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African Journal of Biotechnology

Review

Phytochemical profile and biological activities of *Momordica charantia* L. (Cucurbitaceae): A review

Mozaniel Santana de Oliveira[1*], Wanessa Almeida da Costa[2], Fernanda Wariss Figueiredo Bezerra[1], Marilena Emmi Araújo[1], Gracialda Costa Ferreira[3] and Raul Nunes de Carvalho Junior[1,2]

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This study discusses the bioactive composition, supercritical fluid extraction and biological activities of *Momordica charantia* L. from the last five years. Numerous compounds that have been identified in the extracts of *M. charantia,* including phytosterols, terpenoids, fatty acids, phenolic compounds, phenolic acids and flavonoids were also discussed. Although, several studies reported the use of organic solvents in the extraction of these compounds, this review emphasized on supercritical fluid extraction (SFE), good selectivity, varied fractions in terms of mass yields and chemical composition obtained, in addition to providing a solvent-free extract. Moreover, the biological effects of *M. charantia* extracts, including their antidiabetic, neuroprotective, anti-obesogenic, antimalarial, antioxidant, anti-inflammatory, antimicrobial and allelopathic activities, were discussed. These biological effects of the extracts of *M. charantia* can directly affect human health. The findings of this review are important, as they can guide future studies related to obtaining bioactive compounds from *M. charantia* and its applications.

Key words: Bitter melon, supercritical fluid, bioactive compounds, biological activities.

INTRODUCTION

Momordica charantia L. belongs to the Cucurbitaceae family comprising of 47 species in Africa and 12 in Asia and Australia. All have unisexual flowers, and of the African species, 24 are dioecious, 23 monoecious, while all Asian species are dioecious (Schaefer and Renner, 2010; Dalamu et al., 2012; Rahman, 2013). This plant is known in English as: balsam pear, bitt gourd, African cucumber, wild cucumber, bitter cucumber, bitter melon, bitter apple, carilla fruit, carilla seed, leprosy gourd, basam apple, in Peru as: fun-kua, papailla Central america: cundeamor, balsamina, pepinillo, serosi, in Brazil as: melão de São caetano, melão de São Vicente,

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fruto de cobra, in Philippines as: ampalaya, apalia, palia, paria, pulia, saligun, apape, apapet, amargoso, margoso, in Malasya as: paria laut, duaun periok, in China as: ku gua, k'u hua, chin li chih, lai pu tao, in Japan as: nega-reishi, gôyâ, in Tibetan as: gser-gyi metog, in Mozambique as: nhadzumba, and in Congo as: lunbuzi, lubuzi-buzi, lumbuzi-busi (Quattrocchi, 1999; Abascal and Yarnell, 2005; Zhao et al., 2012). Although it originates from Asia, it is cultivated in several parts of the world, including Central and South America and Africa (Ji et al., 2012).

The São Caetano melon is widely used as a medicine and as food. It has several ethnopharmacological indications, such as antidiabetic (Rahmatullah et al., 2012), immunomodulatory (Deng et al., 2014), anti-dengue (Tang et al., 2012) and antioxidant activities (Aljohi et al., 2016), and has been shown to prevent hepatic fibrosis (Efird et al., 2014); in agriculture, it can be used to promote allelopathic activity (Singh, 2014).

In the Amazon, alternative medicine is very important for traditional communities, and the use of medicinal plants such as *M. charantia* has been widespread in the treatment of diseases such as malaria (Veiga and Scudeller, 2015). Bioactive compounds have been isolated from several parts of the plant, including the fruits, seeds and leaves (Choi et al., 2012; Yaldız et al., 2015). These biological activities are attributed to their complex chemical composition; they are rich in tannins, terpenoids, carbohydrates, resins, saponins, flavonoids, sterols, phylobatamins, anthraquinones, glycosides, amino acids, fatty acids and phenolic compounds (Sathya et al., 2012; Sood et al., 2012). The bioactive compounds are commonly obtained through conventional extraction techniques with many different types of organic solvents (Dar et al., 2014; Tan et al., 2014; Yeo et al., 2014), which may be toxic to human health. Therefore, other forms of extraction are necessary, for example, extraction with supercritical fluids. This extraction technique has great advantages over conventional methods, such as being selective depending on the operating conditions (temperature, pressure, and density) used during the extraction process. It is also considered a "green technique" for obtaining active compounds of plant origin (Coelho et al., 2012; Sánchez-Camargo et al., 2012; Oman et al., 2013). These benefits to human health make *M. charantia* a very important medicinal plant for use in alternative therapies worldwide (Urasaki et al., 2016), as has already been demonstrated in other previous literature reviews (Upadhyay et al., 2015; Tan et al., 2016; Zhang et al., 2016; Janagal et al., 2018).

BOTANICAL TAXONOMY OF THIS PLANT

M. charantia (*Cucurbitaceae*) is Liana or terrestrial creeper found throughout Brazil, and is characterized by the presence of simple, long and pubescent tendrils that present a thin, grooved, and green herbaceous stem. Mature fruits of the wild balsam-pear are 2 to 7 cm in length and 1.4 to 2 cm in width. The leaves are membranous, alternating, and simple with palmatipartite appearance and actinomorphic venation pattern with right lateral insertion, and are obtuse-quadrangular in cross section. They have a hairy surface, mucronate dentate margin, acute apex, lobed base and pubescent surface. The plant also produces pale or whitish yellow monocoic flowers and green berry-like fruits when immature that become yellow-orange when ripe. The seeds are wrapped in a reddish and edible substance. The species, *M. charantia* has diclinous flowers, with diurnal anthesis. The period it can last during flowering is 100 days. In the beginning of the flowering, the species presents dicogamy of the protandry type. The female flowers do not produce nectar, while the male flowers produce nectar during the entire period of anthesis. Fruit formation occurs through crossed-pollination and self-pollination. Figure 1 shows *M. charantia* with some fruits and flowers (Walters and Decker-Walters, 1988; Lenzi et al., 2005; Aguoru 2012; Dalamu et al., 2012; Singh et al., 2014; Giuliani et al., 2016).

PHYTOCHEMICALS PRESENT IN *M. CHARANTIA*

M. charantia contains a large number of bioactive compounds, which were identified and published in the last five years. Results of phytochemical analyses revealed the presence of alkaloids, tannins, saponins, flavonoids, cardiac glycosides and steroids (Mada et al., 2013; Oragwa et al., 2013). The biological activities of these substances are presented in this review.

Phytosterols

Phytosterols are group of sterols naturally found in plants. They are generally found in low concentrations, and have a total of up to 30 carbon atoms (Cherif, 2012). Articles on the identification of phytosterols in *M. charantia* are summarized in Table 1, and their chemical structures are shown in Figure 2. Phytosterols are known for lowering blood cholesterol levels, without altering the high-density lipoprotein or triglyceride levels (Yi et al., 2016). Other pharmacological effects attributed to phytosterols include anticancer, atherosclerotic, anti-inflammatory and antioxidant activities (Ramprasath and Awad, 2015; Uddin et al., 2015; Zhu et al., 2015).

Terpenoids

Terpenoids are diversified class of natural products that have various biological functions in the plant, and are responsible for the growth of the plant (Moses and Pollier, 2013).They also have anti-inflammatory and anticancer applications (Liu et al., 2012; Zhangetal, 2012).

Figure 1. *Momordica charantia* L. fruit and leaves.

Table 1. Phytosterols identified in *Momordica charantia* L. in the last five years.

Identified sterols	Reference
β-sitosterol and Daucosterol	Kim et al. (2013)
Campesterol, Stigmasterol and β-sitosterol	Yoshime et al. (2016)
β-sitosterol	Sen et al. (2012)
25ξ-isopropenylchole-5,(6)-ene-3-O-β-D-lucopyranoside	Liu et al. (2012)
Stigmasterol, β-sitosterol and Diosgenin	Agarwal and Kamal (2013)
Δ5–avenasterol and 25,26-dihydroelasterol	Daliborca et al. (2015)

The effects of six new cucurbitane-type triterpenoids (3-[(5β,19-Epoxy-19,25-dimethoxycucurbita-6,23-dien-3-yl)-2- oxoacetic acid; 3-[(5β,19-Epoxy-19,25-dimethoxycucurbita-6,23-dien-3-yl)oxy]- 3-oxopropanoic acid; 3-[(5-Formyl-7β-hydroxy-25-methoxycucurbita-5,23-dien-3-yl)- oxy]-3-oxopropanoic acid; 3-[(5-Formyl-7β-methoxy-7,23S-dimethoxycucurbita-5,23-dien3-yl)oxy]-3-oxopropanoic acid; 3-[(25-O-Methylkaravilagenin D-3-yl)oxy]-2-oxoacetic acid; 3-[(5-Formyl-7β,25-dihydroxymethoxycucurbita-5,23-dien-3-yl)- oxy]-3-oxopropanoic acid, isolated from the fruits of *M. charantia* on a typical proliferation of vascular smooth muscle cells (VSMCs) were analyzed, and in some cases up to 72.4% proliferation blockade were observed. In addition, these phytochemicals showed no cytotoxicity against the cultured cells studied; thus, *M. charantia* is a potential source of new active biomolecules for the treatment of cardiovascular diseases through the inhibition of VSMC proliferation (Tuan et al., 2017). The terpenoids that have been identified in *M. charantia* are summarized in Table 2, and their chemical structures are presented in Figure 3.

Fatty acids

Fatty acids are organic compounds with a carboxyl group (-COOH) bound to carbonic chains that can be saturated or unsaturated (Campen et al., 2015; Wood et al., 2016). Fatty acids, such as ω-3, can exert functions that are beneficial to human health, and can prevent or reduce the risk of developing cardiovascular diseases (Delgado-Lista et al., 2012). It is also reported that they may act as antimicrobial agents against bacteria (Alva-Murillo et al., 2012) and fungi (Urbanek et al., 2012). These biological activities justify new research on the extraction and applications of fixed oils of vegetable origin. *M. charantia* has high levels of fatty acids, as shown in Table 3. Some examples of their chemical structures are shown in Figure 4.

Phenolic compounds

Phenolic compounds are among the numerous secondary metabolites found in plants. They can be found in the form of simple phenols, phenolic acids, coumarins,

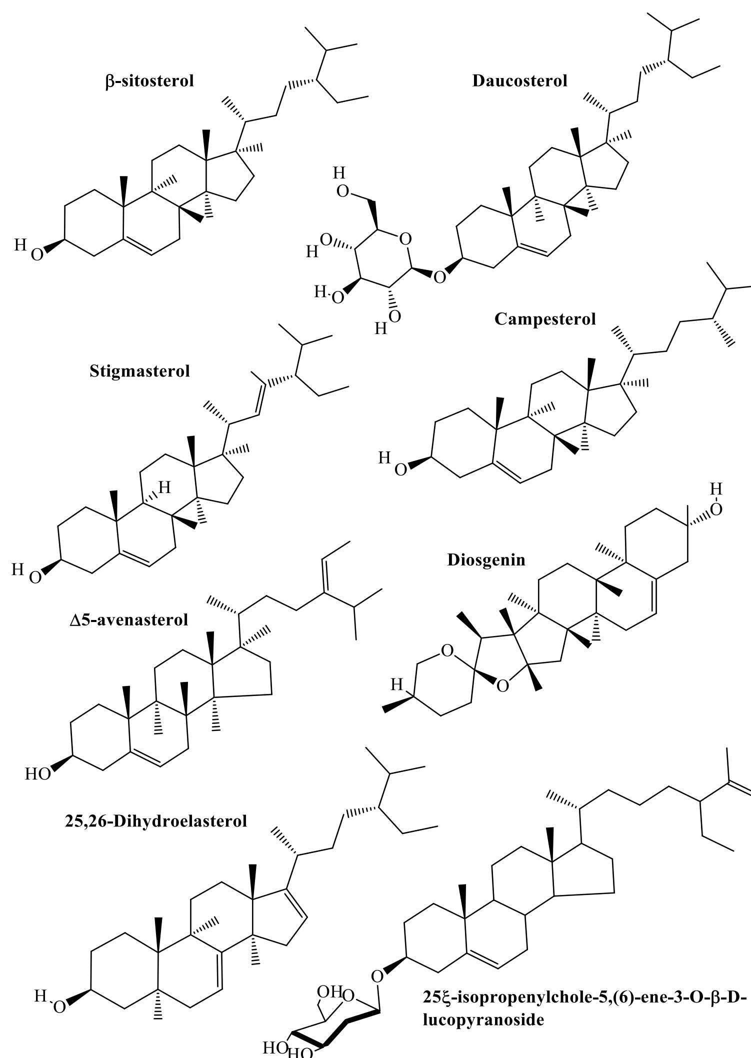

Figure 2. Chemical structures of phytosterols identified in *M. charantia* L. in the last five years.

tannins, lignins, lignans and flavonoids (Žilić et al., 2012; Khoddami et al., 2013). These compounds have several important effects, such as antioxidant, antimicrobial anti-HIV-1, and anticancer activities (Alves et al., 2013; Ghasemzadeh and Jaafar, 2013; Hu et al., 2013; Roby et al., 2013). Species such as *M. charantia* are a rich source of phenolic compounds, as shown in Table 4. The chemical structures of phenolic acids and flavonoids are represented in Figures 5 and 6, respectively.

EXTRACTION OF BIOMOLECULES OF *M. CHARANTIA* WITH SUPERCRITICAL FLUID

Active compounds of plant origin are generally extracted according to their chemical composition, biological activities, and the needs and purposes of the studies; however, some extraction methods can affect the quality of the extracts due to contamination from organic solvents, which can also increase the cytotoxicity of these extracts. In this scenario, supercritical fluid extraction (SFE) has been gaining interest in recent years, because in addition to being considered a "green" extraction method, it has other advantages (Poliakoff and Licence, 2015) such as fractionation selectivity (Chitra et al., 2015), higher yields of bioactive compounds as compared to those of conventional methods (Farías-Campomanes et al., 2013), and the fact that it can be performed at low temperatures, avoiding the degradation of thermosensitive substances. A supercritical fluid is any pure substance at a pressure and temperature above its critical point where distinct liquid and gas phases do not exist (Knez et al., 2014) (Figure 7). In addition, the SFE extraction method has other advantages over conventional methods, such as not using toxic organic solvents, and it usually works with a lower extraction

Table 2. Terpenoids identified in *Momordica charantia* L. in the last five years.

Identified terpenoids	Reference
Charantagenins D and charantagenins E	Wang et al. (2012)
4 new compounds, kuguaosides A−D (1−4), along with 11 known ones, charantoside A (5), momordicosides I (6), F1 (7), F2 (8), K (9), L (10), and U (11), goyaglycosides-b (12) and -d (13), 7β,25-dihydroxycucurbita-5,23(E)-dien-19-al 3-O-β-D-allopyranoside (14), and 25-hydroxy-5β,19-epoxycucurbita-6,23- dien-19-on-3β-ol 3-O-β-D-glucopyranoside (15).	Hsiao et al. (2013)
Phytol	Hsu et al. (2012)
Kuguacin J (Kuj)	Pitchakarn et al. (2012)
5β,19-epoxy-25- methoxy-cucurbita-6,23-diene-3b,19-diol (EMCD)	Cheng et al. (2012)
Charantin A (16), charantin B (17), momordicines I (18) and II (19), 3b,7b,25-trihydroxycucurbita-5,(23E)- dien-19-al (20), and momordicoside K (21)	Zhang et al. (2014)
3*β*,7*β*-dihydroxy-25-methoxycucurbita-5,23- diene-19-al (DMC)	Weng et al. (2013)
28-*O*-*β*-Dxylopyranosyl, (1→3)-*β*-D-xylopyranosyl, (1→4)-*α*-L-rhamnopyranosyl, (1→2)-[*α*-L-rhamnopyranosyl, (1→3)]-*β*-D-fucopyranosyl gypsogenin 3-*O*-*β*-D-glucopyranosyl, (1→2)-*β*-Dglucopyranosiduronic acid (C1) and 28-*O*-*β*-D-xylopyranosyl, (1→4)-*α*-L-rhamnopyranosyl, (1→2)-[*α*-L-rhamnopyranosyl, (1→3)]-*β*-D-fucopyranosyl gypsogenin 3-*O*-*β*-D-glucopyranosyl, (1→2)-*β*-D-glucopyranosiduronic acid (C2)	Ma et al. (2014)
5β,19‑epoxycucurbitane triterpenoids,	Liaw et al. (2015)
Karavilagenin F, karaviloside XII, karaviloside XIII, momordicine VI, momordicine VII, momordicine VIII	Zhao et al. (2014)

temperature, reducing the incidence of degradation of the product; in some cases subsequent purification steps are not necessary (Bagheri et al., 2014; Conde et al., 2014; Nguyen et al., 2015).

In the extraction process, a wide variety of fluids can be used as solvents as shown in Table 5. However, most of these compounds, such as light hydrocarbons, are generally flammable and toxic. On the other hand, carbon dioxide is the only compound that can be used as a "green solvent" and its critical properties are relatively low (Table 5). Carbon dioxide (CO_2) is particularly advantageous for the processing of food materials, because it is an inert gas, in other words, it reacts with the chemical compounds present in the extracts (Tabernero et al., 2012; Conde-Hernández et al., 2017). The critical properties of a pure substance may vary according to the interaction of the chemical bonds (intermolecular forces). As indicated in Table 5, molecules with the highest polarity have the highest critical properties (Pc) and (Tc) (Botelho et al., 2015).

In supercritical fluid extraction, temperature and pressure combinations are linked to the solubility of the compounds (Botelho et al., 2014). The control of pressure and temperature in supercritical fluids is one of the most important operating parameters because the density of the supercritical fluid increases with the pressure at constant temperature and decreases with temperature at constant pressure (Mantell et al., 2013). The density variation may lead to a possible change in the solubility of the compounds present in the raw material (Dias et al., 2012; de Oliveira et al., 2016).

Besides that, the mathematical models of mass transfer are important tool for SFE. These models exploit the kinetic behavior of the dynamic extraction period, and offer parameters such as mass transfer coefficient, diffusion coefficient and diffusivity in the solid phase (Sovová, 2012; Özkal and Yener, 2016). In addition, the modeling of the kinetic curves of extraction makes it possible to suggest scale-up methodology to predict the behavior of the extraction process on an industrial scale (Prado et al., 2012; Wüst Zibetti et al., 2013; Taher et al., 2014).

In this context, in recent years, several authors have used this technique to obtain bioactive

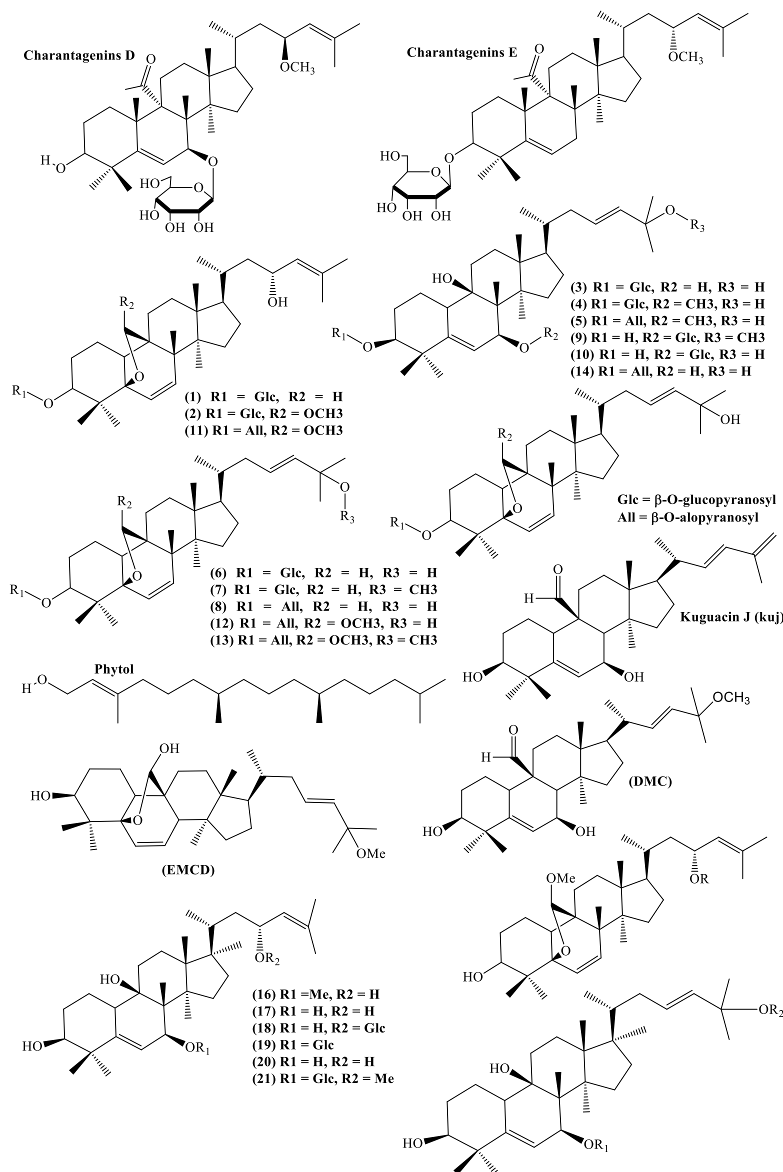

Figure 3. Chemical structures of some terpenoids identified in *Momordica charantia* L.

extracts of *M. charantia* (Ning-Ping, 2013). Supercritical carbon dioxide (SC-CO_2) with ethanol as co-solvent has been used to extract flavonoids from the *M. charantia* fruit, and the influence of parameters such as temperature, pressure and extraction time were verified (Shan et al., 2012). The experimental data showed that pressure, temperature and time had statistically significant effects on the extraction yield, indicating that extraction with SC-CO_2 and ethanol may be an alternative method for the selective extraction of flavonoids from *M. charantia*.

Bitter melon seed oil was extracted with SC-CO_2 to verify the best extraction operating conditions, and the highest yield was obtained at 250 bar/50°C in 100 min report, extraction with SC-CO_2 was performed to improve the efficiency and selectivity of fatty acid extraction, in which the authors found the presence of 42.60% of conjugated linolenic acid (ClNa, cis-9, trans-11, trans 13-18: 3) and 13.17% of conjugated linoleic acid (CLA, cis-9, trans 11-18: 2) (Xu et al., 2016).

BIOLOGICAL ACTIVITIES OF *M. CHARANTIA*

The biological activities of plants traditionally used in folk medicine or as functional foods are the primary motivator for further research (Sihoglu and Tepe, 2015; Heinrich et al., 2016). Several studies on the biological activity of this

Table 3. Chemical composition of fatty acids identified in *Momordica charantia* L.

Fatty acids	Reference
Palmitic, stearic, myristic, pentadecanoic, arachidic, α-linolenic, linoleic, oleic and palmitoleic acids.	Sarkar et al. (2013)
Capric, lauric, palmitic, stearic, oleic, linoleic and arachidic acids.	Ahmad et al. (2012)
Palmitoleic, arachidic, docosanoic, oleic, stearic, heneicosanoic, α-linolenic, myristic, nonadecanoic, lauric, decanoic, linoleic, tridecanoic and pentadecanoic acids.	Sarkar and Barik (2015)
Palmitic, stearic, oleic, linoleic, α-eleostearic, arachidic and gadoleic acids.	Gölükçü et al. (2014)
Decanoic, lauric, tridecanoic acid, myristic, pentadecanoic, palmitoleic, palmitic, heptadecanoic, α-linolenic, nonadecanoic, heneicosanoic, docosanoic and tetracosanoic acids.	Mukherjee and Barik (2014)
Tridecanoic, myristic, palmitic, stearic, oleic, arachidic, α-linolenic, henicosanoic, behenic and lignoceric acids.	Saini et al. (2017)
α-Eleostearic and stearic acids.	Yoshime et al. (2016)

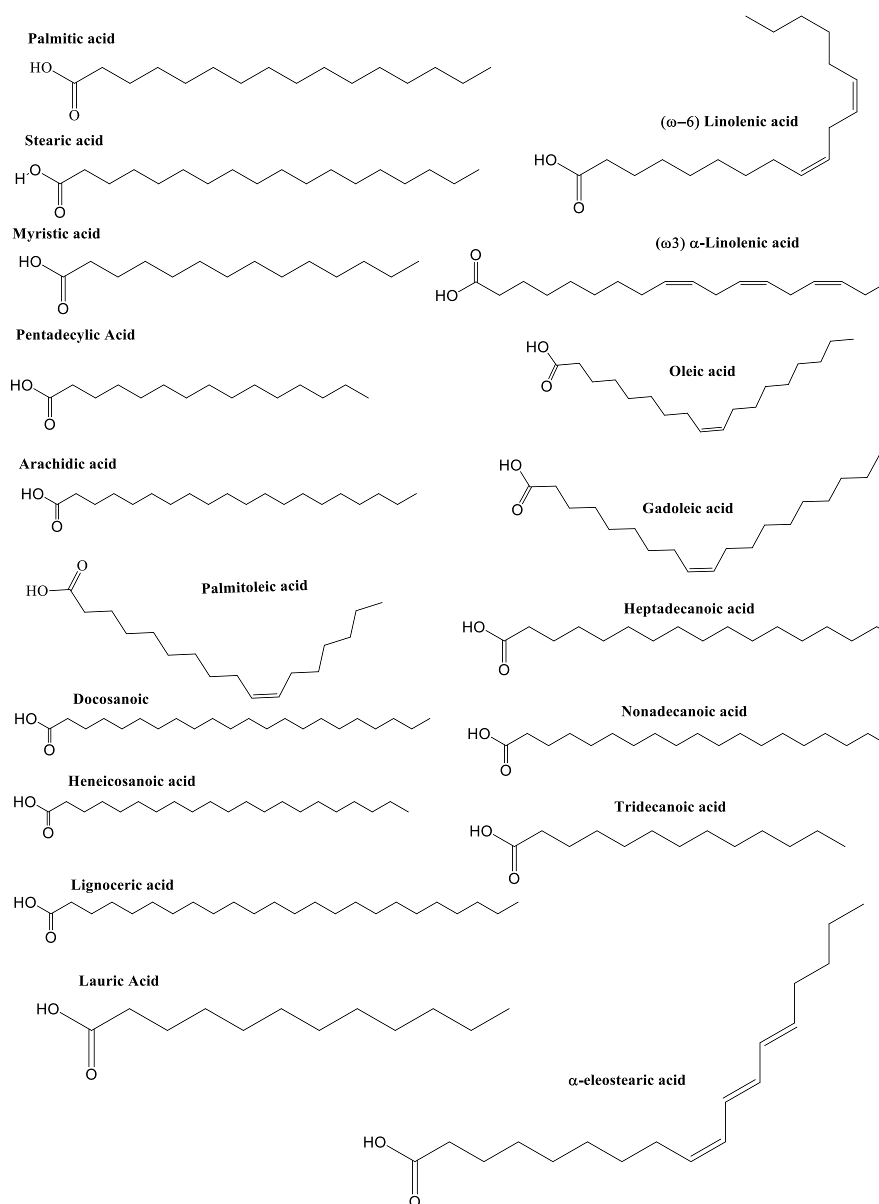

Figure 4. Chemical structures of fatty acids identified in *Momordica charantia* L.

Table 4. Chemical composition related to the phenolic compounds identified in *M. charantia* L.

Phenolic acids	Flavonoids	Reference
Gallic, chlorogenic, caffeic and ellagic acids.	Catechin, epicatechin, rutin, quercitrin, isoquercitrin, quercetin and kaempferol.	Shodehinde et al. (2016)
Gallic, protocatechuic, tannic, p-hydroxylbenzoic, vanillic, caffeic, chlorogenic, p-coumaric and ferulic acids.	Epigallocatechin, epicatechin, gallocatechin gallate, quercetin and kaempferol.	Choi et al. (2012)
Protocatechuic, gallic, chlorogenic, syringic, caffeic, ferulic, 3- coumaric and 4- coumaric acids.	Catechin, rutin, luteolin-7-O-glycoside, naringenin-7-O -glycoside, apigenin- 7-O –glycoside, myricetin, quercetin, kaempferol, luteolin and apigenin.	Kenny et al. (2013)
Gallic, chlorogenic, caffeic, *p*–coumaric, ferullic acids.	Catechin	Lee et al. (2016)
Caffeic, *p*-coumaric, ferulic, *o*-coumaric, chlorogenic, *m*-coumaric, *p*-hydroxybenzoic, gallic, protocatechuic, β-resorcylic, vanillic, syringic, gentisic, salicylic, veratric, *t*-cinnamic and homogentisic acids.	Myricetin, quercetin, kaempferol, catechin, rutin, hesperidin, naringenin, biochanin a, and naringin.	Thiruvengadam et al. (2014)

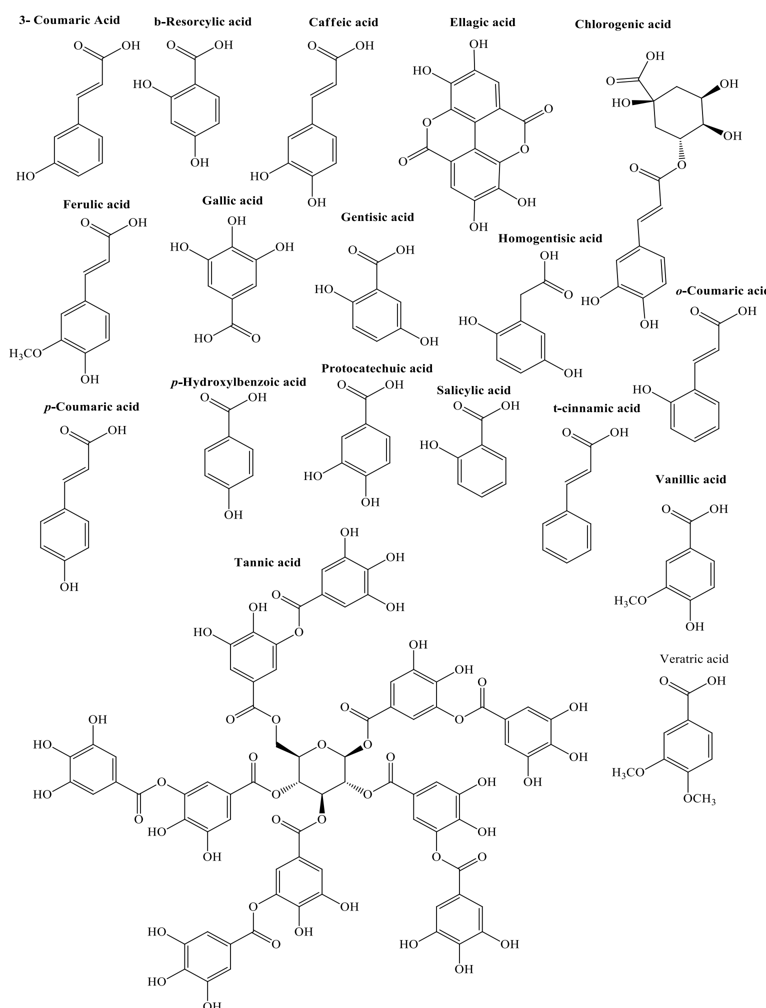

Figure 5. Chemical structures of phenolic acids identified in *Momordica charantia* L.

plant have been published. In this section, the main studies evaluating the biological activities of *M. charantia* were discussed.

Antidiabetic activity

Diabetes is defined as a group of metabolic diseases

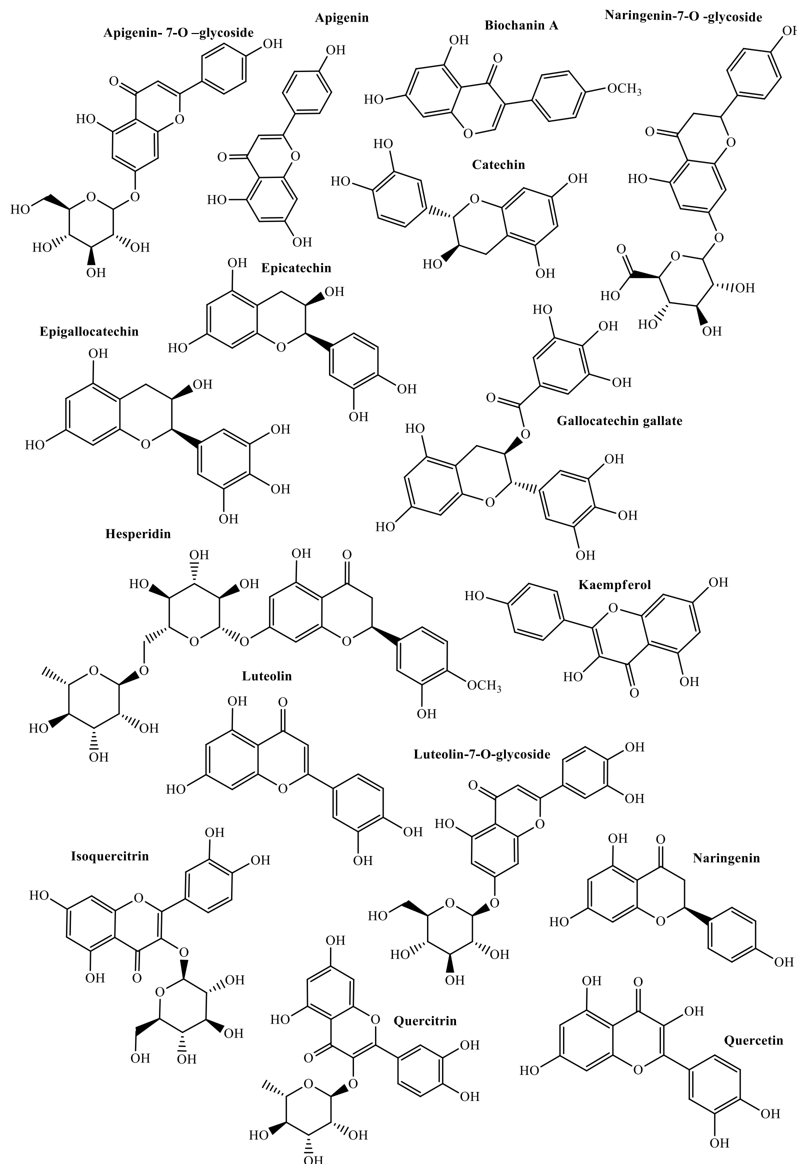

Figure 6. Chemical structures of flavonoids identified in *Momordica charantia* L.

characterized by hyperglycemia caused by defects in insulin secretion, insulin action or both. Chronic (Xu et al., 2014). In addition, the oils showed a high concentration of linolenic and stearic acids. In another hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels (Freinkel et al., 2014; Lo et al., 2014). This disease is common throughout the world, and it is reported that people with this pathology seek alternative treatment with *M. charantia* and other medicinal plants to complement their therapy (Joseph and Jini, 2013).

The extract of *M. charantia* was shown to reduce blood glucose level in rats (Perumal et al., 2015). These results might be related to a study indicating that the extracts of *M. charantia* inhibited the activity of α-amylase and α-glucosidase, reducing blood glucose levels (Poovitha and Parani, 2016). These results can be corroborated by other numerous studies that report antidiabetic activity of the São Caetano melon (Blum et al., 2012; Chaturvedi, 2012; Hasan and Khatoon, 2012; Xu et al., 2015; Mishra et al., 2015; Tayyab and Lal, 2016; Yousaf et al., 2016). Thus, this medicinal herb could potentially be used to treat diabetes. In another scientific report, the effects of *M. charantia* on insulin resistance in diabetic rats were analyzed. The results show that the extract exerts its preventive effects on insulin resistance through the modulation of phospho-NF-κB and phospho-c-Jun N-

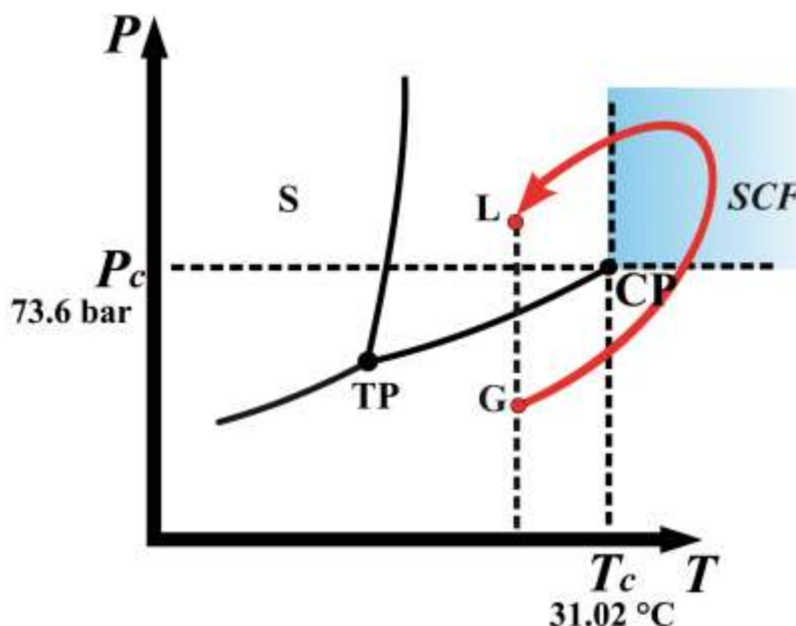

Figure 7. The *pV T* surface for equilibrium states of CO_2. The solid (S) line *GL* is a thermodynamic path where the continuous transformation of the gas (G) into a liquid (L) does not require the phenomenon of condensation to cross the liquid vapor coexistence curve at constant temperature, triple point (TC), critical pressure (Pc), critical temperature (Tc) and critical point (CP). Phase diagram of CO_2, adopted from Zappoli et al. (2015).

Table 5. Critical properties of different compounds. Adapted from Tabernero et al. (2012).

Critical properties of different compounds	Tc (°C)	Pc (bar)
Ethylene	9.35	51
Carbon dioxide	31.02	73.6
Ethane	32.45	49
n-Propane	93.85	43
Chlorotrifluoromethane	111.75	39
Ammonia	132.5	113
Methanol	240.55	79
Benzene	289.05	49
Water	374.45	221

terminal kinase (JNK) pathways (Yang et al., 2015).

Despite these results, there are some reports that *M. charantia* does not have sufficient effect on type 2 diabetes mellitus (Ooi et al., 2012). One study showed that *M. charantia* extract had hypoglycemic effects, and suggested that it has potential to increase insulin sensitivity in rats with type 2 diabetes, instead of protecting against β-cell dysfunction (Wang et al., 2014). However, the majority of studies analyzed in this review indicated that *M. charantia* may act as a complementary treatment for diabetes (Ahmad et al., 2012; Lo et al., 2013; Chhabra and Dixit, 2013; Singh et al., 2014; Duraiswamy et al., 2016; Ekezie et al., 2016; Mahmoud et al., 2017; Wang et al., 2017).

Neuroprotective activity

Cerebral ischemia usually occurs through an obstruction of the arteries in the brain. Although, therapies for restoring blood flow to brain tissues are effective, reperfusion in the ischemic brain leads to a series of pathophysiological changes (Hua et al., 2015), and is a significant cause of morbidity and mortality in patients with aneurysmal subarachnoid hemorrhage (ASAH) (da Costa et al., 2015). The immune response is a great

contributor to stroke pathology, and inflammation occurs due to the involvement of peripheral leukocytes and resident immune cells in the brain (Benakis et al., 2015).

A recent study showed that *M. charantia* could inactivate reactive oxygen species (ROS) present in the area damaged by intracerebral hemorrhage, significantly attenuating thrombin-induced neuronal death in primary hippocampal neurons (Duan et al., 2014). In addition, *M. charantia* inhibited the activation of c-Jun N-terminal kinase 3 caused by intracerebral hemorrhage. These results corroborate those obtained by Gong et al. (2015) who classified *M. charantia* as a plant that has neuroprotective activity, inhibiting the effects of c-Jun N-terminal kinase signaling during ischemia/reperfusion injury. These few scientific reports regarding the neuroprotective activity of *M. charantia* are very important to direct future work in the scientific community.

Obesity reduction

Obesity is considered a worldwide epidemic and is directly related to coronary diseases and diabetes (Yaghootkar et al., 2014; Scherer and Hill, 2016). It can also cause chronic inflammation of adipose tissue (Bluher, 2016). *M. charantia* is also known for its ability to reduce body weight gain (Bao et al., 2013). Therefore, it may be an alternative method for therapies in the control of obesity.

Wang and Ryu (2015a) analyzed the effects on obesity and lipid profile of rats fed fatty acid-rich extracts of *M. charantia*. They found that this plant was anti-obesogenic, and had the ability to modulate lipid proliferation, decrease body weight gain, visceral tissue weight, plasma and lipid concentrations, and lipid peroxidation in metabolism. The weight loss may be related to the increased energy of the rats, demonstrating that a diet rich in *M. charantia* extracts may aid in the treatment of obesity (Bian et al., 2016).

Recent clinical trials have shown that plant extracts, including the São Caetano melon extract, have therapeutic potential against diabetes and metabolic dysfunction related to obesity in animals (Chen et al., 2012; Alam et al., 2015). The effects of this plant on mitochondrial function, during the accumulation of liver fat associated with obesity, were identified by Xu et al. (2014). These authors suggested that *M. charantia* reduces inflammation and oxidative stress, modulates mitochondrial activity, suppresses the activation of apoptosis and inhibits the accumulation of lipids during the development of fat in the liver.

In this context, several articles have shown that *M. charantia* suppresses weight gain in animals, primarily rats (Zeng et al., 2012; Bin and Liu, 2013; Yu et al., 2013; Shih et al., 2014; Wang and Ryu, 2015b; Bai et al., 2016). Some results show that the extracts of *M. charantia* improve the oxidation of hepatic triacylglycerol, which may be one of the mechanisms involved in the decrease of body fat concentration (Senanayake et al., 2012).

Anticancer effect

In many parts of the world, cancer is a large public health problem and represents one of the leading causes of death. In the future, cancer deaths will likely overcome deaths caused by cardiovascular diseases (Siegel et al., 2015). Estimates indicate that one out of four deaths in the United States are due to cancer (Siegel et al., 2013). The main types of cancer are lung, breast and colorectal cancers, and those that cause death most are lung (1.6 million deaths), liver (745,000 deaths) and stomach (723,000 deaths) cancers (Ferlay et al., 2015). However, such deaths can be avoided if the cancer is diagnosed and treated early.

Cancer treatment is generally time-consuming, which risks patients' psychological and physical health (Stanton et al., 2015). In addition, there is risk of acquiring chemoresistance, a great obstacle in clinical management. Therefore, alternative therapies with the use of drugs obtained from medicinal plants including *M. charantia* are of great importance (Comhaire, 2014; Yung et al., 2016).

M. charantia is known to inhibit the growth of cancer cells by inducing of apoptosis (Dandawate et al., 2016). For example, the protein MAP30 present in São Caetano melon seeds, has an effect on liver cancer, HepG2 hepatocellular carcinoma models of human hepatoma and rat cells (Fang et al., 2012). The authors also suggested that the seeds would work as a relatively safe agent for prophylaxis and treatment of this cancer. Other studies also reported the anticancer activity of *M. charantia* (Brennan et al., 2012).

In other studies, the anticancer activity of São Caetano melon was linked to other chemical compounds such as triterpenoids. The effects of Kuguacin J (Kuj), a component of *M. charantia* obtained from the extract of its leaves, were evaluated, and the results showed that this secondary metabolite has a strong inhibitory effect on the growth of prostate cancer in PC3 cell line, with inhibition of up to 63% of cell growth, with no adverse effect on the patient (Pitchakarn et al., 2012). The anticancer activity of terpenoids and sterols found in *M. charantia* are also reported by other authors (Wang et al., 2012; Zhang et al., 2012; Weng et al., 2013). The literatures indicates that *M. charantia* inhibits many types of cancer, including hepatocellular carcinoma (Zhang et al., 2015), lung (Fan et al., 2015), bladder (Lin et al., 2016), colon (Dia and Krishnan 2016) and breast (L. yuan Bai et al., 2016) cancers. The mechanisms of action of bioactive compounds on cancer cells may be related to apoptosis through the regulation of enzymes with a cysteine residue capable of cleaving other proteins

(caspase) and mitochondria (Li et al., 2012; Manoharan et al., 2014; Minina et al., 2017).

Antioxidant activity

There is growing interest in antioxidants of natural origin because of their potential beneficial effect on human health (Gülçin, 2012). Natural antioxidants are very important for maintaining quality of life (Shahidi and Ambigaipalan, 2015), because the human body produces ROS, which can damage cellular structures such as carbohydrates, nucleic acids, lipids and proteins and alter their function, leading to the development of various degenerative diseases (Birben et al., 2012).

Therapeutic agents for the treatment of diseases caused by oxidative stress and metabolic disorders are well known. *Momordica* species have shown good results regarding their antioxidant activity (Nagarani et al., 2014) and may prevent oxidative stress (Sagor et al., 2015). In this way, they may also exert cardioprotective activity (Raish, 2017). Rammal et al. (2012) found that *M. charantia* has the capacity to eliminate ROS; they concluded that the consumption of 100 g of fruit can provide up to 145 ± 1.16 mg of a compound equivalent to vitamin C. Others analyzed the effects of *M. charantia* extracts on the $DPPH^+$ radical, and found an IC_{50} value of up to 0.46 mg/mL (Shan et al., 2012). The antioxidant activities of the extracts of *M. charantia* may be directly related to the method of extraction used to obtain the bioactive compounds. For example, extracts rich in phenolic compounds are shown to have antioxidant activity in different analytical methods: 2,2-diphenyl-1-picrylhydrazyl ($DPPH^+$); 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), and potential iron reducer (FRAP) (Choi et al., 2012; Hamissou et al., 2013; Kenny et al., 2013; An, 2014; Aljohi et al., 2016; Ri Lee, 2016; Hani et al., 2017). In addition to the phenolic compounds mentioned above, some polysaccharides present in *M. charantia* exert antioxidant activities (Liu et al., 2014; Raish 2017). In general, this species has significant antioxidant activity, and can act as functional food aiding in the control of oxidative stress (Sin et al., 2013).

Anti-inflammatory activity

Numerous medicinal plants present scientific evidence of anti-inflammatory effects (Alhakmani et al., 2013; Sagnia et al., 2014; Dzoyem and Eloff, 2015). Among the plant species traditionally used for the control of inflammatory diseases, *M. charantia* can be highlighted. *M. charantia* was found to improve the biological responses against inflammation in rats with sepsis (Chao et al., 2014). Other authors also report the anti-inflammatory activity of this plant (Nagarani et al., 2014; and Liaw et al., 2015). Another report showed that *M. charantia* had anti-inflammatory activity on adipose tissue cells (Bao et al., 2013). Thus, there is great interest in new studies searching for active molecules with anti-inflammatory activity from this plant.

Antimicrobial activity

There is also evidence that the ethanolic extract of *M. charantia* presents low cytotoxicity, with antiepimastigotes and antifungal activities. 46.06 µg/mL was shown to effectively kill 50% of parasites. The extract showed effect similar to metronidazole, which may represent an alternative for the treatment of candidiasis (Santos et al., 2012). Extracts of *M. charantia* also had antimicrobial effects against the microorganisms: *Pasteurella multocida*, *Staphylococcus aureus* and *Salmonella typhi* (Mahmood et al., 2012). The effects of extracts of *M. charantia* on Gram-positive and negative bacteria and fungi are shown in Table 6.

The antimicrobial activity of *M. charantia* L. extract against *S. aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* was evaluated. The extracts at concentration of 100 mg/ml were efficient to inhibit the growth of all bacteria, with different degrees of susceptibility (Mada et al., 2013). Other studies confirm that this medicinal plant has good antimicrobial activities (Ozusaglam and Karakoca, 2013; Shoba et al., 2014; Birla, 2016; Saengsai et al., 2015).

Malaria is one of the deadliest diseases in Africa (Murray et al., 2012). The infection is caused by *Plasmodium falciparum.* However, one of the major problems faced by health professionals is the resistance of parasites to antimalarial drugs. One way to avoid this resistance is by using bioactive compounds from medicinal plants such as *M. charantia*, which represents a potential new source of antimalarial drugs (Olasehinde et al., 2014). Pereira et al. (2016) demonstrated that *M. charantia* has antiprotozoal activity. The methanolic extract of *M. charantia* had an antimalarial effect at doses above 200 mg/kg (Akanji et al., 2016). Other works also reports the antimalarial activity of this plant (Adeyi et al., 2016; Syamsudin et al., 2017). These studies have great relevance for tropical countries, because they contribute to the diffusion of knowledge on alternative methods of controlling diseases caused by parasites such as *P. falciparum.* The antimalarial activity of *M. charantia* can be related to the synergistic and antagonistic effects of chemically active metabolites present in the extracts, such as alkaloid, flavonoid, saponin, tannin, quinone, steroid, triterpenoid and coumarine (Abdillah et al., 2015).

CONCLUSIONS

This review showed that *M. charantia* presents several

Table 6. The effects of different extracts of M. charantia on fungi and bacteria.

Fungi name	Bacteria name	Reference
Candida albicans, Candida neoformans, Candida glabrata, Candida epicola	*Staphylococcus aureus, Staphylococcus albus, Corynebacterium rubrum, Listeria monocytogenes, Micrococcus flavus, Pseudomonas aeruginosa, Pseudomonas stutzeri, Pseudomonas pictorum, Pseudomonas putida, Pseudomonas testosteroni, Pseudomonas syrigae*	Rakholiya et al. (2014)
Candida albicans	*Bacillus subtili, Staphylococcus aureus, Streptococcu pyogenes, Escherichia coli.*	Agyare et al. (2014)
Aspergillus niger subsp, Aspergillus flavus subsp. and *Penicillium* spp.	*Escherichia coli, Pseudomonas* spp., *Bacillus* spp., *Staphylococcus* spp.	Ajitha et al. (2015)
Not reviewed	*Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, Salmonella typhimurium* and *Salmonella enteritidis*	Chang et al. (2017)
Not reviewed	*Escherichia coli, Pseudomonas* sp. and *Salmonella* sp.	Sathya et al. (2012)
Not reviewed	*Enterococcus faecalis* and *Aeromonas hydrophila.*	Malaikozhundan et al. (2016)
Candida albicans, Candida tropicalis and *Candida krusei.*	Not reviewed	Santos et al. (2012)

biological activities, indicating that this species can be a natural alternative to complement the treatment of many diseases and can also act as a bio-herbicide. In addition, its chemical composition is very diverse, and in recent years, new bioactive compounds have been identified, including 25ξ-isopropenylchole-5,(6)-ene-3-O-β-D-lucopyranoside and 28-O-β-D-xylopyranosyl. The use of alternative extraction techniques such as supercritical CO_2 extraction, which can also be modified with co-solvents (ethanol or water), may help in the discovery of new secondary metabolites present in this species.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Enhancement of somaclonal variations and genetic diversity using graphite nanoparticles (GtNPs) in sweet potato plants

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To assay the efficiency of graphite nanoparticles (GtNPs) in sterilizing tissues and their role in enhancing genetic diversity, sweet potato is considered an important crop; hence its explants were used. In this experiment, GtNPs of 200, 400 and 800 ppm concentrations were used for sterilization of MS callus induction and regeneration media in Abees cultivar. The results showed that GtNPs had a good potential for removing bacterial contaminants without having side effects on the explant viability during the sterilization of sweet potato tissue in all their concentrations. Also, the percentage of callus induction increased from 98.67% in control to 100% in all GtNPs concentrations. The number of shoots per callus was enhanced at 400 ppm concentration. RAPD molecular markers and SDS-PAGE analysis were used to assess the genetic diversity of the sweet potato selected plants obtained from somaclonal variations in combination with GtNPs. Five decamer random amplified polymorphic DNA (RAPD) primers generated a total of 96 DNA fragments from the selected variants and their parent. Out of them, 82 polymorphic bands appeared with 85.42% polymorphism. The levels of DNA and protein patterns polymorphism within each treatment varied. RAPD and protein markers revealed that the concentration of 800 ppm showed the lowest similarity average among the ten selected variants and their parent. The obtained results indicated that somaclonal variation with GtNPs can be combined to increase the induced mutations frequency.

Key words: Graphite nanoparticles, somaclonal variation, random amplified polymorphic DNA (RAPD), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

Sweet potato [*Ipomoea batatas* (L.) Lam], belonging to Convolvulaceae family, is a very important crop in the world. It is a good source of proteins, minerals, vitamins and antioxidants (Pfeiffer and Mclafferty, 2007; Bovell-Benjamin, 2007; Tumwegamire et al., 2011). Due to its commercial importance, the genetic improvement of the plant is needed. The essential way to improve this important crop is through the induction of genetic variations, which can be done by biotechnological interventions such as tissue culture. Using plant tissue

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culture makes the production of secondary metabolite, genetically modified and disease-free plants possible (Murashige, 1974; Khosroushahi et al., 2006). In spite of the advantages of tissue culture technique, some methodological problems, such as microbial contamination of explants hinder its importance as an advanced technique for biotechnological research (Cassells, 1991).

In modern science, material nanoparticles (NPs) display completely new or enhanced properties based on their size, distribution and morphology. Scientists suggested positive and negative effects of NPs on plants' growth and development. Many morphological and physiological changes can appear as a result of the interaction between nanoparticles and plants. The chemical composition, concentration, size and physical properties of NPs can determine their efficiency on plants (Ma et al., 2010; Khodakovskaya et al., 2012). Nanoparticles application led to the induction of microbe-free explants and demonstrated the positive role of NPs in callus induction, organogenesis, somatic embryo-genesis, somaclonal variation, genetic transformation and secondary metabolite production (Kim et al., 2017).

Genetic variation resulting from *in vitro* culture, somaclonal variation is considered to be very useful for developing transgenic plants with desirable agronomic traits (Gaafar and Saker, 2006). Detecting genetic variation of transgenic plants is one of the purposes and criteria for their safety assessment. Random Amplified Polymorphic DNA (RAPD) marker has been used successfully as a molecular marker to characterize, identify and determine variations in nuclear genome between sweet potato genotypes (Gichuki et al., 2003; He et al., 2006; Lin et al., 2009; Moulin et al., 2012; da Silva et al., 2014; Galal and El Gendy, 2017). Also, this technique proved to be able to detect variation among individuals and to estimate the genetic diversity of somaclonal variations in various plant species (Hernandez et al., 2007; Sheidai et al., 2008; Khan et al., 2011; Nasim et al., 2012) including sweet potato (Aboulila, 2016).

Among biochemical markers, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a useful and inexpensive tool for describing genetic structure of several plant species (Oppong-Konadu et al., 2005; Salimi, 2013). Because of the importance of plant cell and tissue culture, and to avoid contamination, a dominant barrier in this technique, the aim of this work was to discover the effect of graphite nanoparticles (GtNPs) as antimicrobial agents and their role in enhancing somaclonal variations and diversity in sweet potato plants. Genetic diversity was assessed using RAPD molecular markers and SDS-PAGE analysis.

MATERIALS AND METHODS

This study was conducted at the Laboratories of Genetics Department, Faculty of Agriculture, Kafrelsheikh University, Egypt.

Plant material

Greenhouse-grown plants of sweet potato, Abees cultivar, were considered as the experimental materials *in vitro* using nodal cutting system.

Nano material

Graphite nanoparticles (purity 99.9%) and particle size of 1 to 2 nm) were applied in the present study. GtNPs were diluted in double distilled water at concentrations of 200, 400 and 800 ppm and suspended by sonication for 30 min before use.

Preparation and characterization of GtNPs

GtNPs were prepared using the expanded graphite (EGt) method as described by Yu and Qiang (2012). One gram of EGt was immersed in 1000 mL aqueous solution of 75% alcohol and suspended by sonication for 12 h. GtNPs were purified using a filtration process. They were washed with distilled water and then allowed to dry in a thermo-static vacuum oven at 100°C. Physical characterization and diameters of nanoparticles were noticed and measured by a transmission electron microscopy (TEM). The result of the TEM image of GtNPs (Figure 1) showed that the particle sizes are in the range of nano.

GtNPs treatments, callus induction and regeneration system

To study the effect of GtNPs on callogenic response and antimicrobial effects, stem segments without buds were surface disinfected with 70% EtOH for 30 s, 2.5% NaOCl for 5 min and 0, 200, 400 and 800 ppm GtNPs for 15 min before they were used as explant materials. Explants were cultured on MS (Murashige and Skoog, 1962) medium provided with sucrose (30 g/L), BAP (8 mg/l), myo-inositol (100 mg/l) and three different concentrations of GtNPs (200, 400 and 800 ppm) besides the control. The final pH value was adjusted to 5.8 and the media were solidified with 2 gm/l phytagel. Each treatment consisted of three replicates (five Petri dishes with five explants for each replicate). The explants were incubated at 25±2°C in darkness for 6 weeks with two sub-culturing. Six days after culturing, callugenesis was started. Explants were checked daily for any possible contamination. At the end of callus induction period, microbial contaminants were recorded. Moreover, callus induction percentage was estimated as the percentage of explants that produce callus.

Calli obtained from each treatment were subsequently transferred to shoot induction medium comprising complete MS medium with 100 mg/l myo-inistol and 6.0 mg/l BAP (containing GtNPs graded levels). The observations were recorded after two weeks of incubation for microbial contaminants, percentages of shoot induction (based on number of calli forming shoots) and the number of shoots/callus.

Hardening of in vitro plantlets

Regenerated shoots (3-5 cm in length) were excised from the embryogenic callus, transformed and cultured on half strength MS medium; their pH was adjusted to 5.8. Cultures incubation was done in growth room and maintained under conditions previously mentioned. For acclimatization, the obtained plantlets were hardened as described by Aboulila (2016).

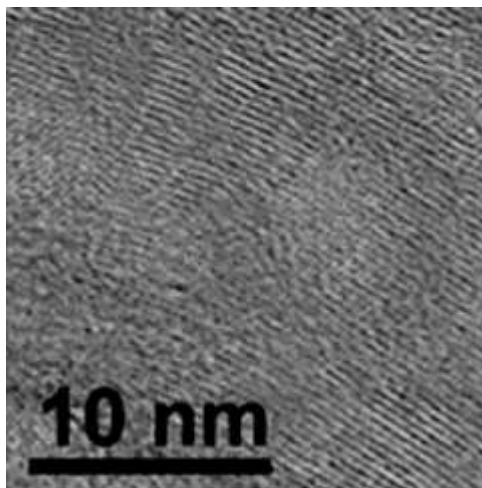

Figure 1. TEM image of graphite nanoparticles.

Table 1. Sweet potato culture response to callus induction and plant regeneration media.

Treatment (ppm)	Callus induction (%)	Shoot induction (%)	Number of shoots/callus	Microbial contamination
Control	98.67±1.33[a]	100[a]	8.67±0.53[b]	(+)
200	100[a]	85.67±4.25[b]	7.87±0.56[b]	(-)
400	100[a]	96.33±1.53[ab]	13.73±1.85[a]	(-)
800	100[a]	63.11±8.74[c]	6.67±0.83[b]	(-)

Molecular analysis

Total genomic DNA was isolated from fresh leaves of the parent plant and ten selected somaclonal variants of each of the control and the three GtNPs treatments using the method of CTAB-chloroform as described by Saghai-Maroof et al. (1984). Random amplified polymorphic DNA analysis using five oligonucleotide decamer primers was applied; OPA-20, OPB-01, OPB-05, OPB-07 and OPB-17 (Bio Basic Inc, Canada). The PCR reaction mixture consisted of 0.75 µl of genomic DNA (40 ng), 0.75 µl of 20 µM primer, 5 µL of 2X PCR Master mix Solution (i-TaqTM, iNtRON's Biotechnology) and 3.5 µL of sterile distilled water in a final volume of 10 µl. Amplification condition was performed according to Galal and El Gendy (2017). Amplification products were separated by electrophoresis and bands were detected on Benchtop UV-transilluminator and photographed using Doc- It[TM] Imaging System. A known 50 bp DNA Ladder ready-to-use (Cat-no: 300003, GeneON) was run against the PCR products.

Biochemical analysis

Total soluble proteins were obtained from 0.5 g fresh leaves of all selected somaclones with their parent. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for total soluble protein was done using 12.5% polyacrylamide gel as described by Laemmli (1970). Molecular weights (MW) for all obtained bands were determined by using pre-stained high molecular weight standard marker (PINK Prestained Protein Marker, Cat. No. MWP02), with molecular weights ranging from 15 to 175 kDa.

Data analyses

Data of recorded traits were analyzed statistically as complete randomized design in three replicates (n=5); the mean values obtained from the treatments were compared by the least significant differences (LSD) test at significance level of $P \leq 0.05$ using the SXW program.

Molecular and biochemical data were introduced to SPSS package program as: Binary value of 1 for visible band and 0 for absent band; genetic similarity was estimated using Jaccard's similarity coefficient (Jaccard, 1901).

RESULTS AND DISCUSSION

Effect of GtNPs on tissue culture

The influence of different concentrations of GtNPs (0, 200, 400 and 800 ppm) was evaluated by adding these concentrations to callus induction and shoot induction media. Results in Table 1 and Figure 2 show that regeneration capacity (shoot induction % and number of shoots/callus) was affected by the concentration of GtNPs. Callus induction percentage did not differ significantly in all treatments, which varied from 98.67% in control to 100% in all GtNPs concentrations. Also, in control treatment all of the calli were regenerable (100%)

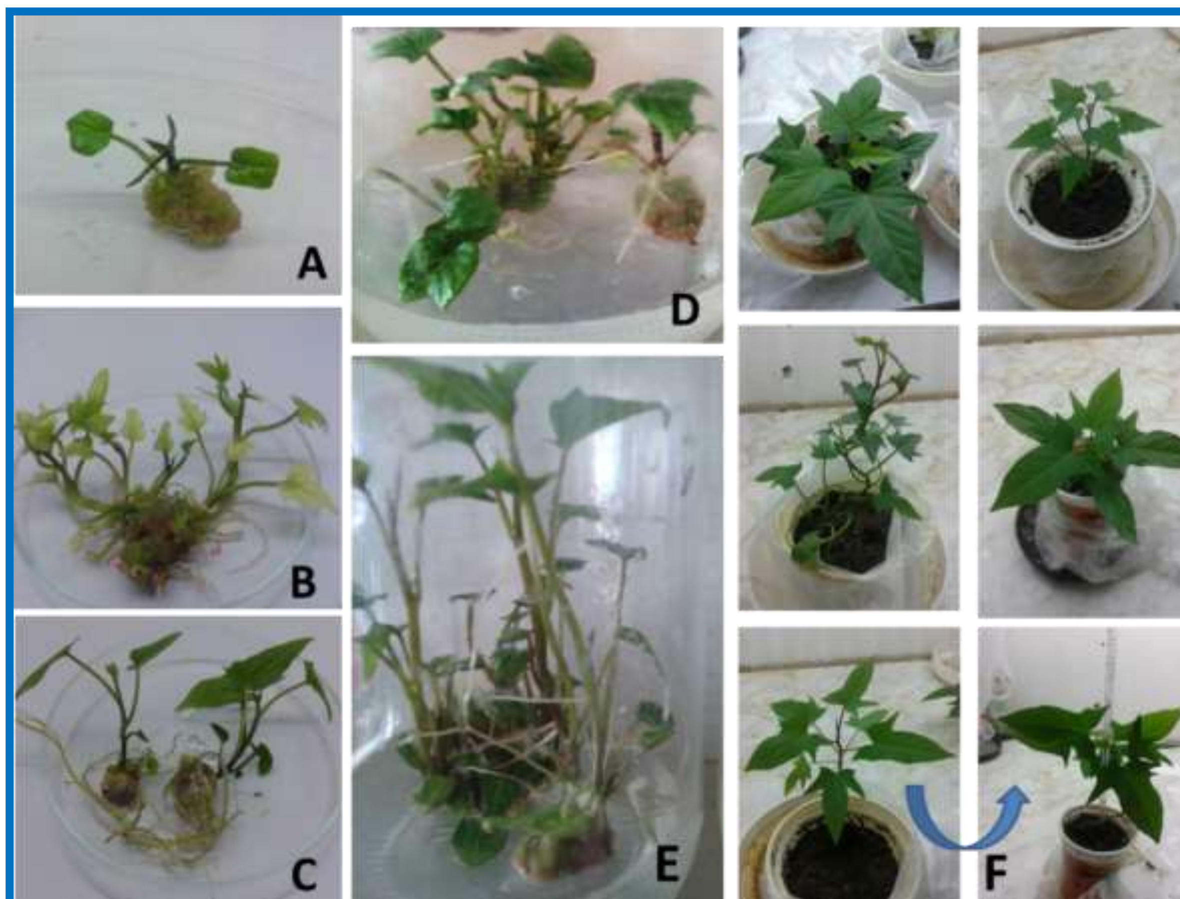

Figure 2. Callus induction and plant regeneration from Abees cultivar. (A) Callus induction; (B) shoot induction; (C) rooting; (D) shoot subculture; (E) shoot multiplication and elongation; (F) hardening of regenerated plants with different phenotypes.

followed by 400 ppm concentration which regenerated 96.33% from all cultured calli on regeneration medium. Plant regeneration and production of multiple shoots from callus were obtained after four to five weeks from callus initiation. While 400 ppm GtNPs recorded the highest number of shoots per callus (13.73) in regeneration medium, the other treatments recorded regeneration capacities ranging from 6.67 to 8.67. These results agree with those of Lahiani et al. (2016) who used carbon-based nanomaterials (CBNs) on tobacco cell culture to increase growth (22-46%) by addition of 50 µg/l.

Moreover, Khodakovskaya et al. (2012) reported that the incorporation of 100 mg/l multi-walled carbon nanotubes into a medium containing 1 mg/l 2,4-D increased callus growth of tobacco explants (64% increase over control). The treatment with carbon nanotubes enhanced callus growth by upregulation of cell division genes (CycB) and water transport (NtPIP1). However, carbon nanotube treatment (10-600 mg/l) decreased cell viability and dry weight in Arabidopsis (Lin et al., 2009). Hence, from all of these reports it can be summarized that the addition of NPs to a plant tissue culture medium affects callus proliferation, shoot multiplication, somatic embryogenesis and rooting by altering antioxidant enzyme activities, gene expression and production of ROS.

Changes observed in developed organs and plantlets are termed somaclonal variation. In this study, some of the obtained regenerated plants after adaptation showed morphological differences compared to their parent, Abees cultivar (Figure 2F). These morphological differences are usually associated with changes in chromosome number, chromosome structure, DNA sequence, DNA methylation and mitotic crossing over (Bairu et al., 2011; Sivanesan and Jeong, 2012).

Positive effects of nanoparticles appeared on callus induction, shoot regeneration and growth in several studies. Aghdaei et al. (2012) found that shoot induction percentage, number of shoots and callus formation increased when culturing the stem explants from *Tecomella undulata* (Roxb) on MS media containing 10 mg/l AgNPs. NPs treatments affect the mitotic index and DNA integrity, and alter the protein and DNA expression in plants (Atha et al., 2012; Landa et al., 2015; Tripathi et

al., 2017).

Identification of anti-microbial effect of GtNPs

The efficiency of GtNPs in decontaminating diverse kinds of explants was evaluated. So, we used different concentrations of GtNPs to find the best sterilization treatment. Sweet potato explants were cultured on GtNPs and GtNPs-free media for callus induction and regeneration to study the elimination of microorganisms in all procedures of tissue culture after four weeks. The results showed that, treating sweet potato stem segments with 200, 400 and 800 mg/l GtNPs for 15 min, there were no contaminants with no effects on organogenesis. Moreover, contaminations were observed only in control treatment (GtNPs-free media) during the different stages in culture media, while microbial contaminants were absent in all GtNPs concentrations. These results showed that GtNPs were effective in the suppression of microbial contaminants in all concentrations.

When GtNPs of 200, 400 and 800 ppm concentrations were applied on sweet potato explant segments, they could function as antimicrobial agents leaving no harmful effect on explants and their viability, and were all able to produce callus. It has been illustrated that the toxicity of GtNPs to microbial cells is apparent even at the range of 200 to 800 ppm concentrations. Several types of NPs such as silver (Ag), aluminum oxide (Al_2O_3), copper oxide (CuO), iron oxide (Fe_3O_4), gold (Au), magnesium oxide (MgO), nickel (Ni), silicon (Si), silicon dioxide (SiO_2), titanium dioxide (TiO_2), GtNPs and zinc oxide (ZnO) have been reported to possess antimicrobial activities against various microorganisms (Liu et al., 2011; Gouran et al., 2014; Beyth et al., 2015).

Genetic diversity in sweet potato using RAPD and protein markers

Genetic polymorphism using RAPD analysis

Five oligonucleotide random primers were employed to study the genomic stability in sweet potato parental cultivar (Abees) along with its somaclonal variants obtained from tissue culture combined with GtNPs (Figure 3). Among the primers used, OPB-17 generated the highest number of total bands (27 bands), while primer OPB-05 produced the lowest (13 bands). All primers generated a total of 96 DNA bands. Fourteen bands were monomorphic and consistent among all selected variants for all treatments; however, 82 bands were observed to be polymorphic with 85.42% polymorphism. The highest level of polymorphism (96.30%) was recorded in primer OPB-17 while the lowest level of polymorphism was 72.22% in primer OPB-07 (Table 2).

On the other hand, the five primers revealed a total of 93 bands in the parental genotype. Out of them, 52, 58, 35 and 47 bands were common in the parental genotype and the somaclonal variants obtained from the four treatments; control, 200, 400 and 800 ppm, respectively (Table 3). The level of polymorphism among the somaclonal variants and the parental genotype varied. The highest number of polymorphic bands (61 bands) was recorded in 400 ppm concentration with 63.54% polymorphism, while the lowest number (37 bands) was noticed in 200 ppm with 38.95% polymorphism. The genetic polymorphism increased in the regenerated plants at 400 and 800 ppm, while it decreased at 200 ppm compared to the control.

Variations observed in the total number of RAPD bands among the parental genotype and plants generated from tissue culture combined with different GtNPs concentrations indicate genetic differences of the variants because of somaclonal variation induced from tissue culture as seen in control treatment, plus the genetic variation induced by GtNPs treatments. This is in agreement with Sheidai et al. (2008) who found that some bands appeared in the parental plants and got lost in regenerated plants because of somaclonal variation. These results proved that RAPD markers were effective in detecting polymorphism which occurs due to insertion, deletions and base substitution that affect the primer-binding site and reflect as the presence or loss of bands. These findings are in agreement with earlier studies using RAPD analysis in describing genetic polymorphism among somaclonal variants in various plant species. Khan et al. (2011) used this technique to determine the genetic variations among micropropagated banana plants.

Genetic polymorphism based on SDS-PAGE analysis

Protein banding patterns were used to detect the genetic variations among the ten selected variants within the four studied treatments (Figure 4). The electrophorotic patterns of SDS-protein revealed marked polymorphism within each GtNPs treatment as shown in Table 3. Polymorphism percentage within the four treatments ranged from 33.33 to 100%. Control treatment revealed the lowest polymorphic percentage (33.33%) indicating that level of polymorphism differed a little within the control treatment. Also, 800 ppm treatment gave the highest polymorphic percentage (100%); a total of nine bands ranging from 2 to 9 were detected and all of them were polymorphic. For the other two treatments (200 and 400 ppm), the profile of variants and their parent exhibited the highest number of protein bands (10 bands for each) which showed 90 and 80% polymorphism, respectively.

Results obtained from many studies have shown that much of the genetic variability generated from plant

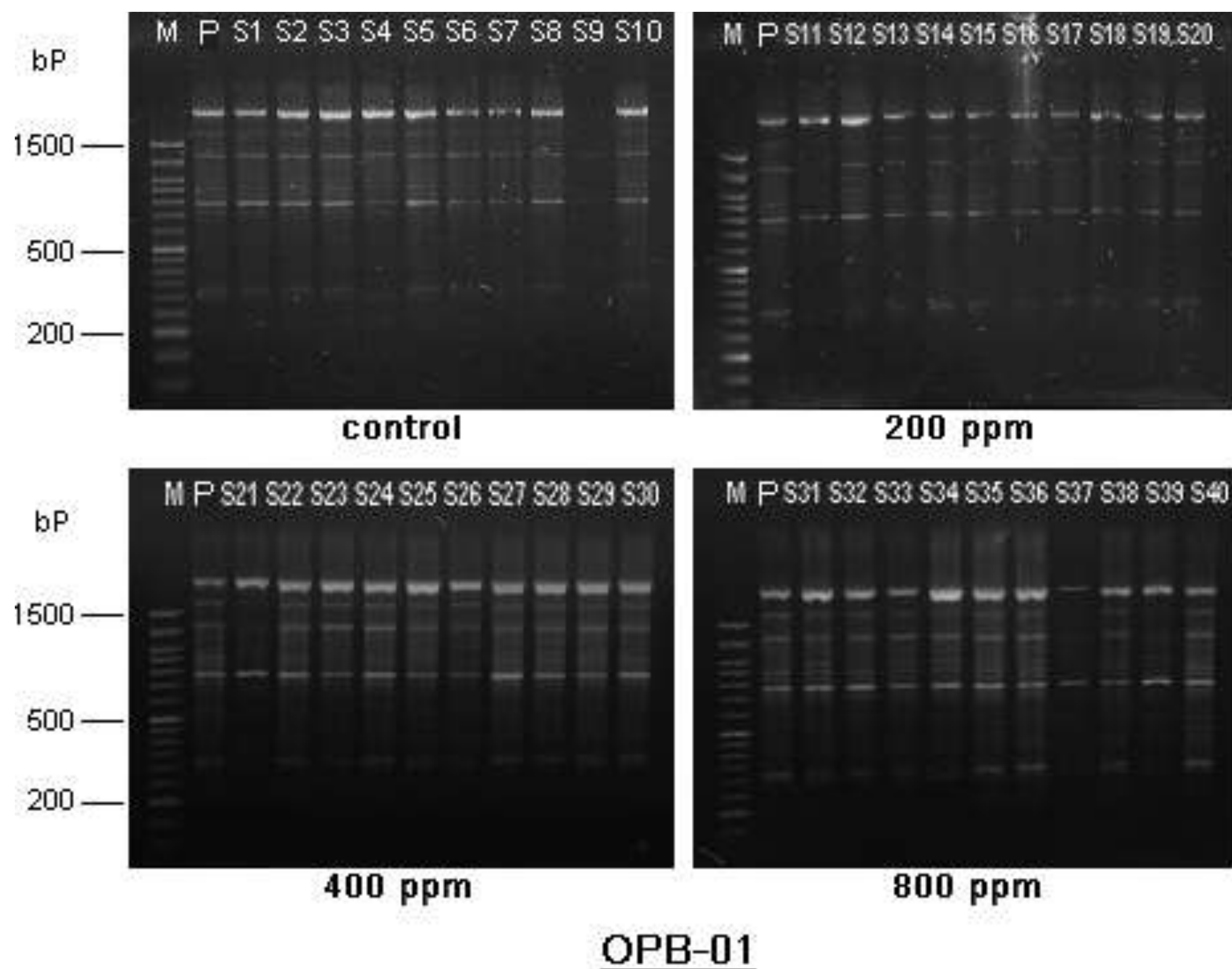

Figure 3. RAPD profile of OPB-1 primer. P, parental plant; S1-S10, S11-S20, S21-S30 and S31-S40: somaclonal variants of the four GtNPs (control, 200, 400 and 800 ppm, respectively). L, 50 bp molecular ladder.

Table 2. Level of polymorphism among the parental genotype and the somaclonal variants treated with different concentrations of GtNPs, based on RAPD analysis.

Primer name	Sequence (5`→3`)	TAF	MB	PB	P (%)	AF				
						Parent	Treatments (10 variants)			
							control	200 ppm	400 ppm	800 ppm
OPA-20	GTTGCGATCC	15	4	11	73.33	15	11-15	11-15	7-15	7-15
OPB-01	GTTTCGCTCC	23	1	22	95.65	23	2-23	12-21	13-23	12-23
OPB-05	TGCGCCCTTC	13	3	10	76.92	13	10-13	11-13	6-13	10-13
OPB-07	GGTGACGCAG	18	5	13	72.22	17	12-18	15-18	16-18	11-17
OPB-17	AGGGAACGAG	27	1	26	96.30	25	22-26	22-26	3-25	20-25
Total	-	96	14	82	85.42	93	95	95	96	94

TAF, Total amplified fragment; MB, Monomorphic bands; PB, Polymorphic bands; P (%), Polymorphism (%); AF, Amplified fragment.

tissue culture may be the result of gene mutation (D'Amato, 1985; Ngezahayo et al., 2007) or epigenetic variation (Kaeppler et al., 2000; Guo et al., 2006; Smulders and de Klerk, 2011). It is likely that these variations are based on the differences in GtNPs concentrations, media used for culture or their

Table 3. Polymorphism percentages generated by RAPD and protein markers within the ten somaclonal variants obtained from each of the four GtNPs treatments.

Treatment (ppm)	TB	Range of bands (ten variants)	MB	PB	P (%)
RAPD markers					
Control	95	69 - 92	52	43	45.26
200	95	79 - 91	58	37	38.95
400	96	68 - 92	35	61	63.54
800	94	66 - 86	47	48	51.06
Protein marker					
Control	6	4 - 6	4	2	33.33
200	10	3 - 10	1	9	90.00
400	10	3 - 8	2	8	80.00
800	9	2 - 9	0	9	100

TB, Total bands; MB, monomorphic bands; PB, polymorphic bands; P (%), polymorphism (%).

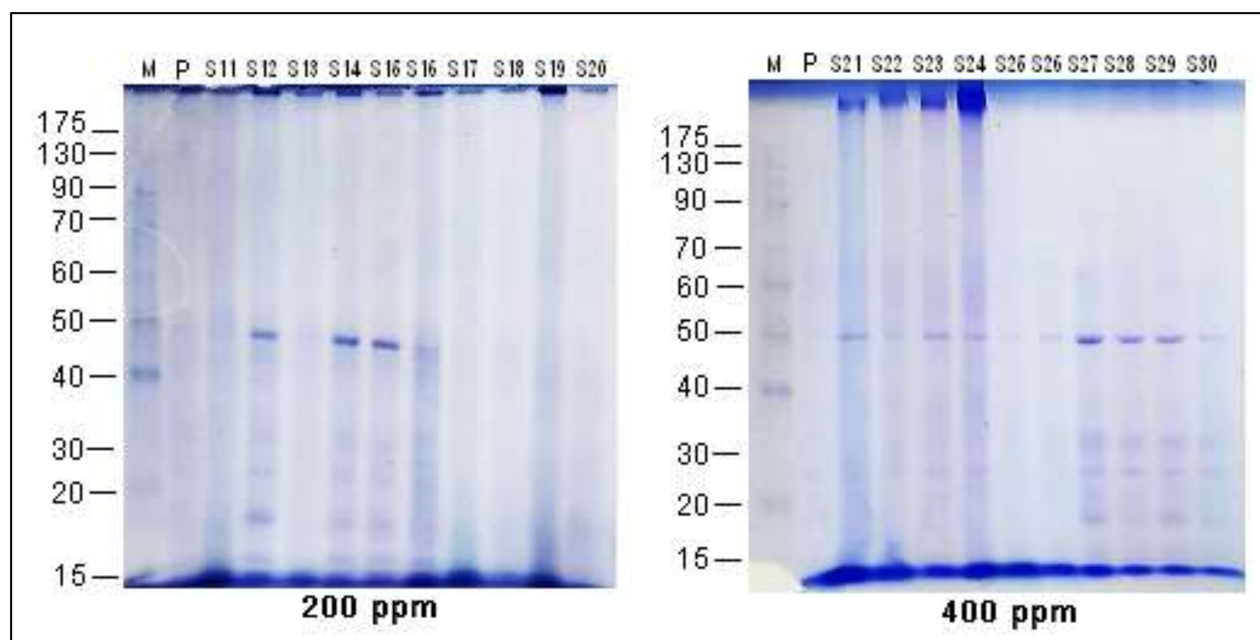

Figure 4. The protein banding patterns of the parental genotype (P) and the ten somaclonal variants (S11-S20 and S21-S30) treated with 200 and 400 ppm of GtNPs, respectively. M, Protein molecular marker from 15 to 175 KDa.

combinations. This is in contrast with the results of Afrasiab and Iqbal (2010), who noted that the recovery of somaclones can be increased by combining micro-propagation with induced mutagenesis *in vitro*.

Genetic similarity

The ranges and averages of similarity values for the ten somaclonal variants and their parent within each of the GtNPs treatments (200, 400 and 800) and control based on RAPD and protein markers are listed in Table 4. RAPD and protein markers revealed that the concentration of 800 ppm showed the lowest similarity average among the ten selected variants and their parent. In the case of RAPD marker, all treatments revealed high similarity averages ranging from 0.816 (800 ppm) to 0.867 (200 ppm), with a mean value of 0.843, indicating high homogeneity within the tested treatments. While, the genetic similarity decreased with GtNPs increase from 200 to 800 ppm, indicating that genetic variations induced in the regenerated plants increase with

Table 4. Similarity ranges and averages within each of the four treatments based on RAPD and protein markers.

Treatment	RAPD marker		Protein marker	
	Range	Average	Range	Average
Control	0.690-0.968	0.864	0.667-1.00	0.850
200 ppm	0.766-0.946	0.867	0.167-1.00	0.563
400 ppm	0.575-0.978	0.825	0.286-1.00	0.605
800 ppm	0.656-0.925	0.816	0.00-1.00	0.416
Mean	0.575-0.978	0.843	0.00-1.00	0.608

the concentrations of GtNPs. Concerning protein marker, the similarity mean value of 0.608 was obtained from all tested treatments. The highest genetic similarity average was found within control treatment (0.850), while the lowest one was observed within 800 ppm concentration (0.416). However, 200 and 400 ppm treatments showed genetic similarity averages with 0.563 and 0.605, respectively.

Therefore, analyses of RAPD and SDS-PAGE appeared to be effective for assessing genetic similarity of sweet potato somaclonal variants and their parent within each GtNPs treatment. These results are in line with those of Metry et al. (2002) who used RAPD markers and SDS-PAGE analysis to identify genetic similarity among transgenic potato cultures.

Conclusion

In conclusion, results showed that genetic variations occurred due to the differences generated from somaclonal variation during tissue culture combined with that generated from GtNPs treatments. Somaclonal variations and GtNPs can be combined to increase induced mutations frequency. Mutation, which changes one or few specific traits of a cultivar, can be utilized for selection of desired traits in sweet potato to crop improvement. The obtained variants could be used for more evaluation to test their commercial biosafety.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparative evaluation of the physicochemical and pasting properties of flour from three varieties of *Brachystegia* spp.

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The chemical compositions, functional and pasting properties of flour from three varieties of *Brachystegia* spp. (*Brachystegia eurycoma, Brachystegia nigerica* and *Brachystegia kennedy*) were studied. Results show that the chemical compositions of flour samples ranged from12.31 to 12.67% (protein), 1.66 to 1.72% (crude fiber), 2.06 to 2.39% (ash), 7.18 to 8.45% (fat), 3.85 to 4.75% (sugar) and 58.45 to 59.62% (starch). Functional properties such as water absorption capacity, oil absorption capacity, swelling power, solubility index, pH and amylose content were in the ranges of 80.14 to 80.77, 84.21 to 84.52, 15.64 to 15.78, 15.44 to 15.98, 5.48 to 6.74 and 20.42 to 20.69%, respectively. In addition, pasting properties values were 85.58 to 89.05°C (peak temperature), 128.54 to 133.45 (peak viscosity), 23.75 to 26.53 (trough viscosity), 419.6 to 449.5 (final viscosity) 53.5 to 59.0 (break down viscosity) and 402.6 to 413.4 (Relative value units, RVU) (setback viscosity). No significant difference ($p > 0.05$) was observed in the functional properties of the flours. The pasting profile showed that peak and hot paste viscosities are the key pasting parameters in characterizing flours from the three *Brachystegia* varieties. The variation in peak viscosity of the *Brachystegia* flours might be due to varietals and geographical influence. The study shows that *B. eurycoma* flour had the best functional and pasting properties results that could be exploited in food formulations such as soup, and sauces.

Key word: *Brachystegia* flour, variety, chemical composition, functional, pasting properties.

INTRODUCTION

There is a need to exploit the food and industrial potentials of *Brachystegia* spp. seeds. However, this requires prior information and understanding of desirable functional properties and the behavior of the material in systems during processing, manufacturing, storage, preparation as well as consumption (Sai-Ut et al., 2009). Over the past 30 years, the use of flour from legume seeds has been on the increase because of greater knowledge of their functional properties, processing and nutritive value (Kisambira et al., 2015). While historically, soy bean and cowpea have had a competitive advantage over other legume seeds, there is a need to identify, develop and explore other legume sources. The *Brachystegia* spp. offers such an unexploited opportunity. *Brachystegia* spp. an underutilized legume crop consumed in Nigeria is a seasonal woody plant mainly found along river banks or swamps in Western and Eastern Nigeria, as well as well drained soils. The crop is

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mainly used for soup making and timber. The gum also impacts certain desirable functional properties when added in other foods. *Brachystegia* spp. is a large tree with irregular and twisted spreading branches.

In Nigeria, there are three major species of *Brachystegia* which include *Brachystegia nigerica*, *Brachystegia eurycoma* and *Brachystegia kennedyi*. *B. nigerica* seed or leaf, (specify) is broad in size, round in shape, dark red in colour; has gummy husk which makes dehulling hard and is commonly found in Katsina, Adamawa in Northern Nigeria. *B. eurycoma* is medium in size, round in shape, dark brown in colour with less sticky husk which makes dehulling very easy. It is commonly found along river banks of the Southern Nigeria. *B. kennedyi* is commonly found in Eastern part of Nigeria. The seed is dark brown in colour, round in shape, broad in size with a gummy seed coat and this makes dehulling very hard. Okwu and Okoro (2006), reported that the fruit of *Brachystegia* spp. ripens from September to January and is released by explosive mechanism. In some states of Nigeria, *Brachystegia* spp. is called 'achi' in Igbo, "akalado" or "eku" in Yoruba; "akpakpa" or "apaupan" by the Ijaws and 'dewen' in Bini (Enwere, 1998).

The lack of information on many basic aspects of three major species of *Brachystegia* in Nigeria hinders their development, diversification and sustainable utilization. There is a need to get more information and understand the characteristics of these crops for their optimal use and application in areas food and allied industries. Therefore, the objective of this study was to determine the nutrient composition, and functional and pasting properties of flour from seeds of *Brachystegia* spp. The data would be of considerable values for food scientists, manufacturers and consumers regarding the selection of suitable *Brachystegia* spp. for preparation of good quality food product and also in the preparation of a much needed food composition table for Nigeria.

MATERIALS AND METHODS

Sample procurement and preparation

Brachystegia seeds samples of *B. nigerica*, *B. eurycoma* and *B. kennedyi* were purchased from Eke-Aba market in Abakaliki, Ebonyi State, Nigeria. The seeds were sorted to eliminate the bad ones. Cleaned seeds were conditioned to 25% moisture content by the addition of distilled water and held for 2 h with occasional stirring. The conditioned sample was sun dried to final moisture of approximately 10%. The dry seeds were dehulled for 2 min using a traditionally manufactured disc attrition mill (No1A Premier). The dehulled seeds were ground in an attrition mill and sieved with American standard sieve number 40 with aperture of 435 µm. The flour was packaged, labeled and stored in a refrigerator at 4°C until use.

Chemical analysis

Protein, fat, ash, crude fibre, starch, amylose and moisture contents were determined for the *Brachystegia* spp. flours. These analyses were carried out according to the AOAC official procedures (AOAC, 2000). The nitrogen was determined with a Kjeldahl method. The protein was calculated by Nitrogen x 6.25. Fat was obtained from 4h extraction with hexane. Ash was calculated from the weight remaining after heating the sample at 550°C for 2 h. Moisture was from the weight loss after oven drying at 110°C for 2 h. The total carbohydrates excluding crude fiber were calculated from the difference. The method of AOAC (2000) was also used to determine the sugar content.

Determination of functional properties

The method of Appiah et al. (2011) was used to determine the water and oil absorption capacities of the *Brachystegia* spp. samples. The swelling power and solubility of *Brachystegia* spp. flour samples were determined according to the methods described by Falade and Olugbuyi, (2010). The method of Xianqiao et al. (2015) was used to determine the amylose content.

Pasting properties determination

The pasting properties of defatted yam bean seed flours were analyzed with a Series 4 Rapid Visco Analyzer (RVA) (Newport Scientific from Australia) with Thermocline for Windows software. The analysis was done using standard one profile. The flour suspensions (6.72 g in 25.28 ml H_2O) corrected to 14% moisture content were exposed to the following time/temperature sequence: 50°C for 1 min, heating from 50 to 95˚C at 12.16˚C /min, maintained at 95°C for 2.5 min, and cooled from 95 to 50˚C at 11.84˚C/min rate. The apparent viscosity was expressed in relative value units (RVU).

Statistical analysis

All experimental analyses in this study were done in triplicates. All the data analysis was done using SPSS version 16.0 Software. Analysis of variance (ANOVA) was performed to generate treatment means and Least Significant Difference (LSD) ($P < 0.05$) values were used to separate the means.

RESULTS AND DISCUSSION

Chemical composition

The results of the chemical compositions of *Brachystegia* spp. are shown in the Table 1. The result showed that there were significant differences in all analyzed components of the seeds of *B. nigerica*, *B. eurycoma* and *B. kennedyi* except for crude fibre and moisture content. The results for crude protein and fat contents in this study are higher than those reported by Ajayi et al. (2014) for all the three species. The results in this study reveal that the levels of protein and fat of *Brachystegia* spp. seed flour are lower compared to that of other legumes like *Afzelia african*a which was reported to have 16.52 and 16.35% for crude protein and fat, respectively Ogunlade et al. (2011), and 21.88 and 23.38% (Igbabul et al. 2014). Kisambira et al. (2015) reported that yam bean flour had 32.16 and 24.14 g/100g crude protein and fat, content, respectively. The *B. kennedyi* flour had the lowest ash

Table 1. Chemical composition of three varieties of *Brachystegia* spp grown in Nigeria.

Variety	Fat percentage (%)	Protein percentage (%)	Ash percentage (%)	Moisture content percentage (%)	Fibre percentage (%)	Sugar percentage (%)	Starch percentage (%)	Carbohydrate content percentage (%)
B. nigerica	8.45[a]	12.45[a]	2.39[b]	10.86[a]	1.72[a]	3.85[c]	60.28[a]	64.13[a]
B.eurycoma	8.16[b]	12.31[a]	2.88[a]	11.35[a]	1.66[a]	4.12[b]	59.52[a]	63.64[b]
B.kennedyi	7.18[c]	12.67[a]	2.08[c]	12.22[a]	1.70[a]	4.75[a]	59.40a	64.15[c]

Values are mean values of triplicate determination. Values with the same superscript in the same column are not significantly different (p>0.05).

Table 2. Functional properties of three varieties of *Brachystegia* spp. grown in Nigeria.

Properties	Variety		
	B. nigerica	***B. eurycoma***	***B. kennedyi***
Water absorption capacity (%)	80.45[a]	80.77[a]	80.14[a]
Oil absorption capacity (%)	84.21[a]	84.52[a]	84.26[a]
Swelling power (%)	15.78[a]	15.66[a]	15.64[a]
Solubility index (%)	15.44[a]	15.89[a]	15.68[a]
Amylose content (%)	20.45[a]	20.74[a]	20.42[a]
pH	5.48[a]	6.74[a]	6.14[a]

Values are mean values of triplicate determination. Values with the same superscript in the same column are not significantly different (p>0.05).

content (2.08%) while *B. eurycoma* had the highest ash content of 2.88%. The ash content obtained in this study is lower than the reported value of 5.0% by Ajayi et al. (2014), and 3.5% for *B.eurycoma* by Uhegbu et al. (2009). The high ash content reflects the high mineral contents of *B. eurycoma*. Ogunlade et al. (2011), reported that *Pachira glabra* and *Afzelia africana* had ash content of 4.34 and 4.03% respectively, while melon seed had 3.3% (Peter-Ikechukwu et al., 2016).

Moisture in foods is actively involved in various metabolic reactions which determine the shelf life and microbial susceptibility of food items. The moisture content of *Brachystegia* samples showed that *B. kennedyi* had the highest moisture content (12.22%) while *B. nigerica* had the lowest (10.86%). The result of the moisture content in this study is in agreement with the one reported by Ajayi et al., (2014). However, the results of the three *Brachystegia* spp. are higher than the (3.21%) value of *Moringa oleifera* leaves reported by Ogbe and John (2012) but extremely lower than the 70.30 to 75.54 range value of some Nigerian pumpkins (*Cucurbita spp*) reported by Blessing et al. (2011). High amount of moisture in crops makes them vulnerable to microbial attack, hence, spoilage. Moisture value obtained in this study were within the range (9 to15%), implying that *Brachystegia* spp. would keep for a long period without spoilage especially in the tropics where wastage of crops is estimated to be around 50% due to high moisture content. The crude fiber content of *B. eurycoma* harms (17.20 ± 0.87) as reported by Ajayi et al. (2014) which was evidently higher than that of *Brachystegia* spp. as reported in this study (1.66 to 1.72%). The total carbohydrate, sugar and starch contents of the *Brachystegia* spp. ranged from 68.05 to 69.16%, 3.85 to 4.75%, and 59.45 to 59.62%, respectively. The difference in the proximate composition of *Brachystegia* spp. might be attributed to the difference in the geographical location, climate and agronomical practices.

Functional properties

Functional properties of food materials play a significant role in manufacturing, transportation, storage, stability, texture, taste and flavor of food products. These properties directly or indirectly depend on type, variety, particle size and chemical composition of flour and type of processing method (Nawaz et al., 2015). The functional properties of flours from three *Brachystegia* varieties are presented in Table 2. The ability to absorb water is a very important property of all flours used in food preparations. Water and oil absorption capacities (WAC, OAC) are useful indices of the ability of the protein in the material to prevent fluid loss from a product during food storage or processing (Kiosseoglou and Paraskevopoulou, 2011). The range of water absorption capacity (80.14 to 80.77%) observed for the different

Table 3. Pasting characteristics of three varieties of *Brachystegia* spp. grown in Nigeria.

Variety	P Temp (°C)	P Time (min)	PV (RVU)	TU (RVU)	FV (RVU)	BD (RVU)	SB (RVU)
B.nigerica	88.25[b]	7.00[a]	130.22[b]	26.53[a]	449.5a	59.0[a]	413.4[a]
B.eurycoma	85.58[c]	5.13[c]	133.45a	23.75[c]	428.8[b]	53.5[c]	402.6[b]
B.kennedyi	89.05[a]	5.40b	128.54[c]	25.61[b]	419.6[c]	56.8[b]	405.4[b]

Values are mean values of triplicate determination. Values with the same superscript in the same column are not significantly different (p>0.05). PV= peak viscosity; TU= Trough viscosity; FV= Final viscosity; BD= Break down viscosity; SB= Set back viscosity; RVU= Rapid viscosity unit.

Brachystegia spp. flours, analyzes was significantly the same (p ≥ 0.05). The *B. kennedyi* had the lowest (80.14%) water absorption capacity than flours from *B. nigerica* and *B. eurycoma*. These results are lower to those reported by Fekria et al. (2012) for defatted ground nut which ranged from 3.03 to 3.07 ml/g for two groundnut varieties. The consistency and stability of viscous foods such as soups entirely depend on WAC of starch and protein present in the flour. The observed water absorption capacity of *Brachystegia* spp. flours in this study might be attributed to their protein and starch contents (Table 1).

The oil absorption capacity (OAC) of the flours from *Brachystegia* varieties ranged from 84.21 to 84.52%. *B. eurycoma* had the highest level (84.52%) of oil absorption capacity while *B. nigerica* had the lowest value of OAC. The result of the oil absorption capacity of the flour samples exhibited no significant (p>0.05) difference from one other. The observed OAC values were lower than 2.87 and 2.93ml/g for defatted ground nut flour (Fekria et al., 2012) and 1.48 and 1.52 g/g for yam bean flour (Kisambira et al., 2015). The low OAC means that, the flour could be used as a coating in deep fat frying to reduce oil absorption by the fried food. The mechanism of oil retention is due to the physical entrapment of oil. Hence, the ability of food to absorb oil may help to enhance sensory properties such as flavour retention and mouth fell, therefore the flour from *Brachystegia eurycoma* may have a high degree of flavour retention and mouth feel.

The amylose content of *Brachystegia* varieties ranged from 20.42 to 20.69% with *B. eurycoma* having the highest apparent amylose content while *B. kennedyi* had the lowest level of amylose content. The amylose content obtained in this study are within the range of values (1.5 to 24%) as reported for rice by Xianqiao et al. (2015). The extractable starch and the amylose contents of the varieties were comparatively different. This observation suggests that the composition of Brachystegia spp is affected by variety and possibly by the location of where it is cultivated. The apparent amylose content of the *Brachystegia* samples were not significantly (p >0.05) different from each other.

The pH levels varied from 5.48 to 6.74. Such pH value of *Brachystegia* spp. shows that they are less acidic. Correlationship was observed between pH and solubility index and items inferring that an increase in the pH tends to increase the water solubility of the components in *Brachystegia* spp. This is true in general as far as the protein solubility is concerned. The pH level of the *Brachystegia* spp. flour samples did not vary significantly (p>0.05) with variety.

The swelling power is a measure of the ability of flour to imbibe water. Food eating quality is often connected with retention of water in the swollen starch granules (Sreerama et al., 2012). The swelling power ranged from 15.64 to 15.78%. *B. nigerica* flour had the highest value (15.78%) while *B. kennedyi* had the lowest (15.78%) value. The values in this study are higher than 0.98 to 1.64% reported by Yellavila et al. (2015) but lower than 2.87 ml/g for *Afzelia africana* (Igbabul et al., 2014). The samples were not significantly different (p>0.05) from each other in terms of swelling power. The *Brachystegia* spp. exhibited restricted swelling/solubility. Sanni et al. (2005) reported that, the swelling index of granules reflect the extent of associative forces within the granule, therefore the higher the swelling index, the lower the associative forces. The extent of swelling of the flour depends on the temperature, availability of water, species of starch, extent of starch damage due to thermal and mechanical processes and other carbohydrates (such as pectins, hemicelluloses and cellulose) and protein.

The solubility index of *Brachystegia* spp. ranged between 15.44 and 15.98%, with *B. eurycoma* variety having the highest value while *B. nigerica* had the lowest value. There was no significant difference (p > 0.05) in the solubility index of the samples. The low solubility index of the *B. nigerica*, might be due to its high amount of protein and fat contents that might have formed inclusion complexes with amylose.

Pasting properties of flours from three varieties of *Brachystegia*

The results of Rapid Visco Analyzer (RVA) of *Brachystegia* spp. flours are presented in Table 3. The processing characteristics of flours can be predicted by testing the rheological characteristics. The pasting properties of *Brachystegia* spp. flour samples namely

peak viscosity, break down viscosity, final viscosity, trough viscosity, set back viscosity, peak time and pasting temperature were analyzed. There were significant differences ($p < 0.05$) in the pasting properties of *Brachystegia* spp. flour samples. The pasting property is important in predicting the behaviour of *Brachystegia* spp. paste during and after cooking.

The pasting temperature is a measure of the minimum temperature required for cooking a given food sample (Ikegwu et al., 2010). It is the temperature at the onset of starch granules swelling and increases in viscosity. The pasting temperature of the samples ranged from 85.58 to 89.05°C with *B. kennedyi* having the highest (89.05°C) pasting temperature while *B. eurycoma* had the lowest (85.58°C) pasting temperature. This implies that *B. eurycoma* flour can form paste in hot water below boiling point. This, at commercial level, is a remarkable cost saving. Varietal differences exist in the pasting temperature of the *Brachystegia* spp. flours at $p <0.05$.

Peak viscosity, which is the ability of starch to swell freely before their physical breakdown, ranged from 128.54 to 133.45 RVU. *B. eurycoma* flour had the highest peak viscosity value of 133.45 RVU and *B. kennedyi* flour had the lowest value of 128.54 RVU. The relatively high peak viscosity exhibited by *B. eurycoma* flour is an indication of high starch content (Table 1) which makes the flour more suitable for products requiring high gel strength and elasticity. Peak viscosity is often correlated with the final product quality and also provides an evidence of the viscous load which is likely to be encountered during mixing.

The trough is the minimum viscosity value in the constant temperature phase of the RVA profile and measures the ability of paste to withstand break down during cooling ranged between 23.73 and 26.53 RVU. *B. nigerica* flour had the highest trough value of 26.53 RVU and *B. eurycoma* flour had the lowest value of 23.73 RVU. Large values of trough viscosity indicate little breakdown of sample starches; this implies that *B. nigerica* will exhibit little breakdown compared to *B. eurycoma* and *Kennedyi* paste during cooling.

The final viscosity which indicates the ability of the starch-based food to form a viscous paste or gel after cooking and cooling ranged from 419.6 to 449.5 RVU. The flour sample from *B. nigerica* had the highest (449.5 RVU) final viscosity value and *B. Kennedyi* flour had the lowest (419.6 RVU) final viscosity. This implies that, *B. nigerica* flour might have the ability to form a viscous paste, while the paste formed from *B. kennedyi* flour maybe less viscous. Thus, consumers who prefer high viscous soup made from *Brachystegia* can use *B. nigerica* flour, while those who prefer less viscous soup can use *B. kennedyi* flour.

The breakdown viscosity which is an index of the stability of starch ranged between 53.50 and 59.00 RVU. The *B. nigerica* flour had the highest break down viscosity (59.0 RVU), while *B. eurycoma* flour had the lowest break down viscosity (53.50 RVU). The ability of a mixture to withstand heating and shear stress that is usually encountered during processing is an important factor for many processes especially those requiring stable paste and low retro-gradation (Adebowale et al., 2008). The higher the breakdown viscosity, the lower the ability of starch sample to withstand heating and shear stress during cooking. Hence, the flour sample from *B. eurycoma* might be able to withstand heating and shear stress.

The setback viscosity ranged from 402.6 to 413.4 RVU. Flour from *B. nigerica* had the highest (413.4 RVU) setback value while flour from *B. eurycoma* has the lowest (402.6 RVU). Lower setback viscosity during the cooling of the paste indicates greater resistance to retrogradation. Hence, *B. eurycoma* flour paste might indicate greater resistance to retrogradation. The peak time, which is a measure of the cooking time, ranged between 5.13 and 7.0 min. The *B. eurycoma* flour was highest with a value of 7.0 min while *B. nigerica* flour has d lowest.

Conclusion

Brachystegia spp flours differed significantly in their chemical composition and pasting properties, with fat, ash and sugar being the key component of variation in chemical composition of *Brachystegia* flour. *B. kennedyi* flour showed lowest peak and final viscosities. The highest setback viscosity value for *B. nigerica* flour indicated the higher tendency of this flour to retrograde. The variations in the functional properties of *Brachystegia* spp. flour were observed to be statistically the same ($p≥0.05$) among varieties. The swelling power of flour from *Brachystegia* varieties studied fall on the group of restricted swelling. This shows that they are good soup thickeners and might be used for the manufacture of value-added products such as composite blends, as they could meet the functional demands of the processors and nutritional requirements of the body of consumers. The study showed that *Brachystegia eurycoma* flour had the best functional and pasting properties results.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Molecular diagnosis of phytoplasma transmission from zygotic embryos to *in vitro* regenerated plants of coconut palm (*Cocos nucifera* L.)

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The aim of this study was to investigate the transmission of the lethal yellowing disease (LYD) of coconut tree caused by a phytoplasma from the zygotic embryo to the regenerated plantlet *in vitro*. From a total of 30 trees, 150 mature coconut nuts where harvested. These nuts were used to extract 150 zygotic embryos. From this package, 96 zygotic embryos were used to regenerate 96 young coconut seedlings *in vitro* and the 54 others were used to extract total DNA. From the stem of the 30 palms at the stage 1 of the LYD, phloem sample were also collected. From the regenerated *in vitro*-plantlets at 6 months age, leaf sample were collected. From the molecular diagnosis by PCR, 80% of the phloem samples carried the 16S rRNA gene of the phytoplasma responsible for LYD. All the zygotic embryos and *in vitro*-plantlets regenerated were healthy. So, coconut zygotic embryos can be used for the safe exchange of genetic material regarding lethal yellowing disease. The regenerated *in vitro* plantlet are free of disease.

Key words: Coconut, phytoplasma, transmission, *in vitro*.

INTRODUCTION

The coconut tree originates from two geographical areas, namely, the islands of Southeast Asia and South India (Gunn et al., 2011). The ancient origins of the local coconut tree bordering the West African coast are probably India and Mozambique (De Nuce and Wuidart, 1979). From its center of origin, the coconut was

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Table 1. Coconut plant material used for the work.

Village of origin	Varieties	Quantity of palms used	Quantity of sampled nuts	Quantity of embryos put in *in vitro culture*	Quantity of embryos used for the molecular analysis
Badadon	PB 121	6	30	20	10
Palmindustrie V1	GOA	3	15	9	6
Groguida	PB 113	3	15	9	6
	PB121	3	15	9	6
Adjadon	GOA	6	30	20	10
Likpilassé	PB113	6	30	20	10
	GOA	3	15	9	6
Total		30	150	96	54

disseminated by flotation of nuts at the mercy of marine currents and, later, by human travel and migration (Harries et al., 2004; Baudouin and Lebrun, 2009). Human migrations were those of Austronesians, Arabs and Europeans. It was introduced from Mozambique (East Africa) to Côte d'Ivoire (West Africa) by Portuguese navigators in the early 16th century (De Nuce and Wuidart, 1979).

Creation of the coconut collection for the purposes of research in the context of the varietal development passes through the exchange of the plant material. With regards to the coconut tree, the exchanges of the genetic material are done using nut in order to create the diversity from the family of brood stocks in a country's collection. However, these exchanges are not easily done because of the volume and mass of the nut, which is the organ usually used (Orozco-Segovia et al., 2003). These nuts that do not have dormancy often carry disease germs like lethal yellowing disease (LYD). Studies on the conservation and transfer of coconut material in the form of embryos have been investigated in several laboratories (Assy-Bah et al., 1989; Danso et al., 2009; Rillo and Paloma, 1991; Cueto et al., 2012; Yoboue et al., 2014).

The lethal yellowing disease threatens the entire world coconut grove. This disease has already destroyed more than thousands of hectares of coconut plantations in several regions of the world such as East Africa, the Caribbean and Central and West Africa (Been, 1981; Oropeza et al., 2005; Dollet et al., 2009; Konan et al., 2013). Phytoplasmas are transmitted to plants during food activity by their vectors, mainly leafhoppers, planthoppers and psyllids (Weintraub and Beanland, 2006).

The embryos contain a completely differentiated vascular system and they can be a source of propagation or transmission of the lethal yellowing disease (Harrison et al., 1995; Cordova et al., 2003). The exchange of germplasm of coconut, usually affected by the embryos, becomes difficult, especially when the embryos come from areas where the disease occurs (Jones et al., 1999).

The disease is known by different names in various countries; in Ghana it is called Wilt disease of Cape Saint Paul (CSPW), in Togo, it is known as Kaïncopé disease, in Nigeria it is Awka and in Cameroon, it is named Kribi disease. The phytoplasmas that cause the diseases are variable from one country to another. Disease appeared in southern Côte d'Ivoire in the department of Grand-Lahou and threatens one of the world's largest coconut collections (Konan et al., 2013).

The presence of the disease in all parts of the world creates mistrust between the coconut producing countries and the exchange of plant material. This has a negative impact on research works and the culture of coconut. The objective of this work was to check the presence or absence of the phytoplasma responsible for lethal yellowing disease in zygotic embryos and regenerated plants obtained from trees affected by this disease. These results will serve as a guide and help to ensure the exchange of plant material between coconut producing countries using zygotic embryos.

MATERIALS AND METHODS

The study focused on zygotic embryos from mature coconut nuts (10-12 months of age) harvested from trees that are visually affected by the LYD and are in stage 1 of the disease. The visual symptoms of LYD are: at stage 0 or apparently breast, the tree does not present symptoms; in stage 1, yellowing of the apical leaves; in stage 2, the fall of immature and mature nuts; in stage 3, leaf and crown leaf loss; at stage 4, only trunks and stems remain. Mature nuts were collected in coconut plantations which contained two types of hybrid (PB121 or MYD x WAT and PB113 or CRD x RIT) and the West African Tall (WAT) variety. The three types of coconut are sensitive to the disease. Sample were collected in five villages of Grand-Lahou Department in Côte D'Ivoire (5° 14'39'' North and 5° 00'11 '' West) that are Badadon, Palmindustrie V1, Groguida, Adjadon and Likpilassé (Figure 1). Sampling is also undertaken for phloem of stem of each palm. A total of 30 trees were sampled including 6 at Badadon, 3 at Village 1, 6 at Groguida, 6 at Adjadon and 9 at Likpilassé (Table 1).

Sampling of phloem, mature nuts, zygotic embryos and plantlets

To check the gene 16s RNAr of the phytoplasma responsible for

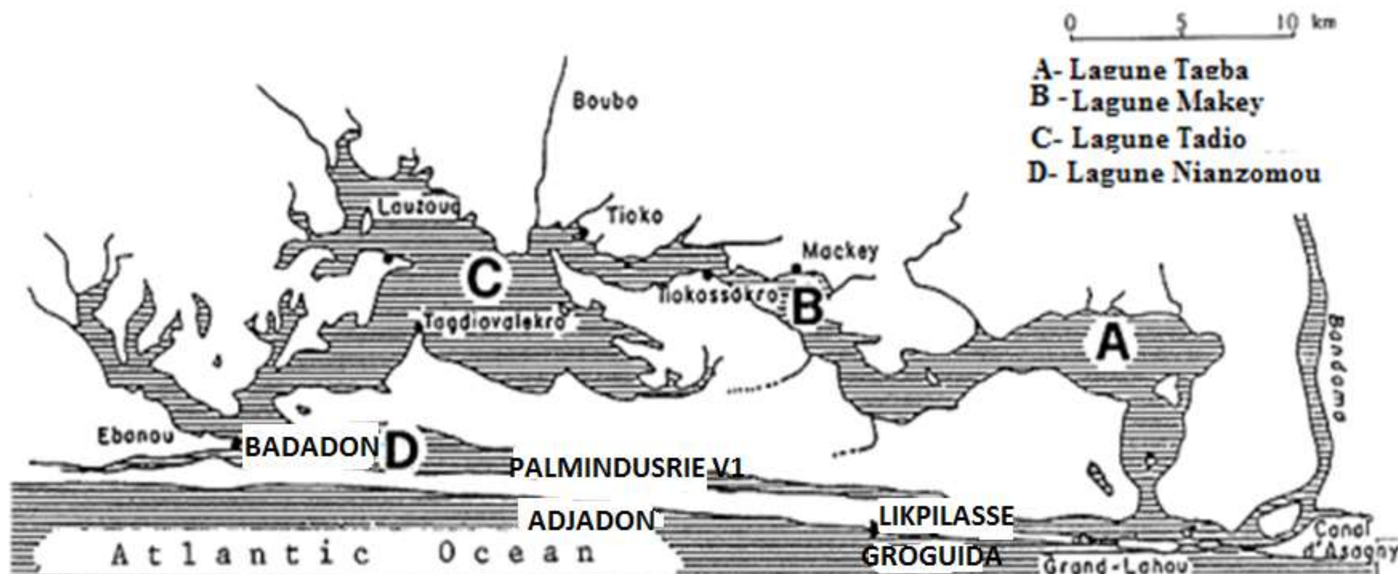


Figure 1. Location of villages visited in the Department of Grand Lahou, Côte d'Ivoire.

Table 2. Primers used for the PCR tests.

Primer codes	Primer sequence	Gene to be amplified	TM (°C)	Size of the gene to be amplified (bp)	Types of PCR
P1	AAGAGTTTGATCCTGGCTCAGGATT	16S RNA 5'	56	1800	Classique
P7	CGTCCTTCATCGGCTCTT	16S RNA 3'			
G813	CTAAGTGTCGGGGGTTTCC	16S RNA 5'	60	600	Nested-PCR
AwkaSR	TTGAATAAGAGGAATGTGG	16-23S	52		

the lethal yellowing disease, one phloem sample per tree was taken for the mother trees; the phloem (Figure 2) was taken with an electric drill. A total of 30 phloem samples from each of the mother trees was collected and stored at -4°C prior for extraction of the total DNA.

Mature nuts, 10-12 months old, recognizable by the brown colour of their epidermis (De Nuce and Wuidart, 1982) or noise of water inside were harvested from the bunches on the mother trees for 5 to 10 nuts. A total of 150 nuts were harvested from the 30 sampled mother trees. The cylinders of the endosperm or solid albumen that contain the zygotic embryos were carefully removed from the nuts and disinfected as recommended by N'Nan et al. (2012) and Yoboue et al. (2014). After disinfection of the endosperm cylinders, extraction and disinfection of the zygotic embryos were carried out in the laboratory under aseptic conditions for operation in air-flow cabinet (ASSY-Bah et al., 1989; Yoboue et al., 2014). The samplings were taken on seemingly healthy trees or in stage 1 trees because during the evolution of the disease, the mature and immature nuts fall early enough; therefore, for these stages (2, 3 and 4), there are no mature nuts on the trees. The zygotic embryos from the nuts (a zygotic embryo/whole nut) from the same mother tree were divided into two batches. For each sampled parent tree, one of the batches of zygotic embryos was used to run tests in order to detect the presence of the phytoplasma DNA. The embryos that compose the other batches were transferred directly to *in vitro* regeneration medium contained in the test tubes as one embryo per tube. The composition of the *in vitro* regeneration medium was continuously modified to successively induce germination of the zygotic embryos and organogenesis (root, stem and leaf) in order to obtain, after 6 months, a complete seedling (Figure 3).

Total DNA extraction

The extractions of the total DNA of phloem from mother trees, zygotic embryos and leaves of regenerated plantlets *in vitro* were done in a buffer CTAB according to the protocol of Harrison et al. (2013). DNA concentrations in the various extracts were read through a spectrophotometer Nanodrop 2000 (thermo-scientific, USA).

PCR and detection of sequence 16S rRNA of the phytoplasma

The conventional PCR was performed with 25 ng of total DNA in a reaction volume of 25 µl containing 1 µM of each P1 universal primer (Deng and Hiruki, 1991) and P7 (Schneider et al., 1995). Concerning the Nested-PCR, the CSPWD G813F/AwkaSR primers (Tymon et al., 1998) were used to amplify the area between the 16s rRNA and 23S gene for detection of West African phytoplasma strain (Nigeria, Ghana and Cote d'Ivoire) of the phytoplasma (N'nan et al., 2014). The characteristics of these primers used are recorded in Table 2.

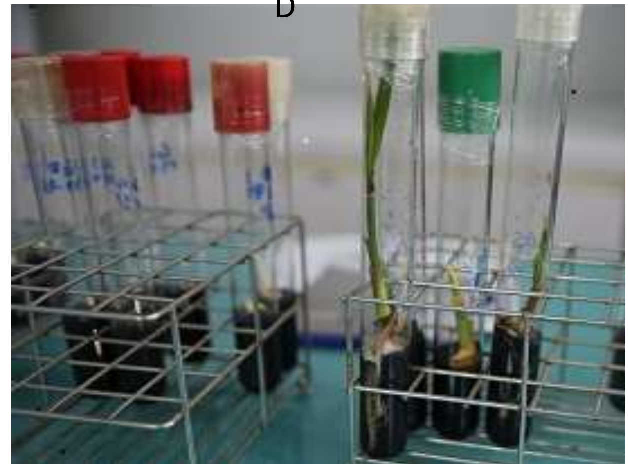
A

B

C

D

E



F

Figure 2. Harvesting of phloem (A, B), embryo (C, D) and young leaves of *in vitro* plants (E, F) for molecular diagnostic of the phytoplasma.

The analysis of the results of the PCR was carried out by electrophoresis on a 1% agarose gel prepared with a TBE buffer at 95 V for 45 min. The agarose gel was pre-saturated with "SYBR Safe DNA" during preparation. Fragment sizes were measured

Figure 3. Results of PCR amplification of the sequence of DNA of the phytoplasma of phloem samples of coconut trees.

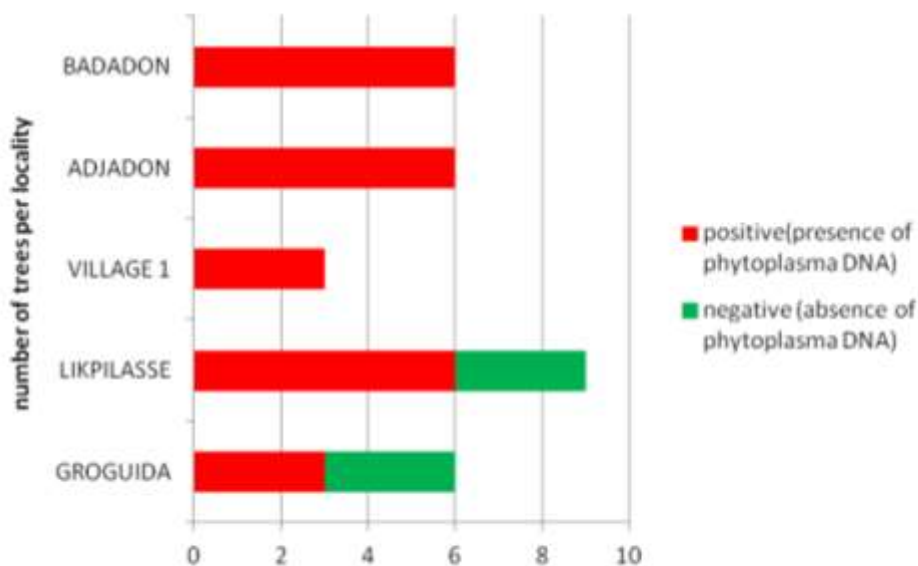


Figure 4. Variability of the proportions of coconut trees that tested positive or not for the presence of the 16S rRNA sequence of the phytoplasma.

using a 100 bp molecular weight marker. After migration, the gel was observed on a screen using a digital imaging system "digidoc-il 120 imaging system".

RESULTS

The display on 1% agarose gel of PCR products obtained from the phloem DNA extracts revealed a total of 80% (24 trees out of 30 tested) positive (presence of the sequence between 800 and 900 bp characteristic of the DNA length of phytoplasma) within the trees (Figure 4). The distribution of trees infected with the lethal yellowing disease revealed that 100% of the individuals tested had the disease in the villages; Badadon, Palmindustrie V1 and Adjadon (Figure 5). A proportion of 20% of trees that tested negative were observed in the villages of Likpilassé (3 trees) and Groguida (3 trees). In the three varieties studied, all PB121 trees and WAT were positive while out of 9 trees of PB 113, 3 were positive and 6 negative (Figure 5).

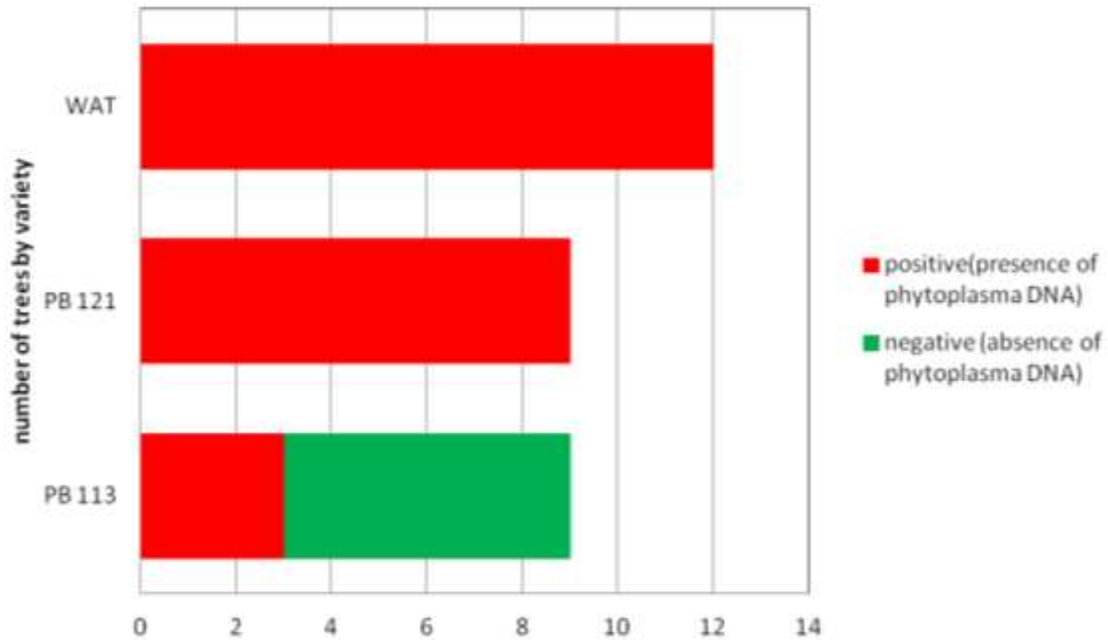


Figure 5. Variability of the number of coconut trees in WAT, PB121 and PB113 varieties tested for the presence of 16S rRNA sequence of the phytoplasma.

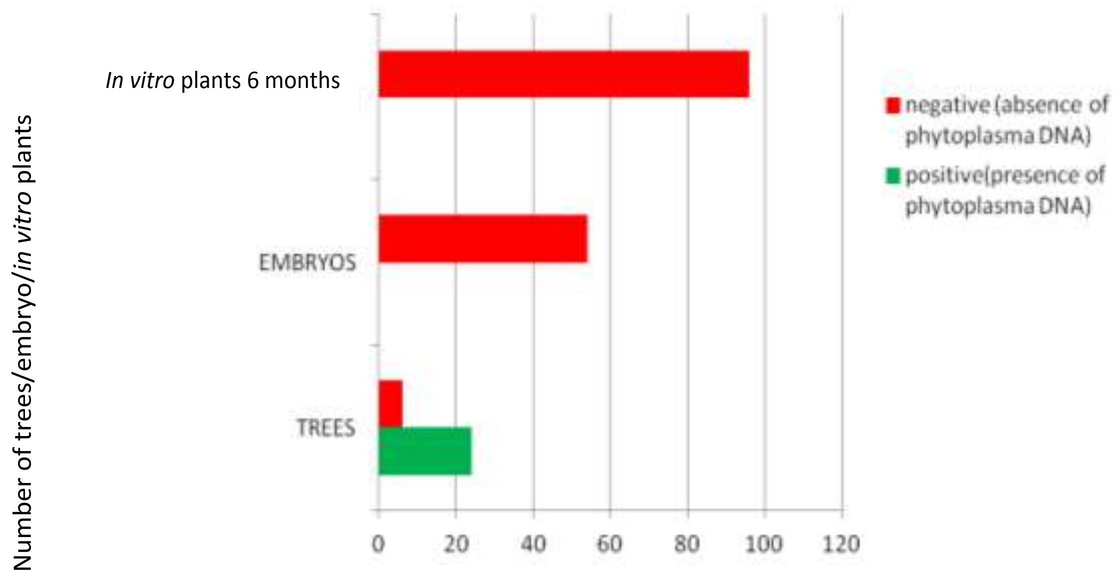


Figure 6. Molecular diagnostic tests by PCR for checking of 16S rRNA sequence of the phytoplasma responsible for coconut LYD disease in trees, embryos and *in vitro* plants.

Molecular diagnosis by PCR carried out for zygotic embryos and *in vitro* plants did not reveal the presence of phytoplasmic DNA on the embryos obtained from the nuts harvested from the trees that tested positive as well as the embryos obtained from the nuts harvested from trees that tested negative at Likpilassé and Groguida (Figure 6).

DISCUSSION

From a total number of 30 sampled trees, 24 trees tested positive to the sequence 16S rRNA of the phytoplasma responsible for the lethal yellowing disease followed up by the molecular diagnosis by PCR. This indicates the presence of the phytoplasma responsible for the lethal

yellowing disease in these trees. It confirms the presence of the disease in the Department of Grand-Lahou as published by Konan et al. (2013) and Yaima et al. (2014). Contrary results were obtained by N'nan et al. (2014) in their works on the lethal yellowing disease in Ghana where the authors did not obtain any amplification for samples taken from trees at the onset of the disease.

When embryo was extracted, the PCR analysis revealed that not all embryos carry phytoplasma because there was no amplification. Similar results were obtained by N'nan et al. (2014) where all embryos tested did not have amplification of the AwkaSR gene. Indeed, during the evolution of the disease, nuts fall very early which could exempt them. Similarly, the seedlings obtained after 6 months of *in vitro* cultures are not carriers of the disease. These results confirm the absence of the phytoplasma in the embryo and the seedling obtained *in vitro*. 80% of the phytoplasma-bearing trees produced nuts with healthy *in vitro* embryos and seedlings, which indicates that the disease is not transmitted from the seed. The absence of DNA of the phytoplasma in embryo demonstrated by the authors also agrees with the work of McCoy et al. (1983) and Cousin (2001). According to the latter, the transmission of the phytoplasma by the embryo contradicts the biological principles which shows that the seeds do not transmit the phytoplasma. The works of Nipah et al. (2007) and Myrie et al. (2011) support this point of view. Indeed, the authors showed that *in vitro* culture of embryos from infected plants leads to healthy plants.

The current work confirms this hypothesis because all the embryos collected in endemic areas are healthy, not carrying the phytoplasma. The methods of extractions and detections were carried out under strict laboratory conditions. Embryos are not transmitters of lethal yellowing.

However, results in disagreement with those reported in this study were previously reported. Harrison et al. (1995) and Cordova et al. (2003) showed the possible presence of phytoplasma in the embryo. The works of Nipah et al. (2007) also reported the presence of phytoplasma from zygotic embryos while working on the Great West Africa (GWA). Cordova et al. (2003) studies on the amplification of 16S rRNA of the phytoplasma gene in the embryo by PCR. According to these authors, the DNA of the phytoplasma is available in the embryo at a very low concentration. Therefore, for the detection of the phytoplasma by PCR, a large number of embryos should be used in order to get a significant quantity of the DNA of the phytoplasma in the total DNA extracted from the embryo. Harrison et al. (1995) have already proposed that several cycles of ultracentrifugation may be useful for amplification of the 16S rRNA gene of the phytoplasma.

Conclusion

This study was conducted to check the phytoplasma responsible for the coconut lethal yellowing disease of coconut tree in embryos and seedlings. The results of the molecular diagnosis by PCR revealed that unlike affected trees, the zygotic embryos from the nuts harvested from these trees and seedlings regenerated from the *in vitro* culture of these zygotic embryos, do not carry the gene responsible for lethal yellowing disease. This is due to the fact that *in vitro* embryo regeneration generates healthy plants. Thus, the use of embryo for the exchange of plant material even from lethal yellowing disease areas is recommended.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antimicrobial activity of metabolites extracted from *Zanthoxylum gilletii*, *Markhamia lutea* and their endophytic fungi against common bean bacterial pathogens

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Antibacterial activity of extracts of *Zanthoxylum gilletii*, *Markhamia lutea* and their fungal endophytes were evaluated against bacterial pathogens of common bean: *Xanthomonus axonopodis* pv. *phaseoli* and *Pseudomonus syringae* pv. *phaseolicola.* The leaves of both plants were dried under shade, ground to fine powder and extracted using methanol. The methanol extracts were fractionated sequentially using ethylacetate and hexane to produce various fractions. Endophytic fungi were isolated from fresh leaves and identified by ITS-rDNA sequence analysis. Antibacterial screening of the fungal endophytes was done by dual culture assay. The most active endophytic fungi were fermented on rice media and extracted using methanol. Pure compounds were analyzed by a combination of mass spectrometry and spectroscopic techniques which included 1D and 2D NMR. Antibacterial activity of all the extracts was determined by disc agar diffusion assay against the test organisms. Twenty-four (24) fungal endophytes were isolated which included: *Fusarium*, *Chaetomium*, *Scopulariopsis* and *Trametes.* Endophytic *Fusarium solani* was the most active against *X. axonopodis* pv. *phaseoli* (20.3 mm inhibition zone) and *P. syringae* pv. *phaseolicola* (18.6 mm inhibition zone). The plant extracts were active against *X. axonopodis* pv. *phaseoli* with an inhibition zone ranging between 8-12 mm except the methanol extract from *Z. gilletii* which did not show any activity. The endophytic extracts were active against both test organisms with a zone of inhibition ranging from 9.3-14 mm. Phenolic compounds present in *Fusarium* species may have contributed to the antibacterial activity of this strain against the test organisms.

Key words: Common bean, medicinal plants, fungal endophytes, antibacterial activity, *Xanthomonus axonopodis* pv *phaseoli*, *Pseudomonus syringae* pv *phaseolicola.*

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is a major legume crop that is largely consumed among various communities in Kenya. It provides cheaper alternative source of protein and household food security to the low-income earners in towns and the rural poor population (Gichangi et al., 2012). However, as noted over the years, its productivity is gradually declining (Katungi et al., 2010). This could be attributed, but not limited, to

bacterial infections such as common bacterial blight caused by *Xanthomonus axonopodis* pv. *phaseoli* and halo blight caused by *Pseudomonus syringae* pv. *phaseolicola*. The effects of these bacteria can be easily spotted in the field which affects the leaves as well as the pods. This leads to a reduction in the productivity of common bean in Kenya. Currently, the methods used to control these pathogens in the field include the use of copper based foliar sprays, synthetic pesticides and antibiotics such as streptomycin. The indiscriminate and intensive use of these pesticides and antibiotics has caused many problems to the environment such as water, animals, soil and food contamination; elimination of non-target organisms; poisoning of farmers as well as selection of phytopathogens, weeds and pests (Stangarlin et al., 2011). There has also been incidences of the occurrence of pesticide residues in the farm produce (Sartori et al., 2004). Therefore, there is need to find alternative ways of controlling these pathogens using extracts from natural sources such as endophytic fungi and medicinal plants which are believed to be easily biodegradable and readily available.

The tropical ecosystem is a host to more than half the number of living species worldwide, and many bioactive metabolites are produced in this ecosystem. Therefore, most plant species in this ecosystem are known to possess medicinal properties (Suryanarayanan, 2011). These plants are inhabited intracellularly by either bacteria or fungi known as endophytes that do not cause any apparent disease symptom (Clay, 1990). The fungal endophytes produce secondary metabolites that have desirable antimicrobial properties such as antibacterial, antifungal, antiviral, antioxidant, somatic fat reducing, blood pressure regulating, anti-inflammatory among others. *Zanthoxylum gilletii* is an evergreen, aromatic deciduous shrub or tree that belongs to the family Rutaceae (Negi et al., 2011) while *Markhamia lutea* (Nile tulip) is an evergreen subtropical, flowering plant that belongs to the family Bignoniaceae (Orwa et al., 2009). Both plants possess medicinal properties and are commonly found and used in Kenya for various medicinal purposes. For instance, *Z. gilletii* is used traditionally for the treatment of urogenital infections, rheumatism and in the management of various parasitic infections (Gaya et al., 2013, Nyunja et al., 2009). *M. lutea* on the other hand has been used in the treatment of earache, skin infections, asthma, cough, gonorrhea as well as alleviation of AIDS symptoms among others (Lamorde et al., 2010). Most of the traditional uses of these plants are based on their importance on the alleviation of human pathogen. Therefore, this study aimed to determine the antibacterial activity of these plants as well as their endophytes against bean bacterial pathogens. This study is significant due to the reduction in the common bean productivity and the need for alternative sources from natural products to control these infections and thereby improving bean productivity in Kenya.

MATERIALS AND METHODS

Collection of plant materials

Fresh leaves of *Z. gilletii* and *M. lutea* were collected from Kakamega Tropical Rainforest which stretches from 0° 10' to 0° 21'N and longitude 34° 44' to 34°° 58'E and an altitude of 1524 m above sea level. The leaves were identified with the help of a taxonomist and were deposited at the Biological Sciences Department, Egerton University.

Isolation of the fungal endophytes

Endophytic fungi were isolated from the leaves of *Z. gilletii* and *M. lutea* within 8 h of collection using the procedure by Zinniel et al. (2002) with slight modifications. Briefly, the leaves were washed under running tap water and blotted dry using filter papers. Thereafter, they were sterilized for 2 min in 70% ethanol, 1% sodium hypochlorite for 3 min and rinsed three times in sterile distilled water. The leaves were then cut aseptically into sections approximately 1 by 4 mm and inoculated in Petri-dishes containing Sabourand Dextrose Agar (SDA) amended with streptomycin sulphate antibiotic (2 g/L). The plates were incubated at 25 ± 2°C for 1 to 4 weeks. Frequent monitoring was done to check for the growth of the endophytic fungi. The first visible hyphal tips were transferred to fresh SDA plates to prepare pure cultures. The cultures were then identified using molecular techniques.

Molecular identification of the isolated fungi

Pure cultures of the endophytes were grown in 30 ml of yeast Malt broth (pH 6.3) and incubated at 28°C on an orbital shaker for 3 to 4 days to allow the fungal mycelia to grow.

DNA extraction

The DNA extraction was performed using the BIO BASIC EZ-10 Genomic DNA kit following manufacturer's instruction. Approximately, 6 to 10, 1.4 mm Precellys Ceramic Beads were added to a 1.5 ml screw cap reaction tube. Approximately, 60 mg of the fungal hyphae obtained from a 3 to 4 day old culture were added to the same tube. The sample was covered with 600 µl Plant Cell lysis buffer (PCB) and homogenized using a homogenizer (Precellys 24 lysis and homogenization, Peq lab, Bertin technologies). β-Mercaptoethanol (12 µl) was added to the sample, vortexed (IKA MS3 Digital) and incubated for 25 min at 65°C in a metal block (MTB 250). Chloroform (600 µl) was added and the mixture centrifuged (5430 R) at 10,000 rpm for 2 min. The supernatant was transferred to a clean Eppendorf tube and the rest discarded. Binding buffer (BD buffer) (200 µl) was added and the

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mixture vortexed, followed by addition of 200 µl ethanol and again vortexed. The mixture was transferred into EZ-10 column placed in a 2 ml collection tube and centrifuged at 12,000 rpm for 1 min. The flow through was discarded and then 500 µl of PW solution was added. The mixture was centrifuged at 12, 000 rpm and the flow through discarded. Then, 500 µl of Wash solution was added and the mixture was again centrifuged at 12,000 rpm for 1 min and the flow through discarded again. The column was again centrifuged at 12,000 rpm for 2 min to remove any remaining wash solution. Finally, the column was transferred into an empty 1.5 ml Eppendorf tube and 70 µl of TE Buffer, pre-warmed to 60°C, added directly at the center of the EZ membrane to increase the elution efficiency. The sample was incubated for 2 min at room temperature and then centrifuged at 12,000 rpm for 2 min to elute the DNA. The DNA was stored at 4°C for further analysis.

Polymerase chain reaction (PCR) amplification

To a PCR tube, the following were added: 0.5 µl of forward primer ITS1F (CTTGGTCATTTAGAGGAAGTAA) and 0.5 µl of reverse primer ITS4 (TCCTCCGCTTATTGATATGC), 12.5 µL of the jump start ready mix that contained 20 mMTris-HCl pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTPs (dATP, dCTP, dGTP and dTTP), stabilizers, 0.1 unit/mL Taq DNA polymerase and JumpStart antibody. This was followed by 9.5 µl of distilled water and 2 µl of the template DNA to make a total volume of 25 µl of the mixture per sample. For a negative control, 2 µl of distilled water was used in the reaction mix instead of DNA template. The amplification was done in a thermocycler (Eppendorf® Mastercycler® nexus Thermal Cycler) under the following conditions; initial denaturation of 5 min at 94°C, followed by 34 cycles of denaturation for 30 s at 94°C, annealing at 52°C for 30 s and elongation for 1 min at 72°C. Then, a final elongation of 10 min at 72°C. The PCR products were pre-stained with midori green dyeand resolved in a 0.8% agarose gel. The visualization was done in a UV transilluminator (Nippon Genetics Europe GMbH) and photographs were taken. The amplified PCR products were purified using BIO-BASIC EZ-10 kit and stored at -4°C for further analysis.

DNA sequencing

The amplified DNA was sequenced by Illumina genome analyzer sequencing machine (applied Biosystems 3730 xl DNA analyzer). The forward and reverse primer sequences obtained from the sequencing were aligned by Genious R7 program to get the consensus sequences. The consensus sequences were deposited in NCBI GenBank and compared with those available in GenBank via BLAST searches. Phylogenetic analysis was conducted using the distance based neighbor joining methods in Molecular Evolutionary Genetics Analysis (MEGA) version 6.06 and the Neighbor joining (NJ) tree constructed using Tamura-Nei distance. All characters were equally weighted and unordered. Gaps and the missing data were treated as complete deletion. Support for the specific nodes on the NJ tree was estimated by bootstrapping 2000 replications. The substitution type was used for nucleotides and the pattern of lineage was homogeneous.

Antimicrobial activity of the isolated fungi

Test organisms

The test organisms used in this study were *X. axonopodis* pv *phaseoli* and *P. syringae* pv. *phasiolicola* which were provided by the Biological Sciences Department of Egerton University.

Dual culture assay

Inhibition of bacterial growth by the endophytic fungi was examined on Muller Hinton (MH) plates using dual culture assay as described by Srivastava and Anandrao (2015) with slight modification. Briefly, 100 µl of bacterial concentration of 5×10^5 CFU/mL was swabbed evenly on the MH media on Petri-dishes using a sterile cotton swab and allowed to dry. A six-millimeter diameter of a 7-day old mycelia plug was placed in the MH media plate inoculated with the test bacteria. A standard chloramphenicol was used as a positive control. The plates were incubated at ± 32°C for 24 h and the zone of inhibition was measured in mm. The experiment was carried out in triplicates. The most active endophytic fungi were subjected to solid state fermentation for secondary metabolites extraction.

Fermentation and extraction of secondary metabolites

Fermentation of the endophyte was carried out using a procedure by Nascimento et al. (2012) with slight modification. The solid-state fermentation was carried out in 21, 500 ml Erlenmeyer flasks containing 90 g of rice in 90 ml of distilled water per flask which were twice autoclaved at 120°C for 40 min. Agar plugs of about 2 × 2 cm were cut from a 7-day old culture of the endophyte and then inoculated in the rice media. One flask without inoculum was kept as a control. The flasks were incubated for 21 days at 25°C under static conditions. The flasks were checked periodically for contamination.

Extraction of secondary metabolites from the endophytic fungi

After the incubation period, the fermentation was ended with the addition of 150 ml of methanol to each of the flasks and left to stand overnight. The cultures were cut into pieces with the aid of a spatula and the flask placed in an ultrasonic cleaner (SB-120 DTN) to allow complete extraction of the secondary metabolites. The mixture was filtered using a Whatman filter paper no. 1 followed by repeated extraction with methanol until exhaustion. The filtrate was evaporated under reduced pressure (BUCHI rotavapor R-205) to yield a methanol extract. The methanol extract was partitioned between hexane and ethylacetate to obtain the respective fractions. The fractions were subjected to antibacterial assay.

Extraction of secondary metabolites from the leaves of the medicinal plants

The collected leaves were dried under a shade for approximately 2 weeks. The leaves were ground into a fine powder and 700 g of each powder was soaked in 1.5 L of methanol overnight. The mixtures were then filtered using Whatman filter paper no. 1 and the filtrate evaporated under reduced pressure. The obtained fractions were partitioned using reverse phase solid phase extraction, followed by thin layer chromatography (TLC) and column chromatography. The fractions obtained were subjected to antibacterial assay.

Antibacterial assay

The antibacterial assay of both the endophytic and plant extracts was performed using agar disc diffusion method as described by Kajaria et al. (2012) with slight modification. The media used in this assay was Muller Hinton Agar (38 g/1000 ml of distilled water). A 24-h bacterial population of 1.5×10^8 CFU/ml (1.0 x 108 – 2.0 x 108 CFU/ml) was spread on the plate containing media and left to dry. All extracts were weighed and a 50 mg/ml concentration of the

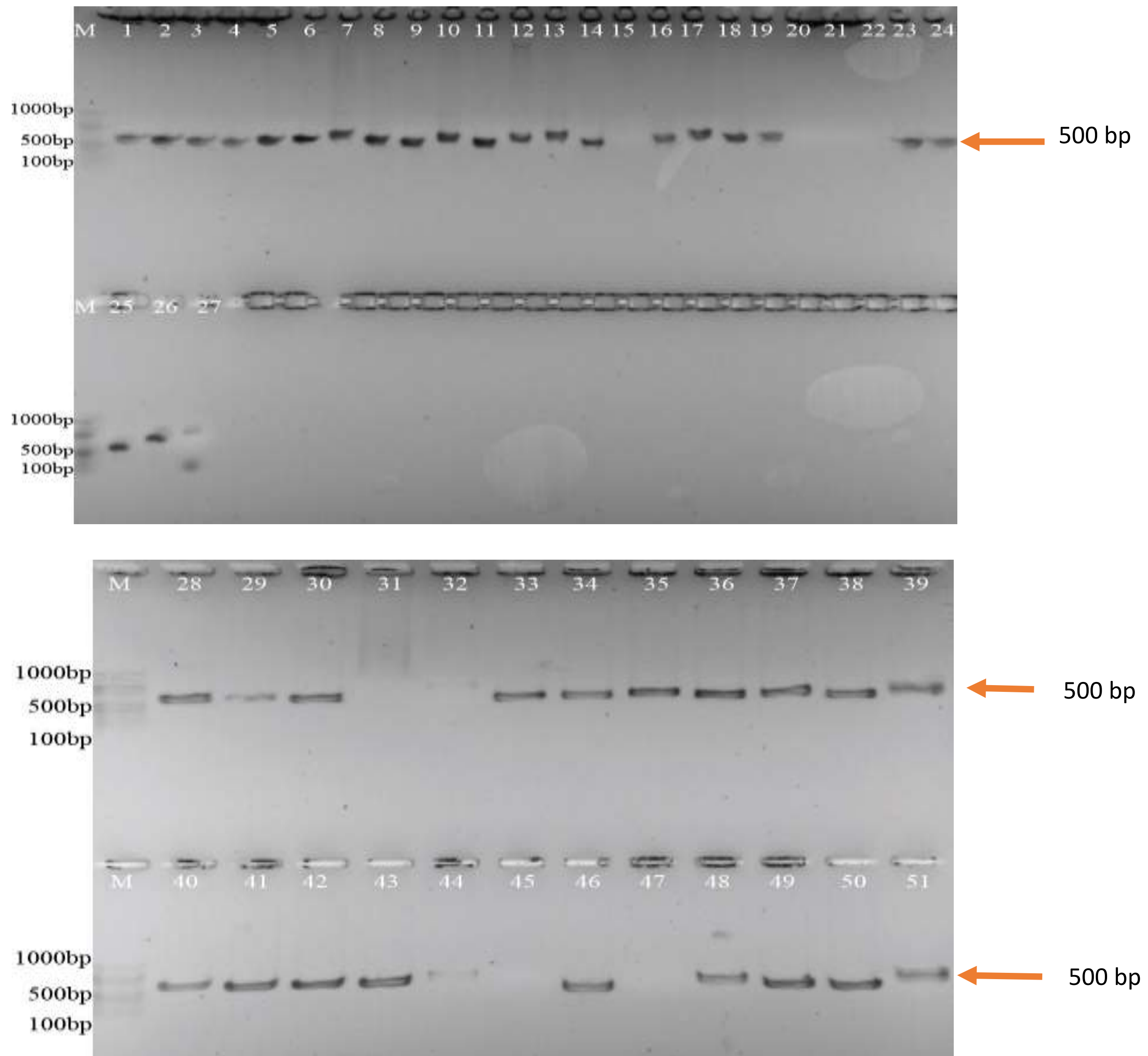

Plate 1. Agarose gel showing ITS PCR products of the isolated fungal endophytes. The molecular weight of the isolated DNA ranged from 500 to 700 bp.

extracts made using DMSO. Blank sterile disc of Whatman filter paper No. 1 of 6 mm in diameter was impregnated with 10 µl of different extracts and plated against the test organisms. A standard chloramphenicol antibiotic was used as a positive control while the negative control was blank sterile disc soaked in DMSO. The plates were incubated at ±32°C overnight and zone of inhibition measured in millimeters.

RESULTS

Isolation and identification of the fungal endophytes

A total of 24 fungal endophytes were isolated from *Z. gilletii* and *M. lutea*, some of which are shown in Plate 1. Optimal PCR products of the isolates were obtained using primer pair ITS1F and ITS4 which varied in band sizes of 500-700 bp (Plate 1). All the 24 identified fungal endophytes belonged to the phylum Ascomycota except *Trametes* aff. *maxima* which belonged to the phylum Basidiomycota. The endophytes were divided into the following groups: 63% *Fusarium* species, 4% *Fusarium solani*, 4% *Fusarium oxysporum*, 4% *Scopulariopsis flava*, 4% *Scopulariopsis brevacaulis*, 13% *Chaetomium* cf. *cochloides*, 4% *Chaetomium* spp. and 4% *Trametes maxima* (Figure 1). The BLAST percentage similarity to sequences in NCBI from the previously identified fungi

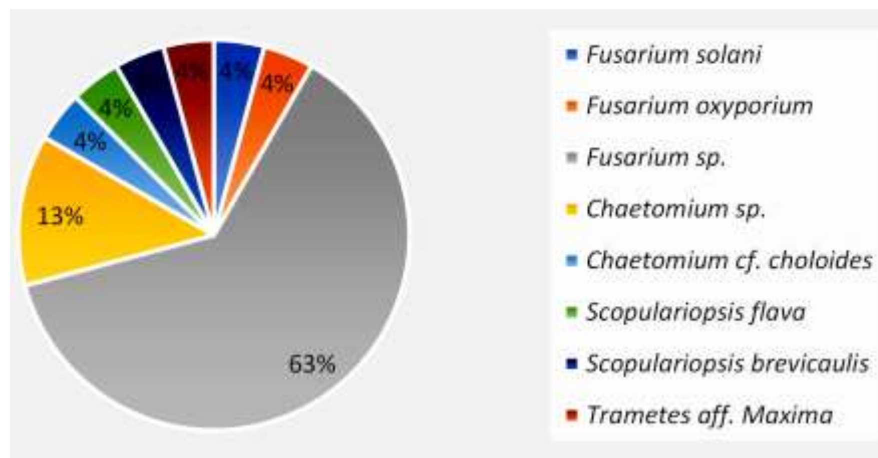

Figure 1. Isolation frequencies of fungal endophytes from the two medicinal plants.

Table 1. Identification and antibacterial activity of the isolated fungal endophytes against the test organisms

Isolate code	Similarity with	Similarity (%)	Accession number	Diameter zone of inhibition (mm)	
				X. phaseoli	***P. syringae***
Ml.1	*Fusarium* sp.	100	EU029589.1	10.6±1.15^{cd}	10.3±1.15^{de}
Ml.2	*Fusarium solani*	100	KM268688.1	20.3±1.5^{a}	18.6±1.15^{a}
Ml.3A	*Chaetomium cf. cochloides*	99	KT895345.1	0±0^{e}	0±0^{f}
Ml.4	*Fusarium* sp.	100	KM268689.1	0±0^{e}	0±0^{f}
Ml.5	*Fusarium* sp.	100	EU750687.1	14±2^{bc}	15±1^{bc}
Ml.6	*ScopulariopsisFlava*	99	LN850790.1	0±0^{e}	0±0^{f}
Ml. 6 A	*Fusarium* sp.	100	AB369907.1	0±0^{e}	0±0^{f}
Ml.7	*F. solani*	99	KM268689.1	15±1^{b}	17±1^{ab}
Ml. 8	*Fusarium oxysporum*	96	KJ573079.1	15.3±1.15^{b}	16.3±1.5^{ab}
Ml.9	*Fusarium* sp.	100	KM889541.1	12±2^{bcd}	9.6±0.5^{e}
Ml. 10	*Fusarium* sp.	99	KM268689.1	15.3±1.15^{b}	16±1.15^{ab}
Ml.11	*Chaetomium* sp.	99	KM520350.1	0±0^{e}	0±0^{f}
Ml.13	*Chaetomium* sp.	99	KM520346.1	0±0^{e}	0±0^{f}
Ml.15	*Scopulariopsis brevicaulis*	99	KP132728.1	0±0^{e}	0±0^{f}
Zg.1	*Fusarium* sp.	99	JN232136.1	10.3±1.15^{d}	11±1de
Zg. 2	*Fusarium* sp.	95	KT313630.1	12±2^{bcd}	11±1de
Zg.3	*Fusarium* sp.	100	KM889544.1	11±1^{cd}	11.3±1.5^{de}
Zg.4	*Trametesaff. maxima*	95	JN164918.1	12±2^{bcd}	12.6±1.15^{cd}
Zg. 5A	*Fusarium* sp.	100	EU750687.1	9.3±0.5^{d}	11±1de
Zg. 5	*Fusarium oxysporum*	100	KM889544.1	9.6±1.15^{d}	10.6±0.5^{de}
Zg.6	*Fusarium* sp.	99	KM889544.1	0±0^{e}	0±0^{f}
Zg.7	*Fusarium* sp.	100	EU750687.1	10±1^{d}	9.7±1.15^{e}
Zg.8	*Fusarium* sp.	99	EU750687.1	11±1.7^{cd}	10.6±0.5^{de}
Zg.10	*Chaetomium* sp.	100	KR012907.1	0±0^{e}	0±0^{f}
Chloramphenicol				20±1^{a}	18.7±1.15^{a}

Within a column, fungal endophytes sharing the same letter(s) are not significantly different in antagonism against the two test organisms while those with different letters are significantly different (α =0.05, Tukey's test). The inhibition zone values are the mean of the triplicates ±S.D. of the mean.

ranged from 95 to 100% (Table 1).

The evolutionary relationships of the isolated fungi were determined by generation of a distance based neighbour joining phylogenetic tree. The neighbour

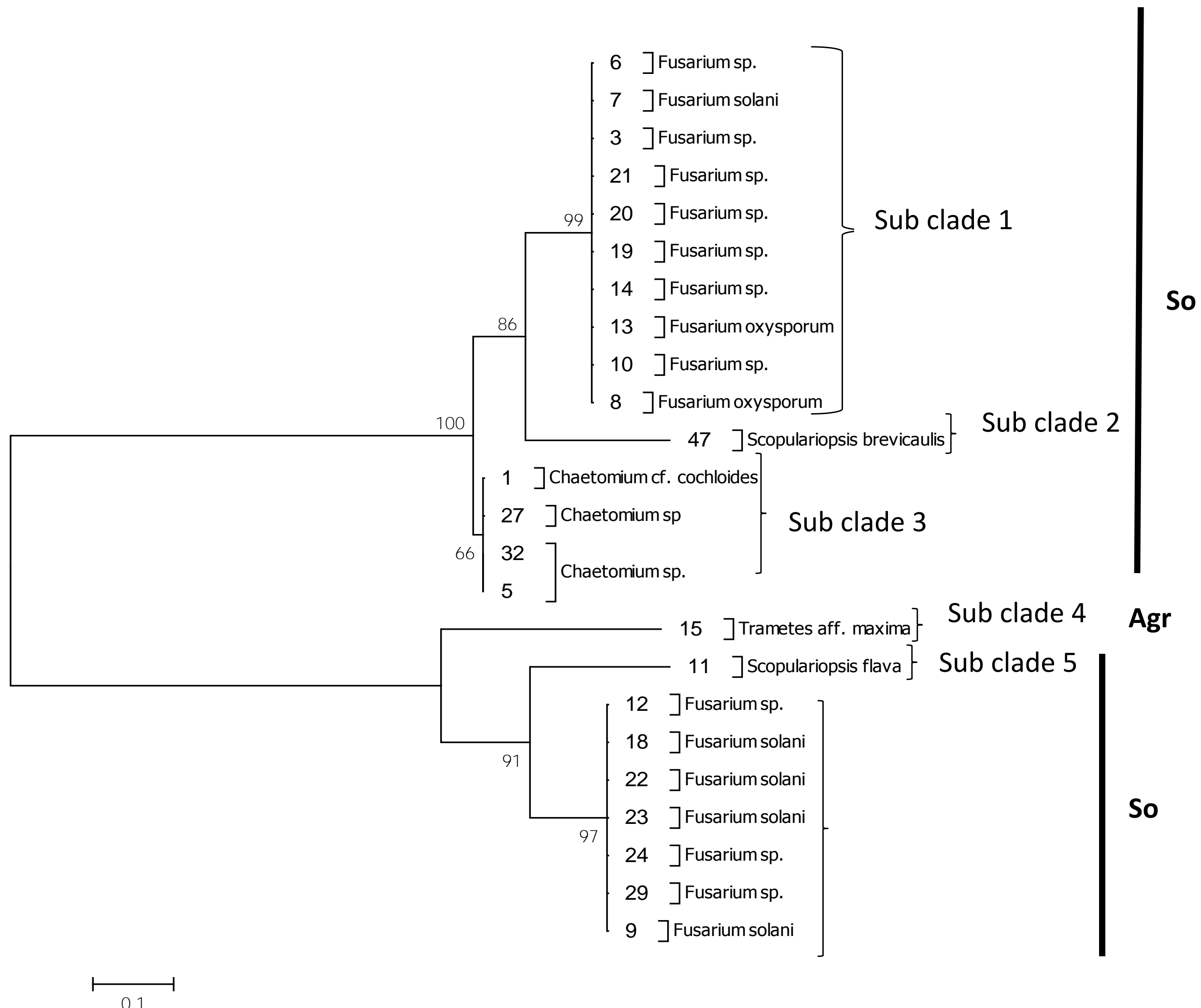

Figure 2. Phylogenetic neighbour joining tree of the isolated fungal endophytes based on ITS analysis (maximum likelihood method; 2000 replicates bootstrap. SO- Sordariomycetes Agr- Agariomycetes).

joining analysis placed the sequences into two groups: *Sordariomycetes* and *Agariomycetes*. The generated tree had two major clades that were divided into six sub clades of distinct species: sub-clade 1- *Fusarium* spp., sub-clade 2- *Scopulariopsis* sp., sub-clade 3- *Chaetomium* sp., sub-clade 4- *Trametes* sp., sub-clade 5- *Scopulariopsis* sp. and sub-clade 6- *Fusarium* spp. Approximately, 75% of the isolated endophytes belonged to the genus *Fusarium* (Figure 2).

Antimicrobial assay of the fungal endophytes

Dual culture assay was used to assess the antagonistic effects of the isolated fungal endophytes against *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *Phaseolicola* (Plate 2). As shown in Table 1, 15 out of 24 endophytes showed antagonistic activity against both *X. axonopodis* pv. *phaseoli* and *P. syringe* pv. *Phaseolicol;* 13 of which belong to the genus *Fusarium*. Endophytic fungus, *F. solani* (MI.2) had the largest inhibition zone of 20.3±1.5 mm against *X. axonopodis* pv. *phaseoli* and 18.6±1.5 mm against *P. syringae* pv. *phaseolicola*.

The one-way ANOVA-Leven's test showed non-homogeneity of variance for the isolated endophytes with a p value of 0.001. The activity of fungal endophyte MI.2 (*F. solani*) against the test organism had no significant difference in activity as compared to chloramphenicol standard.

Disc agar diffusion assay of the plant and endophytic extracts

Secondary metabolites were extracted from both the host plant and the isolated fungal endophytes. Extracts from

Plate 2. Antagonistic test of some selected endophytic fungi against test organisms; *P. syringae pv. phaseolicola* and *X. axonopodis pv. phaseoli.*

Table 2. Inhibition zones (mm) of the plant and endophytic extracts against *X. axonopodispv. phaseoli* and *P. syringaepv. phaseolicola*.

Extract code	Test organism (diameter in mm) n=3	
	X. axonopodis*pv. *phaseoli	***P. syringae*pv.*Phaseolicola***
Z.gMeOH crude	0 ± 0^{f}	0 ± 0^{e}
Z.gMeOH after SPE	0 ± 0^{f}	0 ± 0^{e}
Z.gEtA after SPE	8.3 ± 0.5^{e}	0 ± 0^{e}
Skimianine	12 ± 2^{bcd}	0 ± 0^{e}
M.lMeOH crude	7.6 ± 0.5^{e}	0 ± 0^{e}
M.lMeOH after SPE	8.6 ± 1.5^{e}	0 ± 0^{e}
M.lEthA after SPE	7.6 ± 0.5^{e}	0 ± 0^{e}
M.lEthA after partion	14 ± 2^{bc}	0 ± 0^{e}
Ml.2 EtA	14.6 ± 0.5^{b}	12.7^{b}
Ml.2 Hexane	10.3 ± 0.5^{de}	11 ± 0.8^{cd}
Ml.8 EtA	11.6 ± 0.5^{cd}	9.6 ± 0.5^{d}
Ml.8 Hexane	9.6 ± 0.5^{de}	9.3 ± 0.5^{c}
Chloramphenicol	20 ± 0.5^{a}	18.7 ± 1.15^{a}
DMSO	0 ± 0^{af}	0 ± 0^{e}

Z.g- Extracts from the leaves of Zanthoxylum giletii ; Ml- Extracts from the leaves of *Markhamia lutea*. *The values are the mean of three replicates ±S.D. of the mean. Within a column, the inhibition zones of extracts sharing the same letter(s) are not significantly different while those with different letters are significantly different (α =0.05, Tukey's test).

both *M. lutea* and *Z. gilletii* showed some activity against *X. axonopodis* pv. *phaseoli* but this was not the case against *P. syringae* pv. *phaseolicola* (Table 2). The methanol crude extract of *Z. gilletii* showed no activity against both test organisms. This was also observed from the methanol extract after solid phase extraction (SPE). The ethyl acetate extract after the same procedure showed some activity against *X. axonopodis* pv. *phaseoli* and no activity against *P. syringae* pv. *phaseolicola*. The alkaloid skimmianine isolated from the *Z. gilletii* produced a zone of inhibition of 12±2 mm against *X. axonopodis* pv. *phaseoli* while it showed no activity against *P. syringae* pv. *phaseolicola*. All the extracts from *M. lutea* were active against *X. axonopodis* pv. *phaseoli* with the ethyl acetate extract after partitioning showing the highest activity of 14±2 mm inhibition diameter against *X.*

axonopodis pv. *phaseoli*. However, these extracts did not show any activity against *P. syringae* pv. *phasolicola* as similarly noticed in the extracts of *Z. gilletii.*

The extraction from endophytic fungi *F. solani* MI.2, yielded 0.4 g hexane extract and 1.24 g ethyl acetate extracts after partitioning while that of the second most active MI.8 (*F. oxysporum*) yielded 1.69 g ethyl acetate extract and 0.6 g hexane extract. These extracts were then dissolved in DMSO to make a 50 mg/ml stock solution for the antimicrobial assay. The dual culture results of the endophytes were in line with the results from the extracts of the fungal endophytes with the ethyl acetate extracts of MI.2 (*F. solani*) giving the highest zone of inhibition of 15 mm. This was followed by the hexane extract that produced a zone of inhibition of 10±2 mm (Table 2). The extracts from MI.8 (*F. oxysporum*) showed a low activity as compared to MI.2 given that they both belong to the genus *Fusarium*.

The one-way ANOVA-Levenes test revealed a non-homogeneity of variance by producing a p value of less than 0.005. Turkeys Honestly Significant Difference (HSD) test revealed that both the plant and endophytic extracts had significantly low activity as compared to the standard chloramphenicol with the most active being MI.2 (*F. solani*) ethyl acetate extract as shown in Table 2.

DISCUSSION

Isolation and identification of the fungal endophytes

Fresh leaves of the medicinal plants (*Z. gilletii* and *M. lutea*) were used in this study for the isolation of endophytes in SDA media as well as extraction of secondary metabolites. As compared to this work, several reports have indicated leaf tissues as a source of endophytic fungi (Suryanarayanan et al., 2009). The fungi may penetrate the plant tissues through the aerial interaction making the leaf the most favorable (Banhos et al., 2014).

Twenty-four (24) fungal isolates which are reported in this study were successfully amplified using PCR while the remaining (not reported) were not amplified. As noted by Paterson (2004), the production of PCR inhibitory metabolites such as humic acid and fluvic acid during growth of the fungi inhibits the amplification of the region of interest during PCR. Primer mismatch or bias may also impede PCR amplification (Ihrmark et al., 2012).

Fungal endophyte diversity in plants could be affected by environmental factors, host species as well as the host genotypes (Chen et al., 2010). Various research works show that endophytic fungi mostly consist of members of Ascomycota although some taxa of Basidiomycota, Zygomycota and Oomycote have also been reported (Guo et al., 2001). In this study, 95% of the isolated fungal endophytes were ascomycetes, while 5% were basidiomycetes, showing a combination of both phyla as fungal endophytes. Of all the Ascomycetes obtained, 71% of the fungal endophytes belonged to the genus *Fusarium*. These results correlate with those obtained from a study by Bai et al. (2009), Chen et al. (2010) and Xing et al. (2011) which demonstrated that the dominant fungal endophyte strains isolated so far belong to the genus *Fusarium*. Although, *Fusarium* spp. are always considered as fungal pathogens on plants, they are often isolated as endophytes from various plants and they are also capable of producing various secondary metabolites with medicinal properties (Deng et al., 2009; Tayung et al., 2011). Bacon and Yates (2006) also notes that endophytic *Fusarium* species are capable of inducing plant host resistance to pathogens and increase the plants environmental fitness. This adaptation enables them to produce various secondary metabolites that have medicinal properties such as antimicrobial and anticancer (Shiono et al., 2007). It is worth noting that despite their biomedical importance, various *Fusarium* strains have not been identified to the species level and have not been phylogenetically characterized, hence making their phylogenetic identification quite difficult (Hidayat et al., 2016).

Chaetomium species is another group of fungi isolated in this study though they showed little activity against the test organisms. This group of fungi has been also isolated as endophytes from *Ephedra fasciculate*, *Ginkgo biloba, Aegle marmelos among others (*Bashyal et al., 2005; Qin et al., 2009*). S. flava* and *S. brevacaulis* are other species that were isolated in this study. These species have been isolated as fungal endophytes from lichens and marine sponge, *Tethya aurantium* respectively (Li et al., 2007; Wiese et al., 2011). Finally, *Trametes* aff. *maxima,* which is a white rot fungus, was also isolated as a fungal endophyte from *Z. gilletii*. This group of fungi have been isolated as endophytic fungi from *Theobroma giler*, *T. cocoa*, *Podophyllum hexandrum* and *Taxus globose* (Crozier et al., 2006; Puri et al., 2006; Rivera-Orduña et al., 2011). This study therefore revealed the presences of diverse species of endophytic fungi inhabiting these two medicinal plants.

Antibacterial activity of isolated fungal endophytes and extracts of the plants and the endophytes

Endophytic extracts used in the management of human and plant pathogens have gained a lot of interest (Ibrahim et al., 2017). Fungal endophytes that produce the same bioactive compounds as the host plant have been reported in the literature (Kusari et al., 2012). Different species of the isolated fungal endophytes displayed varied activity against the test organisms. As noted by Gong and Guo (2009) and Vaz et al. (2009), different *Fusarium* species exhibited different rates of activity. This trend was also seen in the activity of the isolated *Fusarium* species in this study. Species such as

Chaetomium and *Scopulariopsis* did not exhibit any significant activity against the test organisms which is in contrast to the results obtained by Momesso et al. (2008) and Rani et al. (2017). Trametes species showed activity against both test organisms. Species of this genus possess secondary metabolites that have broad spectrum antibacterial activity (Waithaka et al., 2017).

The two most active fungal endophytes (*F. solani* MI.2*, F. oxysporum* MI.8) were further examined using solid state fermentation. The two fractions (ethyl acetate and hexane) were both active against the two-test organisms and their inhibition zones were statistically different as compared to chloramphenicol standard. These results are in agreement with those obtained by Devaraju and Satish (2011) and Specian et al. (2012) in which different extracts from fungal endophytes isolated from various plants were active against *X. axonopodis* pv. *phaseoli.*

The fractions obtained from the leaf extracts of the medicinal plants displayed varying levels of activity depending on the fractionation level. For instance, methanol extracts of *Z. gilletii* did not show any significant activity against both the test organisms while the ethyl acetate extract after SPE displayed activity against *X. axonopodis* pv. *phaseoli*. As explained by Tavares et al. (2014), the activity of an extract may depend on the percentage composition of the active secondary metabolite in the sample which may be the case in this instance. Alkaloids from the genus *Zanthoxylum* possess a broad spectrum antibacterial activity. The alkaloid Skimianine obtained from *Z. gilletii* displayed a significant activity against *X. axonopodis* pv. *phaseoli*. The extracts of *M. lutea* showed varying levels of activity for instance, ethyl acetate extract after partition which produced an inhibition zone of 14 mm against *X. axonopodis* pv. *phaseoli* while there was no activity against *P. syringe* pv. *phaseolicola*. All the medicinal plant extracts were not active against *P. syringae* pv. *phaseolicola.* The activity of the medicinal plant extracts against the test organism may depend on the secondary metabolite composition. As much as most of the extracts isolated in this study did not show any significant activity, some extracts from plants such as *Ginkgo biloba have been shown to exhibit antibacterial activity against X. axonopodis pv. phaseoli* (Sati and Joshi, 2011). Garlic extracts have also been shown to inhibit the growth of *P. syringae* pv. *phaseolicola in vitro* (Hassan Eman and El-Meneisy Afaf, 2014). This study therefore demonstrates that extracts of medicinal plants can be applied in the agricultural sector to manage common bean bacterial infections as well as other infections.

Conclusion

This study demonstrated that the leaves of *Z. gilletii* and *M. lutea* are inhabited by different strains of endophytic fungi with promising benefits in controlling *X. axonopodis* pv*. phaseoli* and *P. syringae* pv. *phaseolicola*. The results indicated that *Fusarium* species contains secondary metabolites that can be used as antibacterial agents against these two bacterial pathogens. The leaf extracts of both plants also contain secondary metabolites that can be used directly or incorporated in other available pesticides to control or manage these infections in common bean.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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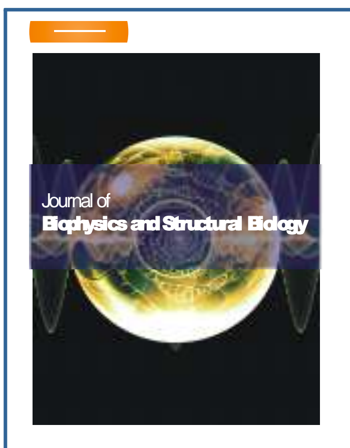

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