentage of contaminated seedlings (CON), oxidated seedling (OXD),number of leaves (NL), aerial part length (APL), base diameter (BD), fresh mass of aerial part (FMAP), fresh mass of roots (FMR), dry mass of aerial part (DMAP), and dry mass of root (DMR) of *C. longa* in function of different asepsis methods after 90 days of *in vitro* cultivation.

Treatments	CON (%)	OXD (%)	NL	APL (mm)	BD (mm)	FMAP (g)	FMR(g)	DMAP (g)	DMR (g)
1	0±00[a]	12.50±2.12[a]	5.00±0.17[a]	95.116±3,00[a]	5.169±0.28[a]	2.376±0.38[a]	1.939±0.38[a]	0.686±0.01[a]	0.174±0.007[a]
2	6.250±1.41[a]	50.00±1.520[a]	3.93±0.20[a]	96.566±1.22[a]	35.427±1.02[a]	0.725±0.07[a]	2.559±1.22[a]	0.177±0.005[a]	0.066±0.000[ab]
3	12.500±2.12[a]	31.25±1.73[a]	3.50±0.23[a]	70.780±0.14[a]	5.055±0.38[a]	1.219±0.15[a]	1.782±0,14[a]	0.126±0.004[a]	0.054±0.002[ab]
4	12.500±2.12[a]	6.25±1.41[a]	4.12±0.39[a]	127.819±0,14[a]	4.085±0.94[a]	1.496±0.11[a]	1.844±0.14[a]	0.153±0.006[a]	0.043±0.006[b]
5	6.250±1.41[a]	18.75±0.7[a]	4.58±0.40[a]	75.004±0.55[a]	6.285±0.04[a]	0.863±0.07[a]	2.598±0.55[a]	0.141±0.008[a]	0.041±0.007[b]
6	18.750±0.7[a]	18.75±0.7[a]	5.04±0.11[a]	86.874±0.11[a]	8.290±0.58[a]	1.608±0.09[a]	2.513±0.10[a]	0.139±0.012[a]	0.029±0.001[b]

* Means with the same letters in the same column are not significantly different at $p \leq 0.05$. Note: cont (%) and oxd (%) analyzed by Kruskal-Wallis's test, test value=5.18, standardized value =14.24

longitudinal diameter of the base (mm), measured by a digital pachymeter; and dry and fresh matter of aerial part and root. To measure fresh matter, fresh seedlings were weighted in an analytical balance; dry matter of the vegetable material was separated into paper bags and subjected to an air circulation oven at 65°C for 4 days until it reached constant weight.

The experimental design performed in this work was a completely random design with six treatments, five replicates, and four flasks per plot, totaling 120 plots. Contamination data and oxidation percentage were analyzed with non-parametric analysis using a Kruskal-Wallis' test at $p \leq 0.05$ of probability. The remaining data were analyzed using analysis of variance (ANOVA) at p ≤0 .05. Averages were compared by Tukey's test at $p \leq 0.05$ of probability, using the SISVAR program (Ferreira, 2011).

Effect of activated charcoal and sucrose

The effect of activated charcoal and sucrose on the development of turmeric was evaluated at Step 2. Shoots were sterilized with sodium hypochlorite (2%) in a laminar flow chamber for 20 min, then washed with deionized water and autoclaved for three times, according to the results of essay 1. After asepsis, the shoots were inoculated in a culture medium prepared using the protocol described elsewhere (Prakash et al., 2008). The characteristics evaluated were: total fungal and bacterial contamination (expressed as percentage), explant oxidation (expressed as percentage), length of aerial part and root (mm); base longitudinal diameter (mm) which was measured by a digital pachymeter, dry and fresh matter of aerial part and roots, and number of shootings and leaves. The experiment was a completely random design in a factorial scheme (2 × 3) with two levels of sucrose concentrations (30 and 60 g/L), and three levels of activated charcoal concentrations (0, 3.0 and 4.5 g/L). A total of six treatments with five replicates and four flasks per plot were prepared. The data were subjected ANOVA at $p \leq 0.05$ and the means were compared using Tukey's test at $p \leq 0.05$, using SISVAR program (Ferreira, 2011).

Salt concentration

The ideal salt concentration for *in vitro* cultivation of turmeric was tested. Shoots were sterilized with sodium hypochlorite (2%) for 20 min in a laminar flow hood, then washed in deionized water and autoclaved three times according to the results of essay 1. The culture medium was prepared according to Prakash et al. (2008). The same characteristics as for previous assays were evaluated: total fungal and bacterial contamination (expressed as percentage), explant oxidation (expressed as percentage, length of aerial part and root (mm), base longitudinal diameter (mm), measured by a digital pachymeter, dried and fresh matter of the aerial part and root, and the number of shootings and leaves. A completely random design was applied to the experiments. The evaluated salt concentrations in MS medium were: 0, 25, 50, 75 and 100%. Five treatments, five replicates, and four flasks per plot were performed. Results were subjected to ANOVA at $p \leq 0.05$. The means were compared by polynomial regression at $p \leq 0.05$, using the SISVAR software (Ferreira, 2011).

RESULTS AND DISCUSSION

Shoot asepsis

The influence of shoot asepsis resulted in statistical differences among treatments for root dry matter (Table 2). Treatment 1 showed the greatest weight average.

Treatments 5 and 6 showed the same average results for root dry matter, which were both greater than that resulting from treatment 2. Overall, it was observed that for some species of the Zingiberaceae family, the highest explant contamination occurred in buds from ginger rhizomes (Faridah et al., 2011), chinese ginger (Yusuf et al., 2011) and mango ginger (Mohanty et al., 2012).

Rhizomes are underground stems in direct contact with soil microorganisms, including pathogens like bacteria and fungi. This probably contributed to a greater infection of the buds and consequently affected the contamination rate at the time of *in vitro* establishment. For other studies that evaluated asepsis in Zingiberaceae

Table 3. Average values of isolated effect of sucrose concentration for base diameter (BD), fresh matter of aerial part (FMAP), fresh matter of root (FMR), dry matter of aerial part (DMAP) and dry matter of root (DMR) of *C. longa.*

Treatment	BD (mm)	FMAP (g)	FMR (g)	DMAP (g)	DMR (g)
30 g/L	5.90±0.50^{b}	0.19±0.18^{b}	8.68±0.17^{a}	0.15±0.05^{b}	0.15±0.03^{b}
60 g/L	7.68±1.9^{a}	0.70±0.00^{a}	2.90±0.77^{b}	0.30±0.04^{a}	0.65±0.11^{a}

*Means with the same letters in the same column are not significantly different at p ≤0.05.

Table 4. Average values of isolate effect of activated charcoal concentration for base diameter of *C. longa* seedlings.

Treatment activated charcoal	Base diameter (mm)
0.0 g/L	5.37 ±0.94^{b}
3.0 g/L	5.49 ±0.80^{b}
4.5 g/L	9.49 ±0.73^{a}

*Means followed by the same letter do not differ among themselves by Tukey's test at (p≤0.05).

buds,stronger substances were required, such as ethanol immersion (70%) for 2 min and two drops of mercury chloride solution at 0.1 (%) in turmeric (Islam, 2004), and mercury chloride solution at 0.1 (%) in wild turmeric (Nayak, 2000). Clorox at 60% was used for 30 min on bitter ginger, then all bud external tissues were removed and the explants were again subjected to asepsis with 20% of Clorox (Faridah et al., 2011).

In this study, shoots from rhizomes but not from buds were used and, probably due to the meristematic origin and adjacent leaf tissues at the borders of the leaves, these factors contributed to a greater protection against pathogens, resulting in less tissue infection (Nair, 2013). Therefore the usual treatments using tissue culture with sodium hypochlorite (at 2%) was sufficient to sterilize explants and also caused less damage to the root system. However, when propagules originated from rhizomes (buds), stronger disinfecting substances like fungicides and mercury chloride should be used for asepsis as used for ginger (Faridah et al., 2011), Chinese ginger (Yusuf et al. 2011) and mango ginger (Mohanty et al., 2012). Although this type of treatment is needed in these cases, it is time consuming and requires significant effort.

Effect of activated charcoal and sucrose

There was an interaction effect between activated charcoal and sucrose concentrations for the factors evaluated in this work (Table 5). For the other assessed characteristics, such as fresh and dry matter of aerial part and root and base diameter, only the isolated effects of sucrose (Table 3) and activated charcoal (Table 4) were observed. It was verified that sucrose solution at 60 g/L favored almost all evaluated characteristics. Only fresh matter of the root was not increased by this treatment (Table 3). The best concentration for activated charcoal was 4.5 g/L. This treatment showed an increase of 4.08 mm of the base diameter when compared to the control sampling. For the usual concentration of sucrose (3.0 g/L), the increase was 4.00 mm. There were no statistical differences between the control and 3.0 g/L of activated charcoal concentration (Table 4).

Sucrose is the most utilized carbon source in most protocols (Ahmadian et al*.,* 2013). Sugars are necessary for the function of several parts of morphogenesis, such as the osmolarity balance of the culture medium, skeletal function for the building of various important factors, heterotrotophy/mixotrophy, and regulation of genes responsible for plant growth (Yaseen et al., 2013). Some studies on plants from the Zingiberaceae family have shown that higher sucrose concentrations of 60 to 70 g/L are more efficient for *in vitro* plant morphogenesis when compared to the usual concentration of 30 g/L. This was verified in assays carried out with wild turmeric in which rooting increased by 70% when compared to the usual concentration (Nayak, 2000). Moreover, at 60 g/L of sucrose in turmeric, a greater number of shoots, number of leaves, and root length (Jala, 2012) were observed.

Beyond sucrose, other carbon sources have been used in the micropropagation, as is the case of sorbitol, mannitol, fructose, galactose (pentoses) and maltose (disaccharide) (Yaseen et al., 2013). Being the first two, more are used in essays involving minimal cultivation or in germplasm collection establishment (Martins et al., 2011). The others may be used in future researches aiming to minimize production costs and the better seedlings development. Few essays evaluated the effect of other sugar sources compared to Zingiberaceae. A comparative study of wild turmeric seedlings cultivated in sucrose, glucose and maltose demonstrated that sucrose was the best source in relation to number of shoots and the length of aerial part (Sharmin et al., 2013). Jalil et al.

Table 5. Average values of sucrose and activated charcoal interaction for contamination percentage (CP%), number of shoots (NS), length of aerial part (LAP) and length of root (LR) in mm of *C. longa*.

Charcoal g/L	Sucrose g/L		Sucrose g/L		Sucrose g/L		Sucrose g/L	
	30	60	30	60	30	60	30	60
	CP %		NS		LAP (mm)		LR (mm)	
0.0	10±0.70^{Aa}	10±0.70^{ABa}	1.30±0.23^{Aa}	1.42±0.11^{Ba}	93.25±1.94^{Aa}	45.42±2.84^{Bb}	59.45±1.60^{Aa}	48.92±0.86^{Ba}
3.0	0±0.00^{Ab}	30±1.41^{Aa}	0.84±0.09^{Aa}	1.15±0.10^{Ba}	86.14±1.80^{Aa}	86.14±1.37^{Aa}	73.89±0.01^{Ab}	121.01±1.44^{Aa}
4.5	0±0.00^{Aa}	5±0.70^{Ba}	0.90±0.19^{Ab}	2.45±0.06^{Aa}	102.66±1.46^{Aa}	102.66±0.76^{Aa}	56.96±2.74^{Aa}	21.42±2.19^{Bb}

*Means followed by the same capital letter in the column and small letter in the line do not differ among themselves by Tukey's test ($p \leq 0.05$).

(2015) also verified that sucrose enhanced cells growth in a ginger suspension when compared with maltose, glucose and fructose. It is important to emphasize that these results corroborate the present study for the same evaluated characteristics, showing that plants from the Zingiberaceae family tend to respond positively to an increase in sucrose concentration in the culture medium. Different results were obtained for two species of *S. tuberosum*, which increase in sucrose concentration limited the number of leaves and the length of the aerial part of *in vitro* seedlings (Bandinelli et al., 2013). In *Orbignya oleifera* embryos, the interaction between sucrose and activated charcoal was not significant. For the assessed characteristics, only the carbohydrate isolated effect was effective for concentrations of 34.5 g/L (Leite et al., 2014). This showed that the response to carbohydrate increase does not depend only on sugar and antioxidant interaction, but also on the genetic plant constitution.

The observed response for contamination percentage, number of shoots, and length of aerial part and root depended on the concentrations of sucrose and activated charcoal together (Table 5). The highest contamination percentage was observed at concentrations of 60 g/L of sucrose and 3.0 g/L of activated charcoal, followed by concentrations of 30 and 60 g/L of sucrose in the absence of activated charcoal 0.0 g/L. There were no contaminated explants at concentrations of 3.0 g/L of sucrose, and 3.0 and 4.5 g/L of activated charcoal. However, the treatment that showed most increase in the number of shoots was 60 g/L of sucrose and 4.5 g/L of activated charcoal. The activated charcoal concentration provided the same independent effect as sucrose concentration when compared to other treatments (Table 5). The growth of the aerial part was most effective in the medium with 30 and 60 g/L of sucrose and 3.0 and 4.5 g/L of activated charcoal. Nevertheless no statistical differences were apparent between the treatments. The smallest growth was observed for the treatment with 60 g/L of sucrose in the absence of activated charcoal. The results for root growth were similar to those described previously, except for the treatment with 60 g/L of sucrose and 4.5 g/L of activated charcoal, which was ineffective (Figure 1A to D). The growth was 5.6 mm smaller than the best treatment (60 g/L of sucrose and 3.0 g/L of activated charcoal) about 17.70% of lower root growth (Table 5).

The use of higher concentration of sucrose (60 g/L) does not necessarily require the use of a greater amount of activated charcoal, as the lengths of the aerial part and root suffer damages in the presence of high sucrose concentrations and absence of activated charcoal. Increase in temperature causes Mailard reaction, which occurs when the sucrose is submitted to high temperatures during the autoclave process and results in the carbohydrate partially hydrolyzing into glucose and fructose. In addition, at the end of the process, it forms a compound known as 5-hidroxymethyl-furfuraldeid, which is toxic to the vegetal tissue (Shibao and Bastos, 2011).
Moreover, when tissue is cut, it can release phenolic compounds that are also undesirable in morphogenesis, as they create polyphenolo-xydase (PPO), which uses ortho-diphenol com-pounds found in a cell vacuole substrate. Finally, when there is a cut in the tissue, quinone polymerization occurs since the substrate is in contact with an enzyme polyphenoloxidase in the presence of oxygen (Thomas, 2008). For example, catechins found in cocoa (Rusconi and Conti, 2010), gallic, cinnamic and caffeic acids were pointed out as substrate to polyphenoloxydase enzyme. These compounds were found in ginger (Ghasemzadeh et al., 2010; Siddaraju et al., 2011). Li et al. (2011) reported at least nineteen different types of curcumins in turmeric as potential substrate to PPO. Quinones and 5-hidroxymethyl-furfuraldeid can be extremely harmful to vegetal development.

Species from Zingiberaceae family have the

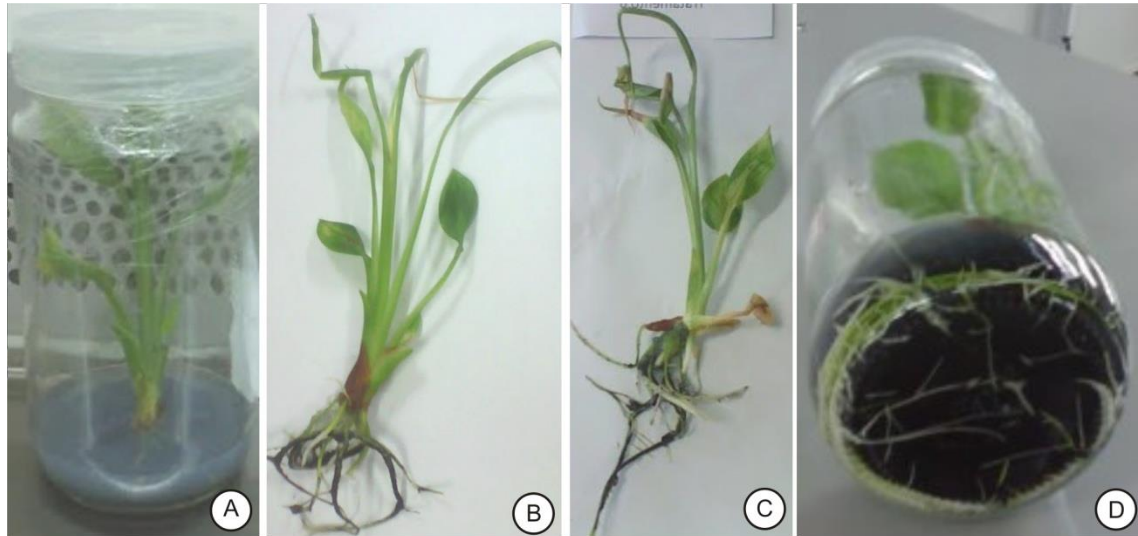

Figure 1. *In vitro* seedlings of *Curcuma longa* in function of the concentrations of sucrose and activated charcoal. A) and D) Inoculation with (60 g/L) of sucrose and (4.5 g/L) of charcoal; B) Inoculation with (30 g/L) of sucrose and addition of (4.5 g/L) of charcoal; C)Inoculation with (60 g/L) of sucrose and addition of (3.0 g/L) of charcoal.

capacity to respond to a higher concentration of sucrose. It was evident in this study that activated charcoal was essential to the utilization of higher sucrose concentrations. This is probably the case because charcoal adsorbs more substances from the culture medium as it is formed by graphically arranged carbons with a thin pore network (Thomas, 2008). However, it is important to establish optimal charcoal concentration because it can also absorb growth regulators, vitamins, and ions such as copper and zinc.

Salt concentration

The salt concentrations affected the evaluated characteristics during the assay, with statistical differences observable at $p \leq 0.05$ (Figure 2). Contamination percentage, oxidation, base diameter, fresh matter of aerial part, and root dry matter were not influenced by salt concentrations in MS medium, and the same effects were observed in all evaluated concentrations.

The quadratic model was used for the number of leaves and shoots. It was observed that, initially, there was an increase in the number of shoots, up to salt concentration of 50%, after which a decrease occurred (Figure 2A), similarly as *Zantedeschia albomaculata*, which presents a greater number of shoots in MS medium at 50% of salt concentration (Chang et al., 2003). The salt concentration at 64.24% influenced the increase in the number of leaves, which was approximately five times greater than the control, as described on (Figure 2B). The length of root and aerial part was better evaluated by the quadratic model. The best concentration for the length of aerial part was 64.81%. At higher concentrations, there was a decrease in approximately 25 mm for this characteristic (Figure 2C). There was an increase in root length at 48.59% concentration with posterior decrease, reaching approximately 20 mm for the highest concentration (100%), according to the (Figure 2D).

The quadratic model described the root fresh mass, and the best average masses were obtained at 60.56%. At higher concentrations, there was a reduction of approximately (3.0 g) of fresh mass. Compared to the control, the fresh mass gain was 4.5 greater (Figure 2E). The linear model was the best for dry matter of aerial part, therefore, the higher the salt concentration, the greater the gain for this characteristic (Figure 2F).

Most studies have shown that higher salt concentrations provide more severe damage to the root system. This seems obvious since the root is responsible for the absorption of ions (macro and micro nutrients) in ideal amounts (George et al., 2008; Khenifi et al., 2011). The same is true for *in vitro* cultivation. In our study, the increase in salt concentration also compromised the number of shootings and leaves. In the same way root system of *Butia capitata* was also damaged at 100% salt concentration and the best root development occurred at 75% of salt concentration (Ribeiro et al., 2011). Erig et al. (2004) observed a greater average length for *Cydonia oblonga* roots using 75% salt concentration in MS culture medium in accordance with Leitzke et al. (2009) who also stated that 75% of salt concentration in culture medium is ideal for *in vitro* rooting for the *Rubus fruticosus* (‘Xavante’ blackberry), producing a greater average

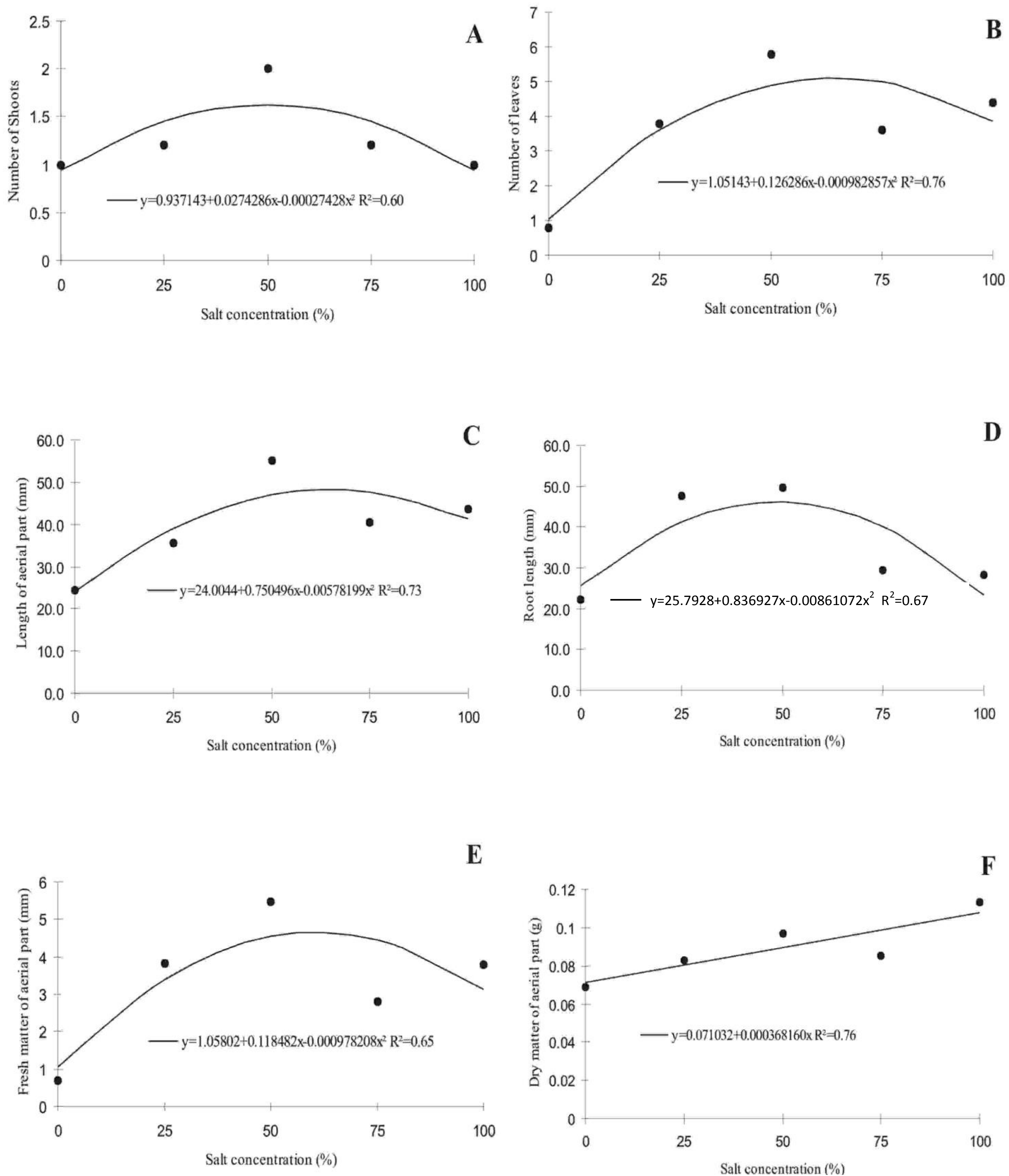

Figure 2. Number of shootings (A); Number of leaves (B); Length of aerial part (C); Root length (D); Root fresh matter (E); Dry matter of aerial part (F). All the characteristics were evaluated at different saline concentrations in Murashige and Skoog (MS) medium after 90 days. Black spots represent the mean values observed.

length of roots.

Ion absorption depends on synergisms and antagonisms. For example, when synergism occurs, the presence and amount of anions may favor the absorption of other anion through the roots. In antagonism the same ion can compete for the same absorption site, limiting the absorption (Silvia and Trevizam, 2015). These equilibrium dynamics may have occurred in the highest salt concentrations for turmeric medium. High concentrations of dissolved phosphate may reduce growth, probably due to the reduced absorption of elements such as Zn, Fe, and Cu. High amounts of K^+ or Ca^{2+} and Mg can result in the decrease of phosphate absorption if the solution pH becomes slightly alkaline (George et al., 2008). One way to maintain pH in the culture medium is through the dynamic equilibrium of ammonium and nitrate absorption. Quick ammonium absorption makes the medium pH decrease to 4.2 to 4.6. When this happens, ammonium capture is inhibited, but nitrate ion absorption is stimulated, causing the pH to increase again (George et al., 2008).

In addition, ammonium concentration may have been toxic to turmeric seedlings due to nitrogen in the form of ammonium nitrite (NH_4NO_3) in MS culture medium. Plants absorb nitrogen as nitrate (NO_3^-) and ammonium (NH_4^+) (Mokhele et al., 2012). The latter is very toxic to vegetal tissues if not assimilated (Britto and Kronzucker, 2002). Plants avoid toxicity from the rapid conversion of ammonium into amino acids (Mokhele et al., 2012). At high concentrations, this ion dissipated protons that are needed for electron transport in photosynthesis and respiration, and metabolites are capture in a vacuole (George et al., 2008). In *Arachis hypogaeaa*, the presence of ammonium over a 25:75 ratio (NH_4^+:NO_3^-) damaged the plant's initial development, but the combination of NH_4^+ and NO_3^- in 25:75 and 0:100 ratios provided greater phytomass production in this species (Ribeiro et al., 2012).

For turmeric, the ideal salt concentration range varied from 50 to 60%, in which the final concentrations of the culture medium for ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) were 825 to 990 mg/L and 950 to 1140 mg/L, respectively. The reduction of ammonium ion concentration (NH_4^+) in the medium contributed to smaller toxicity in the root system of turmeric. This toxicity may have contributed to a decrease in the number of leaves and shoots of the species.

Conclusion

All evaluated asepsis methods presented the same effect on the evaluated characteristics, except for the root dry matter. In that case, the usual asepsis method can be the most effective because it is less costly and demands less time. Therefore, it is recommended that shoots be washed in 2% sodium hypochlorite solution for 20 min, followed by three washings with deionized water. The concentration of 60 g/L of sucrose was the most feasible for turmeric. Nevertheless activated charcoal must be added to MS medium at 4.5 g/L. Although charcoal did not have an effect on all evaluated characteristics, it can be added because it did not compromise seedling development. Salt concentration in MS should range from 50 to 60% of the total amount of salts. When utilized at the highest concentration of 100%, it damages the root system, number of leaves, and shoots.

Conflict of Interests

The authors have not declared any conflict of interest.

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