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# African Journal of Microbiology Research

April 2020
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

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Vol. 14(4), pp. 106-111, April, 2020
DOI: 10.5897/AJMR2020.9323
Article Number: 821C68963431
ISSN: 1996-0808

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*Full Length Research Paper*

# Food hygiene knowledge, attitude and practices among hospital food handlers in Elmanagil City, Sudan

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Received 15 March, 2020, Accepted 7 April, 2020

**Food borne diseases (FBDs) can occur due to the health status of the food handlers. The present study aimed to assess food hygiene knowledge, attitude and practices among hospital food handlers of ElManagil City, Sudan. A cross-sectional study was administered to evaluate knowledge, attitudes, and practices, in food hygiene among hospital food handlers through a questionnaire. The food handlers expressed a level of knowledge, attitude, and practice of 70.1, 63.81 and 74.40%, respectively. The food handlers lacked knowledge regarding the transmission of hepatitis A virus and the working of infected food handler. They demonstrated poor attitude regarding the continuous evaluation of their health status, as well as the importance of training courses in the prevention of food contamination. They exhibited a low practice rate regarding removing nail polish and covering the head while preparing food. Statistically significant differences were found when comparing the educational level with washing the utensils with a detergent, along with wearing gloves while working. It was concluded that the hospitals food handlers in ElManagil expressed moderate level of knowledge, positive attitude, and good practice in food hygiene. Generally, there is an urgent need to improve on food hygiene in health care systems in Sudan. Food hygiene training programs should be implemented among health care food handlers.**

**Key words:** Attitude, Elmanagil City, food handlers, food hygiene, hospitals, knowledge, practice.

## INTRODUCTION

Food-borne diseases (FBDs) have been reported by the World Health Organization to be one of the main causes of morbidity and mortality in developing countries (WHO, 2015).Factors such as unhygienic practices, insufficient sanitation, inadequate cooking, improper storage, cross-contamination and purchasing food from unsafe sources can lead to food poisoning. Improper food handling practices are the most common factor associated with FBD outbreaks (Nyachuba, 2010; Koopmans and Duizer, 2004; Al Suwaidi et al., 2015). In African countries, FBDs have a negative health impact in hospitals. In Sudan, there is a considerable public health burden due to FBDs. Many different pathogens and toxins are known to cause FBDs, with newer ones occasionally emerging, especially among restaurant workers. Pathogenic organisms are carried widely among food handlers in Sudan. In one study, 30.1% of food handlers were found to be carriers of pathogenic organisms, such as *Staphylococci*, *Salmonella*, and *Shigella* in addition to other pathogens (Saeed and Hamid, 2010), hence the food handlers

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**Table 1.** Demographic characteristics of participants.

| Characteristics | Number | (%) |
|---|---|---|
| **Gender** | | |
| Male | 84 | 84 |
| Female | 16 | 16 |
| **Age (years)** | | |
| < 21 | 25 | 25 |
| 21-30 | 32 | 32 |
| 31-40 | 22 | 22 |
| 41-50 | 11 | 11 |
| > 50 | 10 | 10 |
| **Marital status** | | |
| Single | 56 | 56 |
| Married | 44 | 44 |
| **Education** | | |
| Not educated | 1 | 1 |
| Primary | 68 | 68 |
| Secondary | 10 | 10 |
| High education | 21 | 21 |
| **Length of servicing (years)** | | |
| <1 | 26 | 26 |
| 1-5 | 51 | 51 |
| 6-10 | 13 | 13 |
| >10 | 10 | 10 |

may play a key role in the spread and transmission of food communicable diseases. FBDs occur for many reasons, but most cases are related to the health status of the food handler and his/her personal hygiene, knowledge, attitude and practices (Mudey et al., 2010). A food handler is anyone who handles food, whether preparing or serving it (Isara and Isah, 2009). Personal hygiene, such as hand washing, wearing gloves and masks, covering the head and sanitizing/cleaning surfaces, could be a preventive procedure to reduce FBDs in hospitals. Food handlers could also play a significant role by implementing food hygiene measures to ensure food safety (Mudey et al., 2010). In hospitals, medical staffs as well as patients and their relatives consume food from the hospital kitchen, so there should be some level of awareness regarding the health status of food handlers in hospitals (Aycicek et al., 2004). Generally, Food handlers in the hospitals of Sudan, do not have sufficient food-hygiene-related awareness or knowledge regarding the implementation of food safety measures (Honua, 2018). Implementing food safety measures can reduce the risk of FBD occurrences among hospital staffs, patients and even people who eat at hospitals. Therefore, the aim of this study is to assess food hygiene knowledge, attitude and practices among food handlers in ElManagil city, Sudan.

## MATERIALS AND METHODS

A cross-sectional study was performed from July 2016 to July 2018 in ElManagil City, Sudan, to evaluate the knowledge, attitude and practices related to food hygiene among hospital food handlers. ElManagil is a city in Al Jazirah State, Sudan, where the agricultural sector is the main practice of the population. The target population included food handlers in hospital kitchens and cafeterias at ElManagil Teaching Hospital. The distribution of the socio-demographic data for the hospital food handlers at ElManagil included gender, age, marital status, educational level and work experience. A questionnaire was used to gather data on the demographic variables and food-hygiene-related knowledge (22 items), attitude (16 items) and practices (10 items) of the participants. Consent was obtained from all subjects under study, and approval was taken from the National Ethics Committee of the Ministry of Health of Sudan. IBM SPSS Statistics was used for Windows (version 21; IBM Corp., Armonk, NY, USA) for data entry and analysis. Categorical data are presented as numbers and percentages. Chi-squared test also was used to study the associations between the variables of higher levels in knowledge, attitude and practices and the educational level of the food handlers. *P*-value of < 0.05 was considered as statistically significant.

## RESULTS

Table 1 shows the distribution of the socio-demographic data for 100 food handlers at ElManagil Teaching

**Table 2.** Level of food hygiene knowledge of hospital food-handlers in ElManagil city, Sudan.

| Knowledge | Correct (%) | Incorrect (%) | Do not know (%) | Knowledge × education *P*-value |
|---|---|---|---|---|
| The correct storing temperature for foods is 2-5°C. | 70 | 25 | 5 | 0.425 |
| Equipment location allows easy cleaning. | 60 | 10 | 30 | 0.536 |
| Washing utensils with detergent makes them free of bacteria. | 80 | 17 | 3 | 0.000 |
| Separate room for kitchen employees prevents food contamination. | 63 | 12 | 25 | 0.111 |
| Eating and drinking at the work place increase the risk of food contamination | 71 | 15 | 14 | 0.245 |
| Handwashing sink(s) equipped with soap and disposable paper towels will prevent food contamination. | 77 | 20 | 3 | 0.943 |
| Washing hands before work reduces the risk of food contamination. | 88 | 7 | 5 | 0.827 |
| Typhoid fever is a disease caused by contaminated food. | 66 | 15 | 19 | 0.470 |
| Providing storage area for cleaning equipment will prevent food contamination (50%). | 50 | 15 | 35 | 0.491 |
| Using gloves reduces the risk of food contamination. | 90 | 7 | 3 | 0.022 |
| Food items can be obtained from only trusted source. | 73 | 7 | 20 | 0.001 |
| Food Contaminated can be discovered by the changes in color, odor or taste. | 82 | 12 | 6 | 0.553 |
| Adequate ventilation and exhaust systems will improve food quality. | 67 | 21 | 12 | 0.491 |
| Cleaning using detergents will reduce the risk of food-borne diseases | 82 | 5 | 13 | 0.890 |
| Water contamination causes food poisoning. | 87 | 8 | 5 | 0.039 |
| Unwashed vegetables are risk of food-borne diseases. | 86 | 4 | 10 | 0.873 |
| Salmonella is pathogens transmitted through food contamination. | 56 | 14 | 30 | 0.647 |
| Hepatitis A virus is pathogen transmitted through food contamination. | 49 | 10 | 41 | 0.812 |
| Toilets contamination can effect on food quality. | 77 | 13 | 10 | .408 |
| Normal skin and nose of healthy persons are source of food pathogens. | 71 | 24 | 5 | 0.757 |
| The health status of food handlers should be checked periodically. | 54 | 35 | 11 | 0.383 |
| When Injured or infected, the worker to should take leave from work. | 43 | 27 | 30 | 0.786 |
| Mean | 70.1% | 14.7% | 15.2% | |

Hospital as follows; among the study population, 84 (84%) were males and 16 (16%) were females. Our results show that, most of the participants (32 participants, 32%) were in the 21–30 years' age group, followed by the <21 years' age group (25 participants, 25%) and 31-40 years' age group (22 participants, 22%). Also, among the study population, 56 (56%) were married and 44 (44%) were unmarried. Primary education constituted the most frequent educational category investigated (68 participants, 68%), followed by higher education (21 participants, 21%).The most frequent category of servicing years for the food handlers was 1–5 years (51 participants, 51%), followed by less than one year (26 participants, 26%), whereas the least frequent category was more than 10 years (10 participants, 10%), followed by 6-10 years (13 participants, 13%) (Table 1). The food handlers in the study population expressed a moderate level of knowledge, with a mean of 70.1%. Among the study population, according to Table 2, the level of knowledge of the food handlers was high regarding the notion that wearing gloves reduces the risk of food contamination (90.0%), followed by the notions that washing hands before work reduces the risk of food contamination (88%) and that water contamination causes food contamination (87%). Also the knowledge of the food handlers was poor in terms of four food contamination scenarios: (i) when a worker gets injured or infected, he/she should take a leave from work (43%); (ii) hepatitis A virus is a pathogen transmitted through food contamination (49%); (iii) providing a storage area for cleaning equipment prevents food contamination (50%); and (iv) the health status of food handlers should be checked periodically (54%).Differences between the educational levels and knowledge variables (e.g., washing the utensils with a detergent makes them free of bacteria; wearing gloves reduces the risk of food contamination; food should be obtained only from a trusted source) were found to be statistically significant ($p < 0.05$) (Table 2). All food handlers in our study expressed positive attitude with a mean of 63.8%. Among the study population, as shown in Table 3, the level of attitude among food handlers toward food contamination was high regarding the notion that wearing a mask prevents contamination (87%), followed by the notions that wearing gloves prevents contamination (86%), that food contamination is caused by pathogenic bacteria (84%) and that food handlers could be a source of food-borne outbreaks (82%). Our results show that, the attitude of the food handlers was poor regarding the following food contamination scenarios: the health status

**Table 3.** Level of food hygiene attitude of hospital food-handlers in ElManagil city, Sudan.

| Attitude | Agree (%) | Disagree (%) | Do not know (%) | Attitude × Education (Pearson chi test) p-value |
|---|---|---|---|---|
| Well-cooking of foods prevents contamination. | 73 | 15 | 12 | 0.689 |
| Contaminated food is due to presence of pathogenic bacteria. | 84 | 11 | 5 | 0.545 |
| *S. aureus* is one of pathogenic bacteria causing food-borne diseases. | 50 | 12 | 38 | 0.117 |
| Continuous Hand washing can prevent food-borne diseases (74%). | 74 | 11 | 15 | 0.464 |
| Wearing masks prevents contamination (87%) | 87 | 8 | 5 | 0.892 |
| Raw foods should be stored separately from cooked food. | 53 | 7 | 40 | 0.108 |
| Wearing gloves prevents contamination. | 86 | 9 | 5 | 0.774 |
| The health status of workers should be continuously evaluated. | 39 | 20 | 41 | 0.370 |
| Checking the temperature of refrigerators prevents contamination. | 54 | 17 | 29 | 0.803 |
| Wearing caps is an important practice to prevent contamination. | 62 | 25 | 13 | 0.857 |
| The best way of thawing the frozen meat, is using cold water. | 47 | 23 | 30 | 0.300 |
| Knives and cutting boards could be a source for cross contamination. | 63 | 21 | 16 | 0.568 |
| Dish towels can be a source of food contamination. | 72 | 10 | 18 | 0.270 |
| Food handlers can be a source of foodborne outbreaks. | 82 | 5 | 13 | 0.001 |
| Fingernails could be a source of foodborne pathogens. | 50 | 25 | 25 | 0.137 |
| Training courses is important to prevent contamination. | 45 | 15 | 40 | 0.910 |
| Mean | 63.8% | 14.6% | 21.6% | |

**Table 4.** Level of food hygiene practices of hospital food-handlers in ElManagil city, Sudan.

| Practice | Yes (%) | No (%) | Practice × Education (Pearson chi test) p-value |
|---|---|---|---|
| Do you cover your head during food preparation? | 55 | 45 | 0.459 |
| Do you wash your hands properly during your work? | 85 | 15 | 0.429 |
| Do you wear gloves while working? | 86 | 14 | 0.822 |
| Do you use a mask during your work? | 75 | 25 | 0.483 |
| You do not eat, drink, and smoke at your work place? | 80 | 20 | 0.257 |
| Do you remove fingernails paint while working? | 25 | 75 | 0.905 |
| Do you properly clean the surfaces at your work place? | 83 | 17 | 0.428 |
| Do you use the detergents while washing the surfaces at your work place? | 85 | 15 | 0.000 |
| Do you properly wash vegetables and fruits? | 95 | 5 | 0.000 |
| Do you check the expiry date of foods before receiving? | 75 | 25 | 0.876 |
| Mean | 74.4% | 25.6% | |

of all workers should be continuously evaluated (39%); training courses are important to prevent contamination (45%); the best way to thaw frozen meat is to use cold water (47%); fingernails are a potential source of food-borne pathogens (50%); and *Staphylococcus aureus* remain one of the pathogenic bacteria that cause food-borne diseases (50%). The difference between the educational level and the notion that food handlers could be a source of food-borne outbreaks was statistically significant ($p < 0.05$) (Table 3). All food handlers at hospitals in ElManagil expressed good practice in food hygiene, with a mean of 74.4%. Table 4 presents the responses of the food handlers to the practice questions on food contamination. The highest-ranked practice among the respondents was the practice of properly washing vegetables and fruits (95%), followed by the practices of wearing gloves while working (86%); using detergents while washing any surface at the workplace (85%); washing hands properly during work (85%); properly cleaning the surfaces at the workplace (83%) and eating, drinking and smoking at the workplace (80%). In addition, the lowest-ranked food contamination

practice among the food handlers was the practice of removing nail polish while working (25%), followed by the practice of covering the head while preparing food (54%).Differences between the educational level and practice variables(e.g. using detergents while working, washing the surfaces and washing vegetables and fruits)were statistically significant ($p < 0.05$), as shown in Table 4.

## DISCUSSION

Food handlers are regarded as the main source of food contamination, either through manual contact or through respiratory secretions. In particular, food handlers in hospitals may contaminate food and, hence, cause outbreaks of food-borne infections in hospitals(Maguire et al., 2000). The results indicated that the majority of the study subjects were men, which is in contrast to those of many studies that reported a higher proportion of females (Isara and Isah, 2009; Baluka et al., 2015). Regarding age distribution, results from similar studies carried out in India indicated that most food handlers ages were from the15–30 years' age group (Prabhu and Shah, 2014).In total,67.5% of the subjects had an educational level of primary school; thus, the performance of employees regarding food safety might not be satisfactory and, thus, may cause public health problems(Webb and Morancie, 2015). A larger number of participants (51%) had one to five years of experience working in foodservices, followed by participants with less than one year of experience(26 participants, 26%), which is similar to findings from Turkey (Bas et al., 2006). Having good knowledge of food-handling practices and hospital-related infections will definitely improve the experience of workers at hospital kitchens. The food handlers in the study population showed a moderate level of food hygiene knowledge. Similar to our results, it has been found in one study that food handlers had good knowledge regarding personal hygiene and the relationship of food-borne diseases (Hassan and Dimassi, 2014). In general, food handlers had sufficient knowledge regarding the purpose of wearing loves and washing hands and the effect of water contamination, but they lacked knowledge regarding the transmission of hepatitis A virus and whether injured or infected food handlers should work. Similar studies showed that food handlers have poor knowledge regarding many food-hygiene-related issues (Allam et al., 2016; Akabanda et al., 2017).It is important that food handlers at hospitals be knowledgeable about food safety, food hygiene and infection control to make sure that the food is prepared and handled safely to the patients (Mudey et al., 2010; Lestantyo et al., 2017). In our study, food handlers expressed positive attitude towards food hygiene. Similar studies showed that all participants agree that food handlers should be regularly medically examined (Iwu et al., 2017; Bamidele et al., 2015). It is very important that food safety training programs be implemented for food handlers working in the healthcare sector in Sudan, because education and training in hygienic practices may reduce the morbidity and mortality associated with FBDs in hospitals (Ansari and Khan, 2012). Statistically significant associations were found between the educational level and the attitude regarding the notion that food handlers are a potential source of food-borne outbreaks. In Dessie town, Ethiopia, Adane et al. (2018) reported a significant association between the availability of service training and medical check-up with the levels of food hygiene and safety practices. The attitude regarding thawing frozen meat using cold water was found to be poorly rated among the studied food handlers. Re-freezing food causes bacteria to multiply and could result in outbreaks of food-borne illnesses (Julie, 2012). The food handlers in the study population showed good food-hygiene-related practices. Similarly, it has been shown in a study (Park et al., 2010)that food handlers practiced cleaning and disinfection procedures properly. Other studies reported that some food handlers would even keep working even when they get sick (Mudey et al., 2010; Lestantyo et al., 2017). Statistically significant associations were found between the educational level and the practices of using detergents to clean surfaces while working as well as washing surfaces and washing vegetables and fruits before consumption. It can, hence, be concluded that food handlers at hospitals in ElManagil city expressed a moderate level of knowledge, positive attitude and good practices regarding food hygiene. There is an urgent need to improve the level of food safety in the healthcare system across Sudan. It is also important to implement food safety training programs for food handlers working in the healthcare sector to improve their knowledge, attitude and practices.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 14(3), pp. 112-118, April, 2020
DOI: 10.5897/AJMR2019.9064
Article Number: 66AC50263471
ISSN: 1996-0808

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# Physicochemical and microbiological evaluation of water from western part of the Rio Grande does Norte, Brazil

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Received 30 January, 2019; Accepted 6 March, 2020

**The water quality evaluation with reference to parameters of drinkability is made through a series of analyses, which are physicochemical or microbiological. This study aims to evaluate the physicochemical and microbiological parameters of drinking water distribution network in the western part of the Rio Grande do Norte, Brazil. Water samples were collected in several neighborhoods, and divided into 5 zones. In each of those five points two samples were collected: Water receiver of water distribution network and household water collector. In relation to the physical parameters, color and turbidity recorded higher results in the collectors of homes, and identified the lack of maintenance. For the studied microbial tests, the presence of coliform group bacteria was verified, being the major contamination in the receivers. This was justified by the lack of maintenance and prohibition of sealing of these collectors, allowing the access of rodents, insects and other carriers of microorganisms. Therefore, the water consumed at the collection points in the western zone of the RN / Brazil presents adequate physicochemical and microbiological indices in disagreement with the legislation that regulates water portability**.

**Key words:** Water quality, coliforms, microorganisms, portability, water supply.

## INTRODUCTION

Water quality of public supplies, through chemical, physical and microbiological parameters, is an issue monitored by the government through regulatory agencies or consumers (Al-Mudhaf et al., 2009). There are so many reasons for that quality being compromised, such as some chemicals substances persistently remain stable to the conventional water treatment processes or simply by their cumulative effect (Inyang and Dickenson, 2015).

The composition of some chemical products used in the water treatment or its components which are naturally present in the water and their potential adverse health effects associated with long-term ingestion have been studied by Choi et al. (2013); this drives to the importance of policies that regulate issues surrounding water portability parameters for human consumption.

Ordinance N° 2914 of 12 December 2011 of the Ministry of Health (Brazil, 2011) currently holds in Brazil and provides procedures for controlling and monitoring water quality for human consumption and its portability

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standards. This legislation rules establish actions of supplier agencies involved in the water treatment processes and control to provide potable water to the population respecting the proper hygienic and sanitary quality standards, through physical, chemical and microbiological indicators (Morais et al., 2016).

In the assessment of water quality, a set of parameters must be considered as well as their maximum permissible values (MPV) in accordance with Brazilian legislation (Silva et al., 2014). Among these parameters, the physicochemical has fundamental importance because these characteristics can influence the water taste, color and odor, and produce harmful physiological effects (Blank and Vieira, 2014). According to the studies that portray the water quality for human consumption, the main physical and chemical parameters evaluated are pH, hardness, chloride, nitrate, alkalinity, color and turbidity, with standards established by Ordinance 2.914 / 2011 (Sousa et al., 2016; Lima et al., 2016; Fernandes and Scalize, 2015).

Beyond the physicochemical parameters, potable water must be free of pathogenic microorganisms and indicator bacteria which determine contamination, highlighting the coliforms group, total coliforms (TC), thermotolerant coliforms (TTC) and *Escherichia coli* (Fatemeh et al., 2014). These bacteria may be responsible mainly for gastroenteritis which remains a huge concern for public health in developing countries and regions with small financial resources and poor hygiene conditions (Cabral, 2010).

According to Tong et al. (2016), about 88% of gastroenteritis cases in the world are attributed to contaminated water containing these bacteria, which are common in cases of inadequate sanitation or insufficient hygiene. Thus, it is important to develop research into the environmental monitoring and diagnosis, in order to strengthen the scientific basis in the area, thereby allowing the adoption of control measures and management of pollution sources (Oliveira et al., 2009).

Therefore, there is a need for verification on the quality of potable water delivered by the supplier agency to customers of the west RN/Brazil under the following optics: (A) water receiver of water distribution network in the households and (B) household water collector.

## MATERIALS AND METHODS

### Samples and location of sampling

Water samples were collected in several neighborhoods near the municipality of Mossoró / RN, divided into 5 zones (North, South, East, West and Central). In each of those five points were collected two samples – (A) water receiver of water distribution network and (B) household water collector. Samples were collected following the sample storage guidance and preservation, considering the parameter to be analyzed according to Funasa (2006). Ten samples were collected in each recipient at 15 day intervals, in the months of July to November 2016, totaling 100 samples.

### Analyses

Samples were subjected to analysis according to the Standard Methods for the Examination of Water and Wastewater (Eaton et al., 2012). They were processed at the Veterinary Microbiology Laboratory and at Petroleum Engineering Laboratory; both belong to the Federal Rural University of the Semiarid. The following analyses were carried out:

#### *pH*

Using the potentiometer, the electrometric pH measurement principle is the determination of the ionic activity of hydrogen using the standard hydrogen electrode, which consists of a platinum rod on which the hydrogen gas flows at a pressure of 101 kPa.

#### *Hardness*

This is done through the titration process by complexation, using a standard solution of EDTA (ethylenediaminetetraacetic acid).

#### *Nitrate*

This is done by the use of phenol disulfonic acid method, with spectrophotometer reading.

#### *Chlorides*

This was done with titration with Silver Nitrate, where the standard solution of silver nitrate was placed in a 50 mL burette and the titration was carried out until the colour changed from white to red. This procedure was repeated twice and compared with the with test (distilled water).

#### *Alkalinity*

Using titration with sulfuric acid, which is determined by potentiometry is done by titration with standard solution of $H_2SO_4$;

#### *Colour*

The colour determination is made by visually comparing the sample with a coloured glass disk, suitably calibrated with standard solutions of different concentrations of ($K_2PtCl_6$).

#### *Turbidity*

This is determined by the nephelometric method, using a Turbidímetro.

Microorganisms (total coliforms, thermotolerant coliforms and *Escherichia coli*): the most probable number (MPN) technique also known as multi-tube method was used. 25 mL of sample was prepared aseptically and three successive dilutions ($10^1$, $10^2$ and $10^3$) were prepared and for each dilution three tubes containing 10 mL of Sodium Lauryl Sulfate Broth (LST) were used with inverted Durhan tubes, which were subsequently incubated at 35 to 37°C for 24 h. The positivity of the test was observed by the production of gas inside the tubes of Durhan.

### Statistics

The data on physicochemical analyses were performed

**Table 1.** Chemical parameters, pH, hardness, chloride, nitrate, alkalinity of collected water in the areas north, south, east, west and center of the city of Mossoro followed by data extracted from the statistical tests.

| Parameter | pH | | | Hardness | | | Chloride | | | Nitrate | | | Alkalinity | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Score/zone** | **A** | **B** | **X** | **A** | **B** | **X** | **A** | **B** | **X** | **A** | **B** | **X** | **A** | **B** | **X** |
| North | 7.9 | 7.9 | 40.2$^{bc}$ | 112.0 | 118.6 | 63.4$^{b}$ | 48.9 | 51.3 | 30.9$^{bc}$ | 1.4 | 1.0 | 45.5$^{b}$ | 91.0 | 91.2 | 53.1$^{a}$ |
| SD | 0.3 | 0.1 | | 14.6 | 23.0 | | 6.1 | 6.7 | | 0.3 | 0.5 | | 27.0 | 25.7 | |
| South | 8.1 | 7.9 | 53.0$^{ab}$ | 89.2 | 98.4 | 43.8$^{c}$ | 60.3 | 61.6 | 74.1$^{a}$ | 1.9 | 1.6 | 65.9$^{a}$ | 96.2 | 87.0 | 53.5$^{a}$ |
| DP | 0.2 | 0.2 | | 19.3 | 10.2 | | 3.5 | 3.7 | | 0.7 | 0.5 | | 27.9 | 22.8 | |
| East | 8.0 | 8.2 | 65.5$^{a}$ | 29.3 | 34.6 | 10.5$^{d}$ | 52.3 | 54.2 | 41.8$^{b}$ | 2.1 | 1.5 | 65.5$^{a}$ | 94.6 | 89.0 | 49.5$^{a}$ |
| DP | 0.3 | 0.1 | | 13.4 | 8.1 | | 3.5 | 4.1 | | 0.8 | 0.6 | | 24.7 | 26.1 | |
| West | 7.8 | 7.7 | 31.6$^{c}$ | 172.8 | 218.8 | 89.1$^{a}$ | 49.6 | 49.5 | 26.6$^{c}$ | 1.0 | 1.1 | 37.7$^{b}$ | 87.1 | 88.4 | 40.0$^{a}$ |
| DP | 0.3 | 0.2 | | 46.0 | 24.1 | | 9.2 | 4.3 | | 0.4 | 0.2 | | 37.5 | 34.1 | |
| Central | 8.0 | 8.0 | 62.0$^{a}$ | 88.3 | 94.6 | 42.6$^{c}$ | 62.6 | 62.9 | 78.9$^{a}$ | 1.3 | 1.0 | 37.7$^{b}$ | 96.1 | 90.5 | 56.2$^{a}$ |
| DP | 0.1 | 0.2 | | 17.9 | 15.9 | | 6.2 | 3.4 | | 0.4 | 0.4 | | 26.4 | 25.1 | |
| W | | 664.5 | | | 285.5 | | | 442.5 | | | 829.5 | | | 710.5 | |
| P-value | | 0.798$^{ns}$ | | | 0.0007** | | | 0.136$^{ns}$ | | | 0.013* | | | 0.029* | |
| **MAV*** | | **6.0-9.5** | | **500 mg/L** | | | | **250 mg/L** | | **10.0 mg/L** | | | **NC** | | |

(A) Water receiver of the city water distribution network; and (B) water household collector. In the X columns, averages followed by the same letter do not differ among each other, at 5% probability, the Kruskal-Wallis test; $^{ns}$, *, **not significant; significant at 5% and 1% significant, respectively, by Wilcoxon test. *** Ordinance No. 2914/2011(BRAZIL, 2011); NC = not quoted; DP = Standard Deviation; MAV= maximum allowed value.

using two tests for statistical treatment. The Kruskal-Wallis test was used to compare the parameters for the Zones (North, South, East, West and Central). In the samples collected on point A and B, were used the Wilcoxon test – Paired; the hypotheses are tested about equality of means or medians of dependent populations with unknown distributions (Spiegel et al., 2013). The ActionStat v.3.0 software developed by ESTATCAMP was used for the analysis.

In statistical microbiological analyses, data were tabulated in Microsoft Excel 2016 spreadsheet application. Logarithmic transformation was used (LOG) and comparing means was proceeded using the Tukey test, with the level of 5% probability. The software used in the analysis was the SAS (Cody and Smith, 2004).

## RESULTS AND DISCUSSION

The averages of results referring to the chemical parameters pH, hardness, chlorides, nitrates and alkalinity to the North, South, East, West and Central zones are described in Table 1. The averages of the two samples through the test Kruskal Wallis are described in the column identified by the letter X, which tests the statistical difference between the zones. The p-value represents the Wilcoxon test that describes the significance between A and B.

In the Western region, lowest values for pH in both Samples A and in B in the studied households were observed. This may be associated with the nature of the mineral soil, that can be acidic naturally s by a deficiency in the origin soil basis or by processes of soil forming that leads to a removal of basic elements such as K, Ca, Mg and Na. This result differs statistically from the other zones ($p > = 0.05$); however is found to the extent required by law. There was no significant difference statistically in the pH measured between samples A and B ($p = 0.758$).

The default for pH tells that the pipes probably have no corrosion or fouling, suggesting that water used to supply at low pH values less than 4 may contribute to corrosiveness and values greater than 7 increase the possibility of fouling, highlighting the need for control (Sousa et al., 2016).

Hardness recorded in the water samples was between 29.3 and 218.8 mg/L, respectively on the East and West zones. These data are probably due to variation in the multivalent cations concentration in the water solution, either in the sample A or B, being most common calcium and magnesium ($Ca^{+2}$, $Mg^{+2}$). That can originate from nature (dissolution of limestone) or by man

**Table 2.** Physical parameters color and turbidity of the water collected in the areas north, south, east, west and center of the city of Mossoro followed by the data extracted from the statistical tests.

| Parameter | Color | | | Turbidity | | |
|---|---|---|---|---|---|---|
| **Zone/point** | **A** | **B** | **X** | **A** | **B** | **X** |
| North | 3.00 | 6.48 | 41.70[a] | 0.99 | 1.13 | 56.07[a] |
| DP | 2.78 | 9.71 | | 1.04 | 0.89 | |
| South | 5.23 | 4.73 | 50.22[a] | 1.05 | 1.98 | 63.12[a] |
| DP | 5.20 | 3.62 | | 1.10 | 1.80 | |
| East | 3.70 | 3.87 | 46.50[a] | 0.39 | 0.53 | 27.27[b] |
| DP | 2.06 | 2.10 | | 0.15 | 0.35 | |
| West | 6.59 | 9.32 | 59.62[a] | 0.78 | 1.6 | 55.17[a] |
| DP | 8.37 | 11.70 | | 0.41 | 2.64 | |
| Central | 5.06 | 4.75 | 54.45[a] | 0.99 | 0.82 | 50.85[a] |
| DP | 3.85 | 2.35 | | 1.20 | 0.51 | |
| W | | 327.0 | | | 390.0 | |
| P-value | | 0.382[ns] | | | 0.027* | |
| MAV – Legislation *** | | 15uH | | | 5uT | |

A) water receiver of the municipal water distribution network and B) household water collector in the X columns, averages followed by the same letter do not differ at the 5% probability, the Kruskal-Wallis test. [ns], *, **not significant; significant at 5% and 1% significant, respectively, by Wilcoxon test. ***Ordinance No. 2914/2011(BRAZIL, 2011); DP = Standard Deviation; MAV= maximum allowed value.

actions (disposal of industrial effluents). In supersaturation conditions, these cations react with anions in the water, forming precipitates (Brazil, 2011; Sousa et al, 2016).

The values recorded in the study can be said to be classified as not hard (29.37 to 34.69 mg/L) in the East, moderately hard (89.28 to 118.60 mg/L) in the central, South and North regions, and hard (172.82 to 218.82 mg/L) in the Western region. Despite presenting statistical difference between the areas, the observed results are in accordance with the standards of the law.

The statistical difference observed ($p < 0.007$) can be attributed to anthropic action on the receptors of residences with the accumulation of calcium and magnesium in the water pipes. However, Lima (2015) stated that high levels of hardness do not cause health disorders to population.

The result from analysis presented values between 48.9 and 62.9mg/L independently of samples origin A and B, below the limit established by law (250 mg/L) presenting statistically significant differences between zones. Chloride (chlorine in the ion form of $Cl^{-1}$) is one of the most common ions in natural waters and pollution indicator for domestic sewage. This justifies the water portability studied at the reception points and capture in this parameter, and the absence of salt taste with laxative properties (Filho et al., 2015). The amount of chlorides in the samples A and B presented no statistical significant difference ($p = 0.136$). This confirms the research by Vanuchi et al. (2014) and Moreira e Condé (2015) where they studied the presence of chlorides in the cities of Ubá / MG and Ariquemes/RO, which presented similar results to those of this research, with low levels of chloride between 18 and 20 and 19.3 and 35.4, respectively.

Nitrate ion ($NO3^-$) is a byproduct of nitrogen cycle (Stüeken et al., 2016).It is the inorganic contaminant of concern in groundwater which may be from sewage and fertilizer application (Baird and Cann, 2011). The results found in the quantification of nitrate presented a statistical difference, being equal in East and South zones with the averages observed around 1.8mg/L.

The alkalinity results were between 87.1 and 96.2mg / L in zones studied showing that the analyzed water had no major changes by organic matter decomposition processes, effluent discharges or by metabolism of microorganisms through their respiratory activity and release of carbon dioxide.

Although the Brazilian legislation does not mention a maximum permissible value for the parameter, Libânio (2010) describes that the alkalinity of natural waters in the country is less than 100 mg/L of calcium carbonate ($CaCO_3$). This study presented no statistical difference among the regions; however the samples A and B expressed statistically different results, probably due to the presence of $CaCO_3$ originating from rock formations.

The physical parameters shown in Table 2 of the colour of the water supply of the analyzed areas were observed standard values, where the maximum value found was verified in the West region. The result was not statistically different ($p \leq 0.05$) between the zones and between samples A and B studied.

Turbidity is related to the presence of suspended solids

**Table 3.** Values average for microbiological water parameters CT, TTC and *Escherichia coli* collected in the areas North, South, East, West and Center of the city of Mossoro followed by the extracted data in the statistical tests.

| Zone | CT | Parameter X (Averages) | |
|---|---|---|---|
| | | TTC | *E. coli* |
| North | 35.55[a] | 13.87[a] | 2.0[a] |
| South | 75.19[a] | 15.16[a] | 0.9[a] |
| East | 274.19[a] | 15.20[a] | 2.9[a] |
| West | 60.04[a] | 24.64[a] | 2.75[a] |
| Central | 224.89[a] | 6.96[a] | 1.8[a] |
| **POINT** | | | |
| A | 130.41[b] | 15.24[a] | 2.35[a] |
| B | 137.54[a] | 15.09[a] | 2.370[a] |
| VMP – Legislation* | | Absence in 100 mL | |

The measure unit is the Most Probable Number in 100ml of sample (MPN / 100 ml). Averages followed by same letter do not differ in the column, 5% probability by Tukey test. *Ordinance No. 2914/2011(Brazil, 2011).

in the water, which act to reduce the transparency (Paludo, 2010). High turbidity values highlight the undesirable appearance of water and suspended solids can provide shelter for pathogenic microorganisms (Perpétuo, 2014).

The results for the analyzed samples presented a maximum value of 5uT. The East zone presented results statistically difference for the other zones. Statistical difference was found (p = 0.027) in the samples A and B. This can be attributed to the possible presence of solids waste in household receivers of South Zone, which presented the highest rates. The highest value was found in the average of the samples B South Zone, and the lowest averages in sample A of the East Zone. Values below the maximum permissible values were common in the analysis and the highest value observed can be explained by the lack of maintenance of the water with the presence of solid waste. Some researchers analyzed water intended for human consumption and the result observed for turbidity was similar to this research. Vitó et al. (2016) analyzed possible contamination in artesian well water in the state of Rio de Janeiro and obtained average results of 0,77uT as compared to the 5uT obtained in the research.

In relation to the microbiological analysis of total coliforms, thermotolerant coliforms and *Escherichia coli*, there were presence of total coliforms and thermotolerant coliforms in the study areas except *Escherichia coli* in the south. The highest average value of total coliforms was recorded in the East Zone (274.19 NMP) and Central Zone (224.89 NMP), followed by South Zone (75.19 NMP), West Zone (NMP 60.04) and North zone (35.55 NMP) (Table 3).

The water coliform contamination is important to specify the possibility of pathogenic microorganisms that can transmit waterborne diseases (Moura et al., 2009). The highest value of thermotolerant coliforms was positive in the West region (24.64 NMP), followed by East region (15.20 NMP). The lowest average value was found in the central region (6.96 NMP). The presence of thermotolerant coliforms indicates the possibility of fecal contamination and enteric pathogenic microorganisms (Silva et al., 2016).

The abundance of *Escherichia coli* was found in the East region (2.9 NMP), West region (2.75 NMP), North region (2.0 NMP) and the central region (1.8 NMP). The presence of *E. coli* is indicative of contamination that directly compromises the portability of water. The result of TC, TTC, and *E. coli* parameters does not differ statistically between the studied regions. Geldreich (1998) states that the runoff water is the main factor that causes changes in the microbiological quality of groundwater. This is because the water on contact with the ground carries organic matter, animal waste and particulate matter in large quantities to the well; this region still presents exposed cesspools in public via.

The lower results of total coliforms in the North and West region at these places have recently been changed by the water distributor, added to the constant maintenance on the network, reduce occurrences of contamination during distribution, disagreeing with the information presented in the report published by Water and Sewerage Company of Rio Grande do Norte-Brazil. It was observed that the water is distributed to households with compromised sanitary quality. This factor can be explained by the location of the well, probably by water contact with cesspools due to lack of sanitation in urban and rural areas, which is one of the main causes of a

large amount of contaminated water in Brazil (Scapin et al., 2012).

When comparing the means of all samples collected at the receptors (A) and collectors (B) of the households, higher values were observed for B receptors, obtaining 137.54 MPN and 2.37 MPN, for CT and *E. coli*, respectively. The value of the TTC from point B was similar to point A presenting 15.09 NMP. The results of the collection points A and B were statistically different just for CT. Thus, higher values were found at the collectors of the households for this parameter, which indicates lack of maintenance. Regarding the TTC and *E. coli* parameters, the results of samples A and B are worrisome, since *E. coli* has as primary habitat the gastrointestinal tract of humans and other animals and is commonly responsible for urinary infections and diarrhea.

## Conclusion

It can be concluded that, in relation to the physical parameters, color and turbidity recorded higher results in the collectors of homes surveyed, identified the lack of maintenance of these. As chemical analyses of pH, chlorides and alkalinity not described large changes between the studied areas, since the hardness and nitrate analyses were higher in places where there is difficulty in sanitation or where there was a greater chance of contamination water, like sewage in the sky and garbage. For the studied microbial tests, it was verified presence of coliform group bacteria at all points in less than one of the collection, being the major contamination in the receivers. This is justified by the lack of maintenance and lack of prohibition of sealing of these collectors, allowing the access of rodents, insects and other carriers of microorganisms; and leaks in the piping that distributes water throughout the city may also be responsible for contamination.

Thus, the water consumed at collection points in the areas of the city of Mossoró presents physicochemical indices used and microbiological indices in disagreement with a legislation governing portability. So there is need to develop a supply company check critical points of the pipe mesh to minimize bacterial contamination pollution indicators.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 14(4), pp. 119-128, April, 2020
DOI: 10.5897/AJMR2020.9286
Article Number: 9106E5963529
ISSN: 1996-0808

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# Chemical composition and control of *Sclerotium rolfsii* Sacc by essential oils

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Received 12 January, 2020; Accepted 10 March 2020

**Essential oils of medicinal plants show potential to control phytopathogens. Essential oils of *Lippia sidoides* Cham, *Lippia lasiocalycina* Cham, *Lippia origanoides* Kunth - Teresina, *Lippia origanoides* Kunth - Jatobá, *Mesosphaerum suaveolens* (L.) Kuntze, *Croton sonderianus* Muell. Arg. And *Croton zehntneri* Pax et Hoffm. were evaluated regarding their chemical composition and in controlling the *Sclerotium rolfsii* Sacc. The experiments were carried out in a completely randomized design with five concentrations and the control (without oil application): 0.0313; 0.0625; 0.1250; 0.2500 and 0.500 mL $kg^{-1}$ in Petri dishes with PDA medium (potato-dextrose-agar). The evaluations consisted of daily measurements of the colony diameter in diametrically opposite directions, 24 h after the experiment installation and maintained until the radial reach of the colony on the edges of the Petri dish, in one of the treatments. The chemical composition of the essential oils were evaluated by GC-MS and the following compounds were identified: Thymol (33.5%, *L. sidoides*); piperitenone oxide (67.7%, *L. lasiocalicyna*); borneol (19.2%, *L. origanoides*); borneol carvacrol (34.4%, *L. origanoides* – Jatobá); sabiene (30.3%, *M. suaveolens*); β-sabinene (30.5%, *C. sonderianus*); and estragole (90,1%, *C. zehntneri*). The *S. rolfsii* fungus is highly sensitive to *L. sidoides, L. origanoides - Jatobá* and *C. zehntneri* essential oils, suggesting its use in the management of Sclerotium wilt in cowpea.**

**Key words:** Antifungal activity, alternative control, Sclerotium wilt, *Vigna unguiculata.*

## INTRODUCTION

The cowpea (*Vigna unguiculata* (L.)Walp.) is very important in human nutrition because it is a natural source of proteins, calories, vitamins and minerals (Freire Filho et al., 2011). However, although rustic, *V. unguiculata* is affected by some diseases, among them, the slerotium wilt. This disease is caused by the

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polyphagous and cosmopolitan fungus *Sclerotium rolfsii* Sacc, that causes high yield loss in infected plants (Bedendo, 2011).

In cowpea, the disease occurs in the stem, and present as a more representative symptom a tangle of white mycelium, which later develops into yellow tones and, under this tangle, there is an intense disruption of the parasitic plant tissue (Athayde et al., 2005). The disease can be lethal, regardless of the phenological stage of the crop, leading to the reduction of the stand, with direct effects on grain yield (Cardoso, 1994).

Among the management practices aiming the disease control, the use of resistant cultivars (Adandonon et al., 2006; Silva et al., 2014); crop rotation (Bedendo, 2011); and the use of biocontrolling microorganisms (Punja et al., 1985; Singh et al., 2002; Adandonon et al., 2006; Pacheco et al., 2016); the use of alternative products based on plant extracts and vegetable oils (Brum et al., 2014) and the use of chemical fungicides (Punja et al., 1985; Sales Júnior et al., 2005; Athayde et al., 2005; Bedendo, 2011) can be highlighted.

The lack of registered fungicides for the crop has stimulated the search for alternative products for the management of Sclerotium wilt. Substances such as essential oils, crude extracts and tinctures from plants have been studied because they have in their composition molecules with fungicidal properties (Matos, 1997). In addition to being characterized as secondary plant metabolites and of low toxicity to humans, they are extensively tested in *in vitro* and *in vivo* control of phytopathogens and seed treatment (Rodrigues et al., 2006).

The studies have reported biological properties for essential oil, rich in thymol and carvacrol, as antimicrobial, anti-inflammatory, antioxidant and larvicidal properties (Damasceno et al., 2011; Carvalho et al., 2013; Guimarães et al., 2014).

Considering the studies that report the antimicrobial activity of essential oils of plants existing in the ecosystems of the Mid-North region of Brazil, associated with the sensitivity of *S. rolfsii* to essential oils, the objective of this research was to identify the chemical composition of essential oils of native plants of the Mid-North region of Brazil, evaluating the mycelial sensitivity and its potential use in the control of *S. rolfsii*, agent of Sclerotium wilt in cowpea.

## MATERIALS AND METHODS

### Experiment management, isolation and pathogen maintenance

The experiments were carried out in the Plant Pathology Laboratory at Embrapa Mid-North, where the *S. rolfssii* fungus was isolated from cowpea plants with typical symptoms of the disease. Disinfestation was performed with 70% alcohol for 30 s and 1.5% sodium hypochlorite for 2 min. To remove excess hypochlorite, the material was immersed in two consecutive portions of distilled and sterilized water. The tissue fragments were then transferred to Petri dishes containing PDA medium (Potato, Dextrose and Agar) and subsequently incubated at 25°C for 7 days. After this period the maintenance was performed through re-pricing in Petri dishes with PDA medium.

### Extraction and analysis of the chemical composition of essential oils

The essential oils of six botanical species belonging to the Brazilian Mid-North region (Table 1) were obtained. All studied species are spontaneously occurring shrubs or sub-bushes; none of them are still cultivated, being all vegetatively propagated.

The extraction of the essential oils was carried out by the hydrodistillation method, using the Clevenger apparatus, coupled to the heating mantle used as heat source for the system (Gomes et al., 2014). In each extraction, 200g of dehydrated and previously crushed leaves were used. The inflorescences were also used for *L. origanoides*.

The gas chromatography coupled to mass spectrometry (GC-MS) was performed on a Shimadzu GC-17A / MS QP5050A apparatus with electron impact ionization at 70 and V. Mass spectra were obtained from 43 to 350 m/z. The temperature of the injector / detector and the thermal program were maintained at 240°C. The carrier gas used was helium. Identification was made by comparison with standard spectra of the internal data library and retention times based on the linear retention index. The GC-FID chromatogram was used to determine the relative concentration using the peak areas in the Agilent 5975C system, which method of analysis was similar to the GC-MS system, previously described. It was used a DB-5 capillary column (30m 0.25mm di, 0.25µm film; J & W Scientific, Folsom, CA, USA) and hydrogen was used as carrier gas.

### Evaluation of fungal sensitivity to oils

The experiment was conducted in a completely randomized design, with five concentrations and one control (without oil): 0.0313; 0.0625; 0.125; 0.2500 and; 0.5000 ml $kg^{-1}$ in PDA culture medium with four replicates, each replicate being represented by three Petri dishes with 90 mm in diameter.

The essential oil was deposited in the center of the Petri dishes on the solidified medium and evenly distributed with Drigalsky's Spatula. Immediately after the oil distribution, a 5 mm diameter disk containing fragments of the four-day old *S. rolfsii* culture grown in PDA (Veloso et al., 2012) was deposited in the center of each plate. The plates were then sealed with plastic film and incubated in BOD at 25°C and photoperiod of 12h. The control group received the disc with fungal structures. The evaluation consisted in the daily measurement of the diameter of the colonies in diametrically opposite directions, with the aid of a millimeter rule, obtaining an average for each repetition. Evaluations were initiated 24 h after the experiment set up and maintained until the radial growth of the colony reached, in one of the treatments, the total diameter of the Petri dish. With the obtained data, the growth rate of the fungus was determined in the different concentrations tested, using the formula adapted from Lilly and Barnet (1951). $Tx = (Cn + 1 - Cn) / T$, where: Tx = growth rate, Cn = growth at incubation time "n", Cn + 1 = growth at incubation time n + 1 and T = time interval considered, in this case, 24 h.

The mycelial growth values were transformed into percentage of inhibition, and the inhibitory concentrations of 50% of the mycelial growth (IC 50) were obtained from the logarithmic regressions for each oil / species. For that, the statistical software Costat (Tonin et al., 2013) was used. From the IC 50 data it was possible to classify the sensitivity of the fungus to the oils in: highly sensitive (HS) when $IC_{50}$<1mg $L^{-1}$; (1 mL $kg^{-1}$ ≤ IC50 ≤ 40 $mlkg^{-1}$) and non-sensitive

**Table 1.** Species, origin, altitude, registration numbers and part of the plant used to obtain the tested essential oils. Teresina, PI, 2018.

| Specie | Origin | Altitude (m) | Register | Part of the plant |
|---|---|---|---|---|
| *Lippia sidoides* | Teresina | 72 | CEN 92438 | Leaf |
| *Lippia lasiocalycina* | São João do Piauí | 228 | CEN 92437 | Leaf |
| *Lippia origanoides* | Gilbués | 481 | CEN 92436 | Leaf and inflorescence |
| *Lippia origanoides*- Jatobá | Jatobá do Piauí | 240 | CESJ 70120 | Leaf |
| *Mesosphaerum suaveolens* | Parnaíba | 5 | IPA 57264 | Leaf |
| *Croton sonderianus* | Parnaíba | 5 | CEN 92500 | Leaf |
| *Croton zehntneri* | Valença | 208 | TEPB 30944 | Leaf |

(NS) (IC50 ≥ 40 ml $kg^{-1}$), defining the fungitoxicity (Edgington et al., 1971; Tonin et al., 2013).The data obtained for mycelial growth of *S. rolfsii* were submitted to variance analysis and polynomial regression to define the model that best explains the behavior of the studied variable, with the aid of the Assistat software, version 7.7 beta (Assis and Silva, 2013).

## RESULTS AND DISCUSSION

The chemical composition of essential oils obtained by gas chromatography coupled with mass spectrometry (CG-EM) identified 73 compounds in all evaluated oils. For some species, it was not possible to identify all the constituents present in the essential oil. The results of this study revealed that only the oils of *L. origanoides* - Jatobá and *C. zehntneri*, had 100% of their constituents identified. The major compounds identified were thymol, piperitenone oxide, borneol, carvacrol, sabinene, β-sabinene and estragol, for *L. sidoides*, *L. lasiocalicyna*, *L. origanoides* - Teresina, *L. origanoides* - Jatobá, *H. suaveolens*, *C. sonderianus* and *C. zehntneri*, respectively. The essential oils of *L. sidoides*, *L. origanoides* and *Croton zehntneri* have demonstrated properties of epidemiological interest, with potential to control plant diseases. Such characteristics are usually associated with the presence of major compounds in their chemical composition.

As highlighted in Table 2, the main constituent of the *L.sidoides* essential oil, thymol (33.2%), is associated with its excellent level of control (Lemos et al., 1990; Gonçalves et al., 2015; Athayde et al., 2005) observed in the control of the fungus *S. rolfsii* (Figure 1). In addition, a representative example of the GC chromatogram with peak assignments is shown in Figure 2. For *L. origanoides* - Jatobá, the main constituent detected was carvacrol (54.4%), which also showed exceptional bioactivity on the mentioned fungus, the result of which is in addition to those obtained by other authors who had demonstrated bioactivity of this compound on some microorganisms (Lorenzi and Matos, 2002; Queiroz et al., 2014).

The chemical composition variability of each essential oil is of extreme importance for its effective action, since the antimicrobial activity is directly related to the synergism of its chemical composition, where compounds like alcohols, phenols, terpenes and ketones are pointed out as the main responsible ones by toxic properties (Sellamuthu et al., 2013). Terpenes (Table 3) are considered to be the secondary metabolites most produced by plants with applications in different areas, such as in the pharmaceutical and solvent industries (Wang et al., 2016). According to Sangwan et al. (2001), the genotypic characteristics associated with the environmental factors can be determinant in the biosynthesis of the secondary metabolites, including the essential oils. For example, the climatic conditions may influence the enzymatic activities of a particular plant species (Barros et al., 2009).

Regarding this species (*L. origanides* - Jatobá), no records were found in the researched literature of any effect of its essential oil on filamentous fungi - this being the first record. However, some authors have demonstrated bioactivity of this essential oil against *Staphylococcus aureus* (Queiroz et al., 2014) and *S. aureus* and *Escherichiacoli* (Sarrazin et al., 2015).

In this work, the results obtained by essential oils extracted from plants of the genus *Croton* also deserve to be highlighted,especially *C. zehntneri*, which showed excellent fungicidal action on *S. rolfsii*, similar to those revealed by *L. sidoides* and *L. origanoides* - Jatobá (Figures 3 and 4). Although there are no results in the researched literature showing the effect of essential oils extracted from *Croton* on *S. rolfsii*, there are reports that demonstrate the action of *C. conduplicatus* in the control of *Lasiodiplodia theobromae* (Peixinho et al., 2017) and *C. rhamnifolioides* acting against fungi of the genus *Candida* (Vidal et al., 2016). In view of the bioactivity demonstrated by the essential oils studied against the fungus *S. rofsii*, it proved reasonable to evaluate its efficiency individually.

The essential oil of *M. suaveolens*has sabinene (3.0%) and 1.8-cineole (12.0%) as the main constituents with different composition from those observed by other authors (Martins et al., 2006; Branquinho, 2015).The sensitivity of fungi to a particular toxic substance can be expressed, according to Tonin et al. (2013) by $ED_{50}$

**Table 2.** Retention index (RI) and chemical composition of essential oils of *Lippia sidoides* (Ls), *Lippia lasiocalycina* (Ll), *Lippia origanoides* (Lo), *Lippia origanoides* Jatobá (Lo-J), *Mesosphaerum suaveolens* (Ms), *Croton sonderianus* (Cs)and *Croton zehntneri* (Cz).

| RI[a] | RI[b] | Compound | *Ls* | *Ll* | *Lo* | *Lo-J* | *Ms* | *Cs* | *Cz* |
|---|---|---|---|---|---|---|---|---|---|
| 850 | 866 | Z-3-Hexaneol | 0.5 | 0.3 | 0.4 | - | - | - | - |
| 924 | 921 | α-Thujene | 1.1 | - | - | 1.1 | 0.9 | - | - |
| 932 | 928 | α-Pinene | 0.5 | 0.5 | 0.6 | 0.3 | 2. 8 | 0.6 | - |
| 946 | 943 | Camphene | - | - | 1.8 | - | - | - | - |
| 969 | 967 | Sabinene | - | - | 0.8 | - | 30.0 | - | 0.2 |
| 974 | 971 | Octen-3-OL | 0.7 | - | 1.1 | - | 7.0 | - | - |
| 988 | 984 | β-Myrcene | 2.2 | 0.2 | 0.5 | 2.0 | 0.2 | - | - |
| 1002 | 1001 | α-Phellandrene | | | | 0.1 | - | 1.2 | - |
| 1014 | 1011 | α-Terpinene | 1.6 | - | - | 1.7 | 0.2 | - | - |
| 1020 | 1019 | Para-Cymene | 13.1 | 0.5 | - | 13.3 | 3.3 | 0.5 | - |
| 1024 | 1023 | Limonene | 0.8 | 11.9 | 1.4 | 0.4 | 1.2 | - | - |
| 1025 | 1023 | β-Phellandrene | - | - | - | - | - | 1.3 | - |
| 1026 | 1025 | 1,8-Cineole | 2.3 | - | 6.9 | 0.2 | 11.3 | 2.9 | 2.9 |
| 1032 | 1030 | Z-Ocimene | 0.2 | 0.5 | - | - | - | - | - |
| 1044 | 1040 | E-β-Ocimene | - | 0.2 | - | - | 0.2 | - | 0.6 |
| 1054 | 1052 | γ-Terpinene | 4.3 | 0.5 | - | 7.8 | 0.3 | 0.3 | - |
| 1065 | 1060 | Z SABinene Hidrate | 0.4 | - | - | - | - | - | - |
| 1083 | 1083 | Fenchone | - | - | - | - | 1.0 | - | - |
| 1086 | 1083 | Terpinolene | - | - | 0.4 | - | - | 0.5 | - |
| 1095 | 1093 | Linalool | 0.6 | 0.4 | - | - | 0.3 | 0.7 | - |
| 1114 | 1109 | Fenchol | - | - | - | - | 0.5 | - | - |
| 1119 | 1115 | ρ-Mentha-E-2,8-Dien-1-Ol | - | 0.2 | - | - | - | - | - |
| 1133 | 1129 | Z-ρ-Mentha-2,8-Dien-1-Ol | - | 0.2 | - | - | - | - | - |
| 1140 | 1144 | E-Verbenol | - | 0.2 | - | - | - | - | - |
| 1165 | 1161 | Borneol | - | - | 19.2 | - | - | 0.4 | - |
| 1174 | 1172 | 4-Terpineol | 1.6 | - | 0.6 | 0.8 | 2.6 | 1.1 | - |
| 1186 | 1185 | α-Terpineol | 0.8 | 0.5 | 0.7 | - | 0.3 | 0.3 | 0.8 |
| 1195 | 1195 | Estragolee | | | - | - | - | - | 90.1 |
| 1232 | 1229 | Thymol Methyl Ether | 2.8 | - | - | 5.5 | - | - | - |
| 1241 | 1239 | Carvacrol, Methyl Eter | - | - | - | 0.4 | - | - | - |
| 1289 | 1290 | Thymol | 33.2 | 1.2 | - | 3.0 | - | - | - |
| 1298 | 1297 | Carvacrol | 0.6 | | - | 54.5 | - | - | - |
| 1335 | 1331 | δ-Elemene | - | - | - | - | - | 0.6 | - |
| 1339 | 1332 | E-Carvil Acetate | - | 0.2 | - | - | - | - | - |
| 1340 | 1336 | Piperitenone | - | 2.2 | - | - | - | - | - |
| 1349 | 1348 | Thymol Acetate | 0.3 | - | - | 0.5 | - | - | - |

**Table 2.**Contd.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1366 | 1366 | Piperitenone Oxide | - | 67.2 | - | - | - | - | - |
| 1374 | 1370 | α-Copaene | - | - | 0.9 | 0.6 | - | 0.4 | - |
| 1387 | 1379 | β-Bourbonene | - | - | - | - | 1.1 | 2.3 | - |
| 1388 | 1396 | Iso Jasmone | - | 0.2 | - | - | - | - | - |
| 1389 | 1386 | β-Elemene | - | - | 1.4 | - | 1.0 | 12.7 | - |
| 1410 | 1407 | α-Cedrene | - | - | 0.4 | - | - | - | - |
| 1417 | 1414 | Trans-Caryophyllene | 17.4 | - | 2.6 | 4.0 | 5.1 | 0.4 | 2.1 |
| 1419 | 1419 | β-Cedrene | - | - | 1.3 | - | - | - | - |
| 1431 | 1423 | β-Gurjinene | - | - | - | - | - | 3.8 | - |
| 1432 | 1430 | α-Bergamotene | | | | 0.3 | - | - | - |
| 1437 | 1433 | α-Guaiene | - | - | 0.4 | - | 2.2 | - | - |
| 1439 | 1433 | Aromadendrene | 1.2 | - | - | - | - | 2.3 | - |
| 1452 | 1448 | α-Humulene | 0.9 | - | 16.0 | 0.1 | 0.3 | 1.7 | - |
| 1453 | 1446 | Geranyl Acetone | - | 6.4 | - | - | - | - | - |
| 1454 | 1450 | β-Farnesene | - | 0.2 | - | - | - | - | - |
| 1458 | 1356 | Alloaromadendrene | - | - | - | - | 4.3 | 1.3 | - |
| 1464 | 1455 | α-Acoradiene | - | - | 0.6 | - | - | - | - |
| 1483 | 1471 | α-Amorphene | - | - | - | - | - | 7.2 | - |
| 1484 | 1476 | Germacrene-D | 1.1 | - | 3.9 | - | 5.9 | 2.0 | - |
| 1489 | 1481 | β-Sabineno | - | - | 2.4 | 0.8 | - | 30.5 | - |
| 1494 | 1491 | Biciclogermacrene | 2.8 | - | - | - | - | 0.4 | - |
| 1496 | 1488 | Valencene | - | - | 2.2 | 1.6 | - | - | - |
| 1498 | 1494 | α-Selinene | - | - | 1.5 | - | - | - | - |
| 1500 | 1491 | Bicyclogermacrene | - | 1.3 | - | - | 0.3 | 0.5 | 3.3 |
| 1505 | 1502 | β-Bisabolene | - | 0.5 | 0.3 | - | - | - | - |
| 1508 | 1500 | Germacrene A | - | - | 0.7 | - | - | 0.3 | - |
| 1513 | 1509 | γ-Cadinene | - | - | - | - | - | 2.1 | - |
| 1522 | 1518 | δ-Cadinene | 0.4 | - | 2.6 | 0.6 | 0.8 | 0.3 | - |
| 1539 | 1536 | α-Copaene-11-Ol | - | - | 0.5 | - | - | - | - |
| 1577 | 1573 | Spathulenol | 0.8 | 0.2 | - | 0.3 | 9.0 | 12.7 | - |
| 1578 | 1579 | Epiglobulol | | | | | | 3.4 | - |
| 1582 | 1578 | Caryophillene Oxide | 3.7 | 0.2 | 0.5 | 0.4 | 2.2 | - | - |
| 1586 | 1586 | Isospathulenol | - | 1.2 | - | - | - | - | - |
| 1592 | 1587 | Viridiflorol | - | - | - | - | 0.4 | 0.7 | - |
| 1606 | 1604 | Humulene Epoxide | - | - | 2.5 | - | - | - | - |
| 1651 | 1649 | Pogostol | - | - | - | - | 3.9 | - | - |

IR[a]: relartive retention index, calculated by the Van den dool equation. %: Compound percentage. The compounds of the table are in ascending order of column Rxi-5HT, 30 m × 0,25mm elution. IR[b]: experimental retention indexes.


**Figure 1.**(A) Cowpea plant with disease symptom; (B) *S. rolfsii* in PDA medium; (C) Visualization with optical microscope.

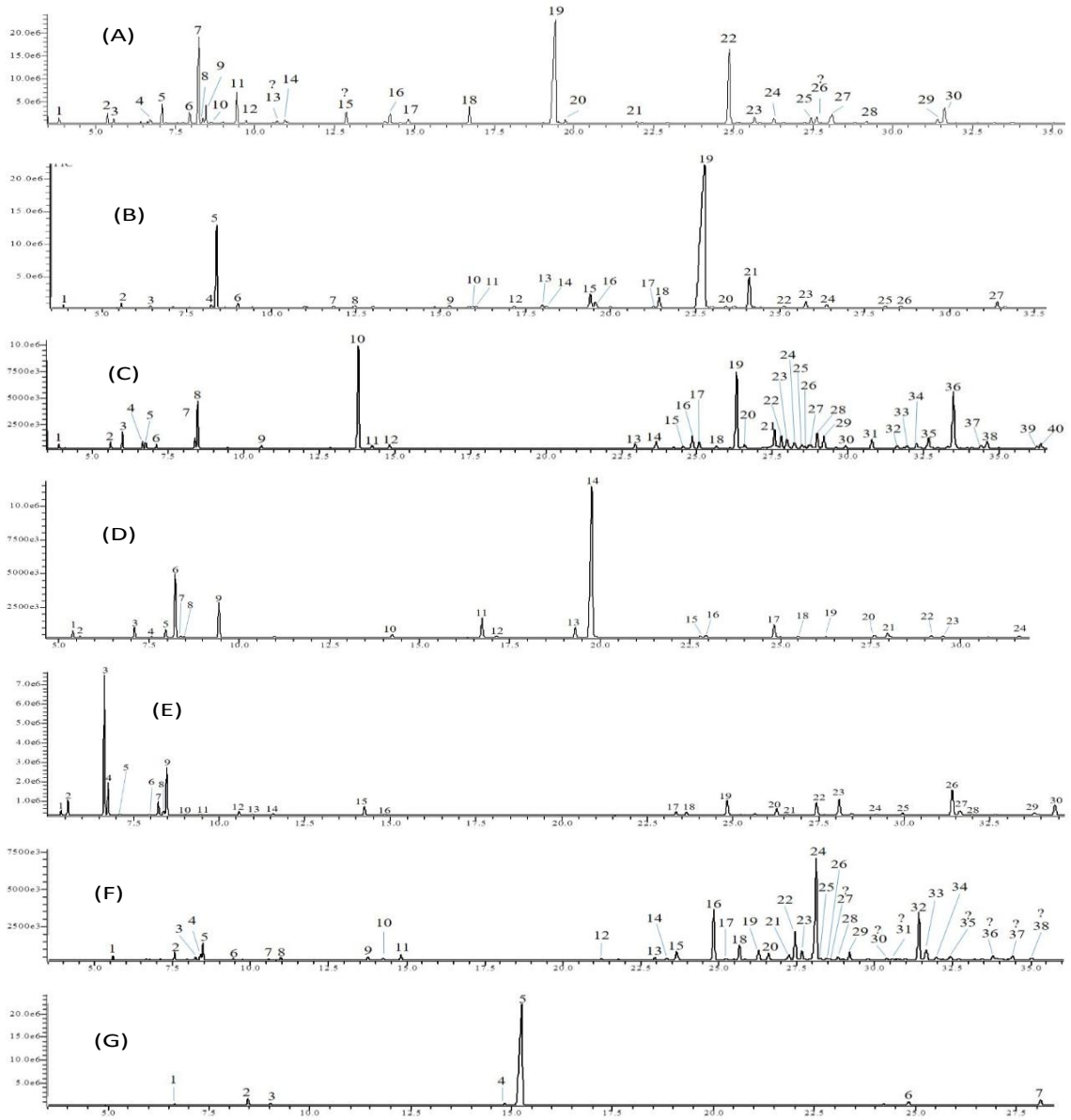


**Figure 2.** GC chromatogram of essential oils of *L. sidoides* (A), *L. lasiocalycina* (B), *L.origanoides* (C), *L.origanoides* Jatobá (D), *M. suaveolens* (E), *C. sonderianus* (F)and *C. zehntneri* (G) with peak assignments.

**Table 3**.Percentage of chemical compounds by group from essential oils of *L.sidoides* (Ls), *L. lasiocalycina* (Ll), *L. origanoides* (Lo), *L. origanoides* Jatobá (Lo-J), *M. suaveolens* (Ms), *C. sonderianus* (Cs)and *C. zehntneri* (Cz).

| Group of compounds | % | | | | | | |
|---|---|---|---|---|---|---|---|
| | ***Ls*** | ***Ll*** | ***Lo*** | ***Lo - J*** | ***Ms*** | ***Cs*** | ***Cz*** |
| Monoterpenes hydrocarbons | 57.25 | 14.52 | 4.19 | 26.52 | 46.14 | 4.36 | 0.84 |
| Oxygenated monotherpenes | 10.39 | 72.36 | 29.86 | 64.75 | 16.04 | 5.37 | 93.75 |
| Sesquiterpenes hydrocarbons | 23.94 | 2.02 | 37.24 | 8 | 20.91 | 68.7 | 5.41 |
| Oxygenated sesquiterpenes | 4.55 | 8.05 | 3.9 | 0.73 | 15.54 | 16.79 | v |
| Other | 0.48 | v | 0.4 | v | v | v | v |
| Not identified | 3.39 | 3.05 | 24.41 | v | 1.37 | 4.78 | v |

v: trace (<0.05%).

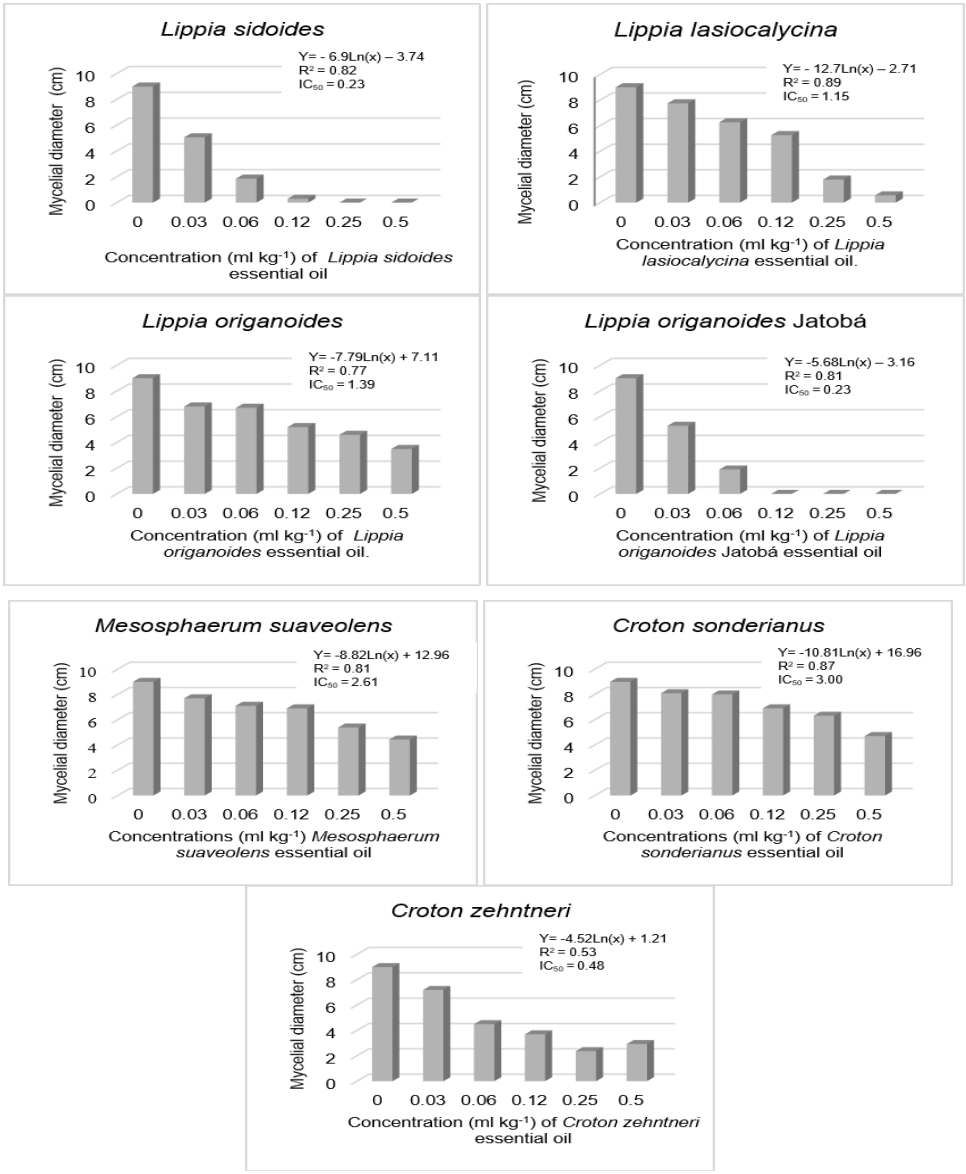


**Figure 3.***In vitro* mycelial growth of *Sclerotium rolfsii* in six concentrations (mL $kg^{-1}$) of *L. sidoides*, *L. lasiocalycina*, *L. origanoides*, *L. origanoides* Jatobá, *M. suaveolens*, *C. sonderianus* and *C zehntneri* essential oils. $IC_{50}$ = Concentration that inhibits 50% of the mycelial growth.

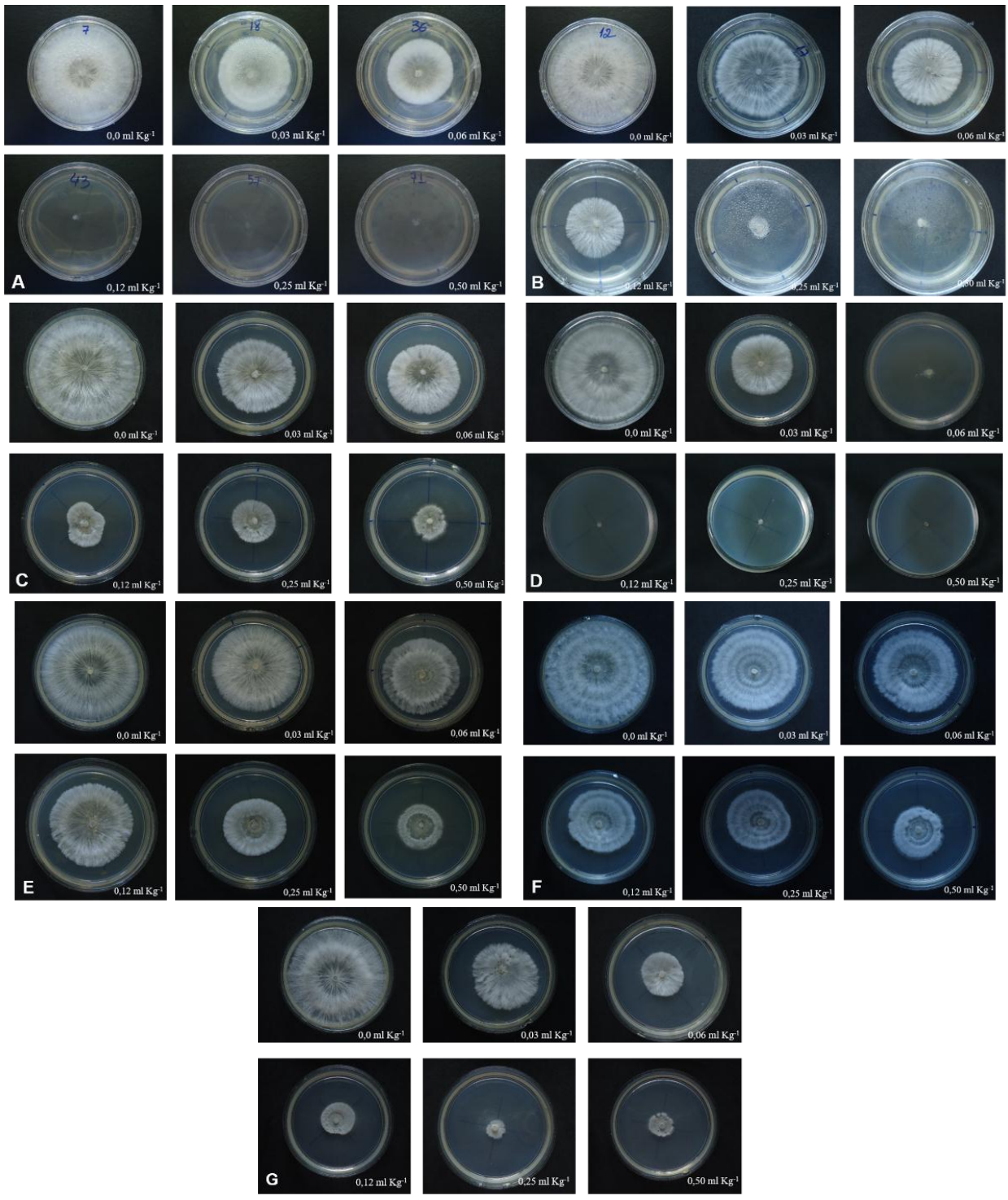


**Figure 4.** *In vitro* mycelial growth of *Sclerotium rolfsii* in six concentrations (mL $kg^{-1}$) of *L. sidoides* (A), *L. lasiocalycina* (B), *L. origanoides* (C), *L. origanoides* Jatobá (D), *M. suaveolens* (E), *C. sonderianus* (F) and *C. zehntneri* (G)essential oils.

(effective dose), $EC_{50}$ (effective concentration) or $IC_{50}$ (inhibition concentration). If a fungus is sensitive to a given substance, its fungitoxicity becomes apparent; otherwise it will be innocuous (non-toxic). On the other hand, if a substance does not present fungitoxicity the fungus is considered insensitive (Reis et al., 2007). The

oils that showed high toxicity, demonstrated by the high levels of inhibition ($IC_{50}$<1 ml $kg^{-1}$) were *L. sidoides, L. origanoides* - *Jatobá* and *C. zehntneri* (Figure 2).

The antifungal properties of the essential oils are due to their lipophilic characteristics (Bakkali et al., 2008), which occur with the presence of compounds such as monoterpene phenols, especially thymol, carvacrol and eugenol (Barrera-Necha et al., 2008). Thus, the hydrophobicity of the essential oil allows an interaction between it and the lipids of the cell membrane, interfering in its permeability and causing changes in its structure (Costa et al., 2011). Some of these alterations were confirmed by optical microscopy, where the *Syzygium aromaticum* essential oil significantly interfered in the mycelial growth of *Rhizoctonia solani, Fusarium solani* and *F. oxysporum*. In this study, several morphological alterations were observed, such as the presence of vacuoles, disorganization of cellular contents, decrease in cell wall sharpness, intense fragmentation and less turgidity of hyphae (Costa et al., 2011).

As previously shown (Figure 3), among the tested oils, those of *L. sidoides, L. origanoides Jatobá* and *C. zehntneri* deserve special mention in the control of *S. rolfsii,* for which the fungus showed high sensitivity. These three species presented yields of, respectively, 1.38; 0.4 and; 1.20%, whose results reinforce the possibility of its *in vivo* use. None of these species has been cultivated and its use has now been made by extractivism.

In this way, the great antifungal potential of the essential oils is evident. Its broad spectrum of action encourages further studies, in order to better understand the physiology of plants, especially the factors that influence the synthesis of its compounds. The next stage should focus on the development of an industrial scale production process aiming at obtaining alternative inputs based on essential oils plant diseases control.

In summary, the results show that the chemical composition of the essential oils identified the following major constituents: thymol (*Lippia sidoides*); piperitenone oxide (*Lippia lasiocalicyna*); borneol (*Lippia origanoides*); carvacrol (*Lippia origanoides* - Jatobá); sabinene (*Mesosphaerum suaveolens*); β-sabinene (*Croton sonderianus*) and estragole (*Croton zehntneri)*, being *Sclerotium rolfsii* highly sensitive to the essential oils of *Lippia sidoides, L. origanoides* - *Jatobá,* and *Croton zehntneri*.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 14(4), pp. 129-135, April, 2020
DOI: 10.5897/AJMR2020.9321
Article Number: 5379E4863532
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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# Formulation and pathogenicity of a bioherbicide for wild poinsettia control

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Received 13 March, 2020; Accepted 16 April, 2020

**Adequate formulation of bioproducts represents one of the most challenging aspects of bioproduct development. The incorporation of adjuvants with bioagents can positively influence product development. However, it is indispensable to evaluate the sensitivity of bioagents to these adjuvants. The aim of this study was to determine the toxicity of seven adjuvants at different concentrations to *Bipolaris yamadae* (*Bipolaris euphorbiae*) fungus, to select a product compatible with this phytopathogen for a wettable powder formulation and to evaluate the pathogenicity of the formulation against wild poinsettia (*Euphorbia heterophylla*). The powder fraction of the formulation was made up of 1% anti-wetting silicon dioxide mixed with *B. yamadae* conidia to a final concentration of $10^7$ conidia.ml$^{-1}$. The aqueous fraction was composed of 0.1% Geropon T36 compatibilizer, 0.075% silicone, the dispersant, 0.1% Tween 80 or tensioactive and 0.5% PVP K30 or spreading agent. The incidence of disease was observed in 83.6% of the plants inoculated with the formulated fungus, which was 79.0% higher than that in the plants inoculated with the bioagent only. These findings strongly suggest that the new formulation successfully controls *Euphorbia heterophylla* and greatly increases the pathogenicity of the fungus.**

**Key words:** *Bipolaris yamadae*, *Euphorbia heterophylla*, adjuvants, biological control, phytopathogenic fungi.

## INTRODUCTION

Weed control plays an important role in the management of economically important crops (Green, 2014; Zhu et al., 2020). The spread of weeds with biotypes that areresistant to chemical herbicides, concerns about environmental issues and the necessity of reducing production costs are the main factors that drive the search for new weed control strategies (Caldwell et al., 2012). The use of specific phytopathogens as bioherbicides is a potential strategy for weed management due to its practicality and environmental safety.

One factor that limits the advancement of this kind of

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weed control is the difficulty of obtaining adequate formulations. This limitation is caused by the need to add compounds to increase the efficiency and stability of the biocontrol agent, allowing it to remain in the environment and increasing the possibility of reaching and attacking the target plants (Caldwell et al., 2012; Loureiro et al., 2003).

Adjuvants are commonly used in agriculture to increase the action of chemical pesticides. The incorporation of these adjuvants into biological formulations can positively influencethe performance of the formulations, especially fungal conidia formulations, by maintaining low levels of available water to prevent conidial germination during storage, promoting adhesion and uniform spreading, protecting the phytopathogenfrom UV radiation and retaining the pulverized droplets on the foliar surface (Wraight et al., 2001). In addition, the adjuvants are able to modify the morphology of the epicuticular wax or promote injury to the leaf tissue, favouring microbial action in harming weed growth and development (Greavesand MacQueen, 1990; Womack and Burge, 1993; Prasad, 1994; Falk et al., 1994). However, these adjuvants can be toxic to the phytopathogen, so it is advisable to evaluate their toxicity to these microorganisms (Wyss et al., 2004; Keswani et al., 2016).The fungus *Bipolaris yamadae* (*Bipolaris euphorbiae*) (Marin-Felix et al., 2017) is a host-specific phytopathogen of *Euphorbia heterophylla* L. (Euphorbiaceae), commonly known as wild poinsettia. It is an invasive weed of great economic importance for crops, especially soybean. *B. yamadae* can be used as a bioagent to control this noxious weed; however, to enhance *B. yamadae* efficacy and survival, the development of effective formulations with proper additives is necessary.The present study aimed:

(1) to evaluate the toxicity of certain adjuvants to the fungus *B. yamadae*,
(2) to select certain compatible additives for this phytopathogen to compose a wettable powder formulation and
(3) to evaluate the pathogenicity of the developed formulation on *E. heterophylla* under green house conditions.

## MATERIALS AND METHODS

### Fungal strain and solid-state cultivation

This study was performed using the FCAV#569 fungal strain of *B. yamadae*. The strain was grown in Petri dishes containing Pontecorvo minimal medium according to Pontecorvo et al. (1953); it was modified by supplementation with peptone at 2 $g.L^{-1}$ and with starch replaced with glucoseat10 $g.L^{-1}$ (Penariol et al., 2008). The Petri dishes were kept at 25 ± 0.5°C for 10 days. In addition, the strain was preserved by ultrafreezing at -80°C in 2% malt broth containing 10% glycerol. Cultivation in the solid medium was performed to obtain a significant amount of *B. yamadae* conidia. The growth substrate was composed of a mixture of soybean hulls and sorghum in 60:40 m/m. The mixture was distributed in 250 ml Erlenmeyer flasks and then sterilized by autoclaving for 20 min at 121°C. The flasks were inoculated with three mycelial discs of 8mm diameter taken from fungal colonies grown on Pontecorvo minimal medium. The flasks were incubatedunder the same conditions described above.After incubation, the colonized mixture was dried, and the conidia were extracted according to Machado et al. (2013).

### *Bipolaris yamadae* compatibility with chemical adjuvants

The list of the adjuvants evaluated for the fungal formulation and their respective concentrations and functions are presented in Table 1. Predetermined quantities of the adjuvants (Table 1) were added to flasks containing liquefied Pontecorvo minimal medium (Pontecorvo et al., 1953), and then the medium was poured into Petri dishes measuring 90 × 15 mm. For the control treatment, adjuvants were notadded to the minimal medium. After solidification, one mycelial disk of 5 mm diameter of precultured fungi was transferred to the centre of the plates.

In order to determine the adjuvant toxicity profile to the fungus, the biological index (BI) model proposed by Rossi-Zalaf et al. (2008) was used. This model takes into consideration parameters such as vegetative growth, sporulation and germination. It was calculated according the formula:

$$BI = \frac{47[VG] + 43[SPO] + 10[GER]}{100}$$

In which:BI = biological index; VG = percentage of vegetative growth after 10 days of incubation compared to that in the control; SPO = percentage of sporulation after 10 days of incubation compared to that in the control; GER = percentage of conidial germination after 7h of incubation. The BI toxicological classification of the adjuvants was made using the scale described by Rossi-Zalaf et al. (2008), where BI values between 0 and 41 were considered toxic to the fungus; BI values between 42 and 66 were considered moderately toxic; and BI values above 66 were considered compatible with the fungus.

Vegetative growth (VG) was analyzed by measuring in mm two perpendicular diameters on the $10^{th}$ day of incubation. After this period, the conidia produced on the surface of the colony were removed by scraping and transferred to a test tube containing 9 ml of Tween 80® solution in 0.1% v/v. The amount of conidia or SPO was determined by counting in a Neubauer chamber. The conidial viability or GER was determined as described by Francisco et al. (2006).

### Pathogenicity trials of *B. yamadae* formulation on wild poinsettia

Based on the results of the compatibility test, the adjuvants and their respective concentrations were selected to compose the *B. yamadae*-based formulation and to evaluate its efficiency on weed plants.

In the greenhouse, *E. heterophylla* seeds were sown into plastic trays containing an organic substrate. After 20 days, the seedlings were transferred to 400 mL volume plastic pots containing sieved soil. Three seedlings were transferred to each plastic pot, and these were considered repetitions within each treatment to obtain a total of 15 plants per treatment.The plants at the four- to six-leaf stages were sprayed with 50.0 mL ofwater or control; adjuvants only or mixture; fungus formulation or *B. yamadae* + adjuvants and fungus onlyor *B. yamadae* with no adjuvants. Immediately after spraying, the plants were covered with plastic bags for 24 h. The incidence of disease was evaluated 10 days after spraying by scoring the

**Table 1.** Chemical adjuvants evaluated for compatibility with *B. yamadae* to compose a fungal bioformulation.

| Adjuvant | Concentrations (%) | Function |
|---|---|---|
| Silicon dioxide | 0.01; 0.05; 0.1; 0.5; 1; 2; 3 | Anti-wetting agent |
| Supragil WP® | 0.01; 0.05; 0.1; 0.2; 0.3; 0.4; 0.5 | Dispersing agent/ Wetting agent |
| Supragil MNS 85® | 0.01; 0.05; 0.1; 0.5; 1 | Dispersing agent |
| Geropon T 36® | 0.01; 0.05; 0.1; 0.2; 0.3; 0.4; 0.5 | Dispersing agent, Compatibility agent |
| Silicone oil | 0.05; 0.075; 0.1; 0.15; 0.2 | Dispersing agent, Spreading agent |
| Geropon SDS® | 0.5; 1; 1.5; 2; 3 | Wetting agent for dry formulations |
| Rhodapon LS 94 RPB® | 0.5; 1; 1.5; 2; 3 | Anionic surfactant |

**Table 2.** Anti-wetting effect of silicon dioxide at several doses on the viability, mycelia growth, andsporulation of *B. yamadae* and the respective toxicological classifications.

| Concentration | Germination (%) | Growth (mm) | Sporulation ($x10^6$ g substrate$^{-1}$) | BI | Toxicological classification |
|---|---|---|---|---|---|
| Control | 100 | 90 [a] | 47.9 [a] | | |
| 0.01% | 100 | 90 [a] | 68.5 [a] | 127 | C |
| 0.05% | 100 | 90 [a] | 39.1 [a] | 97 | C |
| 0.10% | 100 | 90 [a] | 37.7 [a] | 96 | C |
| 0.50% | 100 | 90 [a] | 55.5 [a] | 115 | C |
| 1.00% | 100 | 90 [a] | 43.2 [a] | 101 | C |
| 2.00% | 100 | 85 [a] | 59.0 [a] | 115 | C |
| 3.00% | 100 | 68 [a] | 53.5 [a] | 126 | C |
| F test | - | 2.17 ns | 1.47ns | - | - |
| C.V. (%) | - | 3.56 | 9.78 | - | - |

Original values and statistical analysis of sporulation and germination performed with log x and arc sin (x/100) data transformation, respectively. Means followed by the same letter in the column do not differ by the Tukey test ($p \geq 0.05$). ns: Not significant. BI: Biological index; C.V.: coefficient of variation; C: compatible.

number of leaves with symptoms and the total number of leaves on each plant according to De Nechet et al. (2006).

#### Statistical analysis

All data were submitted to variance analysis by the F-test, and the means were compared by Tukey's test with 5%probability, using AgroEstat software (Barbosaand Júnior-Maldonado, 2015).

## RESULTS AND DISCUSSION

Anti-wetting silicon dioxide was previously identified by Machado et al. (2016) as a wettable powder for *B. yamadae* bioformulation due to its texture, which allows an increase in the preparation volume of conidia + anti-wetting and creates a homogeneous mixture. In the present study, the concentration used by these authors was extrapolated to verify the level of fungal tolerance to the product. All the evaluated parameters did not differ significantly from the control. Fungal sporulation varied between 37.7 and 68.5 x $10^6$ conidia.g substrate$^{-1}$, and all the concentrations were considered compatible with the phytopathogen according to the BI model as shown in Table 2.

Different concentrations of the dispersant agents led to significant differences in all the parameters evaluated, except for *B.yamadae* germination and sporulationwith Geropon T 36®as presented in Table 3.When both Supragil WP® and Supragil MNS 85® were added to the culture medium, the biological parameters evaluated were inversely proportional to the increase in the product concentration in the medium, starting from 0.3 to 0.5% and fungal growth and development were completely inhibited (Table 3). These products were considered moderately toxic and toxic to the fungus, except Supragil WP®at 0.01% concentration.

For the compatibilizer agent Geropon T36®, even though a reduction in the diameter of the colonies was observed after ten days of incubation, the fungal sporulation was approximately $10^6$ conidia.ml$^{-1}$for all evaluated concentrations. However, according to the BI,

**Table 3.** Effects of dispersants at several doses on the viability, mycelial growth, and sporulation of *B. yamadae*, and their toxicological classifications.

| Product and concentration | Germination (%) | Growth (mm) | Sporulation ($x10^6$ con g substrate$^{-1}$) | BI | Toxicological classification |
|---|---|---|---|---|---|
| Supragil WP® | | | | | |
| Control | 99.5$^a$ | 90.0$^a$ | 2.89$^a$ | - | - |
| 0.01% | 99.6$^a$ | 90.0$^a$ | 2.64$^a$ | 96 | C |
| 0.05% | 97.7$^a$ | 38.0$^b$ | 1.55$^{ab}$ | 42 | MT |
| 0.1% | 91.9$^a$ | 29.7$^c$ | 0.85$^b$ | 48 | MT |
| 0.2% | 35.1$^b$ | 20.0$^d$ | 0.25$^c$ | 18 | T |
| 0.3% | 0.0$^c$ | 0.0$^e$ | 0.0$^d$ | 0 | T |
| 0.4% | 0.0$^c$ | 0.0$^e$ | 0.0$^d$ | 0 | T |
| 0.5% | 0.0$^c$ | 0.0$^e$ | 0.0$^d$ | 0 | T |
| F test | 106.56* | 38762.18** | 88.38** | - | - |
| C.V. (%) | 15.13 | 0.87 | 19.63 | - | - |
| Supragil MNS 85® | | | | | |
| Control | 99.6$^a$ | 90.0$^a$ | 41.3$^a$ | - | - |
| 0.01% | 97.8$^b$ | 22.3$^b$ | 24.3$^{ab}$ | 47 | MT |
| 0.05% | 77.2$^c$ | 18.3$^{bc}$ | 6.4$^b$ | 24 | T |
| 0.1% | 44.1$^d$ | 15.8$^c$ | 8.8$^{ab}$ | 22 | T |
| 0.5% | 0.0$^e$ | 0.0$^d$ | 0.0$^c$ | 0 | T |
| 1.0% | 0.0$^e$ | 0.0$^d$ | 0.0$^c$ | 0 | T |
| F test | 1445.96* | 1995.12** | 23.10** | - | - |
| C.V.(%) | 3.49 | 3.67 | 35.81 | - | - |
| Geropon T36® | | | | | |
| Control | 100 | 90.0$^a$ | 30.3$^a$ | - | - |
| 0.01% | 100 | 90.0$^a$ | 22.3$^a$ | 89 | C |
| 0.05% | 100 | 78.0$^b$ | 23.8$^a$ | 84 | C |
| 0.1% | 100 | 70.4$^c$ | 19.3$^a$ | 74 | C |
| 0.2% | 100 | 68.3$^c$ | 13.3$^a$ | 64 | MT |
| 0.3% | 100 | 52.8$^e$ | 13.8$^a$ | 57 | MT |
| 0.4% | 100 | 57.9$^d$ | 11.5$^a$ | 60 | MT |
| 0.5% | 100 | 50.0$^e$ | 17.5$^a$ | 61 | MT |
| F test | - | 170.59** | 0.68ns | - | - |
| C.V. (%) | - | 0.83 | 25.09 | - | - |
| Silicone oil | | | | | |
| Control | 99.7$^a$ | 90.0$^a$ | 24.3$^a$ | - | - |
| 0.05% | 98.9$^b$ | 90.0$^a$ | 26.0$^a$ | 103 | C |
| 0.075% | 98.9$^b$ | 90.0$^a$ | 12.8$^{ab}$ | 80 | C |
| 0.1% | 98.9$^b$ | 59.3$^b$ | 6.7$^b$ | 53 | MT |
| 0.15% | 98.6$^b$ | 36.0$^c$ | 5.2$^b$ | 38 | T |
| 0.2% | 98.3$^b$ | 35.0$^c$ | 1.1$^c$ | 30 | T |
| F test | 6.85** | 1347.01** | 25.17** | - | - |
| C.V. (%) | 1.37 | 0.6 | 22.68 | - | - |

Original values and statistical analysis of sporulation and germination performed with log x and arc sin (x/100) data transformation, respectively. Means followed in the column by at least one of the same letter do not differ by the Tukey test ($p \geq 0.05$). ns: Not significant. BI: Biological index; C.V.: coefficient of variation; C: compatible; MT moderately toxic; T toxic.

only concentrations of 0.01, 0.05 and 0.1% were considered compatible with the fungus (Table 3). The compatibilizing function of this product in the formulation is essential, as it improves the homogeneity of the mixture and the uniformity of application (McMullan, 2000), ensuring that all plant leaves got into contact with the inoculum.

Silicone at concentrations of 0.05 and 0.075% was also

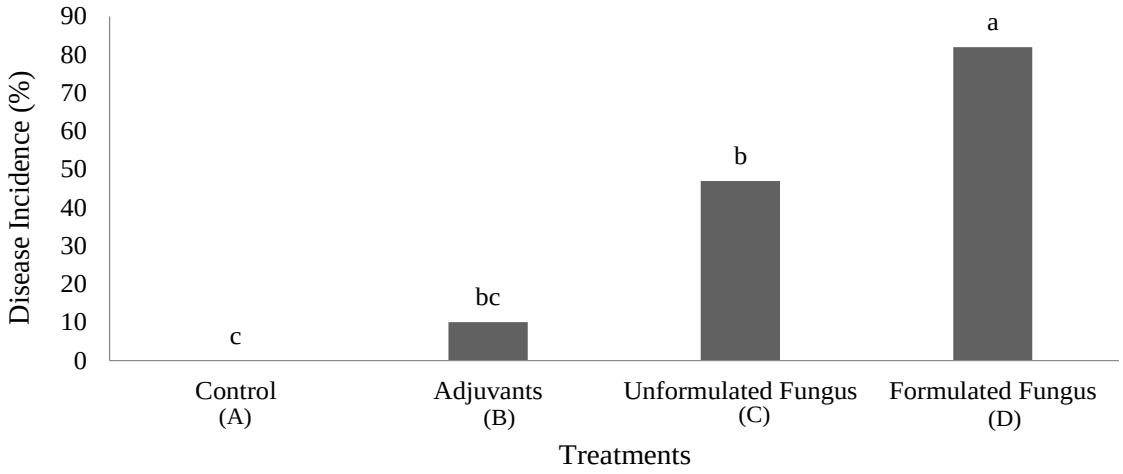


**Figure 1.** Effect of *B. yamadae* bioformulation on *E. heterophylla.* Disease incidence on *E. heterophylla* (%) after 10 days of spraying with solutions without infectious propagules of *B. yamadae* (A and B) and containing *B. yamadae* (C and D).

classified as compatible with the fungus and could be used in bioherbicide formulations (Table 3).The products Geropon SDS® and Rhodapon LS 94® at concentrations of 0.5 to 3.0% completely inhibited conidial germination in *B. yamadae* and, consequently, the subsequent stages of fungal development (data not shown). For the chemical control of weedy plants, the presence of adjuvants in the spraying tank is essential; even though adjuvants do not directly affect the efficacy of theherbicide, they improve the efficacy of pesticides by reducing or minimizing any negative effects at the time of application (McMullan, 2000). These effects are desirable in bioproduct development for pest control and justify the importance and benefits of adjuvants associated with biopathogens of interest. However, there are no guidelines for the selection of adjuvants to be used in association with biocontrol agents, which leads to the necessity of investigating and selecting compatible products for different phytopathogen-weed systems (Sanyal et al., 2008).

Several studies have shown the association effects of adjuvants and pathogens on the control of different target weeds (Gronwald et al., 2002; Borges-Neto et al., 1998; Borges-Neto and Pitelli, 2004). However, these studies did not evaluate the effects of these products on the bioagent, which can explain the failure of weed control in some cases. Only a few studies showed that the adjuvants had negative or toxic effects on the biopathogens, and this was the main motivation for the present study.

Similarly, Zhang et al. (2003) evaluated the effects of different surfactants and adjuvants on the germination and mycelial growth of *Colletotrichum* sp. and *Phoma* sp. in order to compose bioformulations with these fungi as phytopathogens. The results showed varied effects on the evaluated parameters, with increased or decreased effect depending on the situation.Based on the results of the present study, the wettable powder formulation with *B. yamadae* was composed of two distinct fractions: (1) a powder fraction and (2) a water fraction. The powder fraction was composed of the active ingredient, or conidia,at a final concentration of $10^7$ conidia.$ml^{-1}$ with anti-wetting silicon dioxide at 1% and inert kaolin, added only to increase the volume of the fraction. The water fraction was composed of 0.1% Geropon T36® the compatibilizer agent, 0.075% siliconeor dispersant, 0.1% Tween 80 or surfactant and 0.5% PVP K30® or spreading agent. The latter two products were identified as nontoxic to *B. yamadae*in previous studies (Machadoe et al., 2013, 2016).

In the greenhouse, the variance analysis was significant ($p>0.05$) in the test that evaluated the fungal pathogenicity on poinsettia. The incidence of disease was observed on 46.5 and 83.6% of the plants sprayed with a solution containing the bioagent alone and the formulated bioagent, respectively (Figure 1). Additionally, the mixture containing only the adjuvants did not cause a notable incidence of disease, but only 11.2% when sprayed on the plants, confirming that the adjuvantswere not toxic to this weed thus reaffirming the phytopathogenic action of the fungus (Figure 1).

The plants in the treatments containing the biological agent presented necrotic spots on leaves and stems within 48h after spraying. Moreover, during the evaluation period, intense defoliation thatresultedinto the death of the aerial partsof the plants was observed. Among the adjuvants that are being researched for use in fungal-based formulations, surfactants, when added to the spray solution, promote the suspension, dispersion, deposition, wetting, adhesion and retention of the conidia, thereby increasing the toxicity to the target (Costa et al., 2003).

Comparing the treatments in which the poinsettia

weeds were sprayed with solutions containing formulated and non-formulated microorganisms, the chemical adjuvants incorporated with the fungus were able to increase the contact and interaction of the fungus with the target plant, allowing significantly more effective control (79.0%). These products might have contributed in changing the morphology of the epicuticular wax or caused leaf tissue injuries, thereby facilitating *B. yamadae* entry and development.

*B. yamadae* produces a specific phytotoxin against wild poinsettia that causes negative effects during germination and affects susceptible leaves, promoting intense defoliation, but it does not affect non-host crops (Barbosa et al., 2002). It has been reported that fungal phytotoxins may also interact with plants other than the specific host (Hudson, 1986); however, this was not the case for *B. yamadae*in the present study as reported by Barbosa et al. (2002). This fungus did not affect soybean germination, and no disease symptoms were observed during soybean development (data not shown).

## Conclusion

*Bipolaris yamadae* development was influenced by the tested adjuvants, including by varying the fungal response at different concentrations of these products. After the compatibility tests, a *B. yamadae* bioformulation was developed containing 1% silicon dioxide or anti-wetting, 0.1% Geropon T36® as compatibilizer agent, 0.075% silicone or dispersant, 0.1% Tween 80®or tensioactive agent and 0.5% PVP K30®or spreading agent. The test of the bioformulation against *E. heterophyllla* showed that weed control was improved when the plants received formulated *B. yamadae* compared to that under its direct, unformulated inoculation. Therefore, this study presents the development of a new phytopathogenic fungus-based formulation with great efficiency as a bioherbicidal agent for the control of poinsettia weeds.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENT

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) [Finance Code 001].

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Vol. 14(4), pp. 136-147, April, 2020
DOI: 10.5897/AJMR2019.9086
Article Number: E55022F63587
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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# Chemical composition and *in-vitro* antimicrobial activity of essential oil of African nutmeg (*Monodora myristica* (Gaertn) Dunal on microorganisms isolated from smoke-dried catfish (*Clarias gariepinus*)

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Received 28 February, 2019; Accepted 12 December 2019

**Dried seeds of *Monodora myristica* were obtained from Oba market in Edo State, ground into fine powder and stored in an air tight plastic container at room temperature until when used. Essential oils were extracted using steam distillation and characterised using the gas chromatography-mass spectroscopy (GC-MS) technique. Microbial isolates obtained from smoke dried fish samples purchased from Oba Market, Benin City, Edo State. Fish samples were put in perforated polythene bags kept in plastic baskets and stored for four (4) weeks. Identification and characterisation of isolates were carried out following standard microbiological procedures. The antimicrobial activity was determined using agar well diffusion method. Data obtained were subjected to a one-way analysis of variance. Significant means were separated using Duncan Multiple Range Test at 95% confidence level. The result showed that *M. myristica* oil contained twenty-five components which were mainly monoterpene hydrocarbons constituents consisting of limonene (17.6%), α-phellandrene (16.3%), α-pinene (12.2%), β-myrcene (11.2%), 3-Thujene and β-Linalool (7.3%). The oil showed bacteriocidal activity at minimum ihhibitory concentration/minimum bactericidal concentration (MIC/MBC) of 150 mg/mL against *Staphylococcus epidermidis* PM221, *Micrococcus luteus* NCTC 2665, *Bacillus subtilis* 6051-HGW and *Pseudomonas aeruginosa* AAU2 and fungicidal activity at MIC/minimum fungicidal concentration (MFC) of 300 mg/mL against *Aspergillus flavus* and *Rhizopus* species. In view of the aforementioned, the expressed antimicrobial activity is a demonstration of the efficacy of the oil against fish spoilage organism and use in the fishery industry**.

**Key words:** Chemical compositions essential oils, antimicrobial activity, *Monodora myristica*, agar well diffusion method, *Clarias gariepinus*.

## INTRODUCTION

African nutmeg (*Monodora myristica*), is a perennial edible plant that belongs to the family Annonaceae. It is a berry commonly found in the evergreen forests of West Africa (Burubai et al., 2009). It can sometimes be referred to as Iwor (Itsekiri), Ikposa (Benin), Ehuru or Ehiri (Igbo), Ariwo (Yoruba), Uyengben (Edo), Guijiya dan miya (Hausa), Ehiriawosin (Ikale) and (Feyisayo and Oluokun, 2013; Enabulele et al., 2014). The fruit of is smooth,

green and spherical with a diameter of 20 cm which becomes woody at maturity. It is has a long stalk which is up to 60 cm long. It is composed of numerous oblong, pale brown seeds which are about 1.5 cm long and are enclosed by a whitish fragrant pulp. The seed extract contains important pharmacological compounds like alkaloids, flavonoids, and vitamins A and E as well as numerous significant lipids (George and Osioma, 2011). The seed of the plant is very popular as a result of its nutritional and medicinal qualities (Corbo et al., 2009). The seeds are used in Eastern Nigeria as condiment and have been shown to have anti-sickling properties (Uwakwe and Nwaoguikpe, 2005). Additionally, the seeds can be utilized for treating individuals with elevated cholesterol level in light of the fact that have the capacity to lower cholesterol (Onyenibe et al., 2015; Ekeanyanwu et al., 2010). The essential oil from the leaves has been shown to contain *β*-caryophyllene, *α*-humulene and *α*-pinene, whereas that from the seeds contains *α*-phellandrene, *α*-pinene, myrcene, limonene and pinene (Fournier et al., 1999). Owokotomo and Ekundayo (2012) reported that the essential oil of the seeds contained germacrene D-4-ol (25.48%), tricyclo[5.2.1(1,5)dec-2-ene (13.35%), δ-cadinene (11.09%) and linalool (15.10%) while the major constituents of the stems-bark oil were γ-cadinene (31.31%), α-elemene (17.98%), α-cubebene (6.70%) and γ-muurolene (5.94%). Recently, essential oil from this plant attains to researchers interest especially food industry because of their antimicrobial and anti-oxidative properties. However, literature report on the essential oils in terms of composition and antimicrobial activity from this plant is scarce especially in Nigeria. As a result, this study aims to evaluate the essential oil chemical composition and *in-vitro* antimicrobial activity of essential oil of African nutmeg (*M. myristica*) on microorganisms isolated from smoke-dried catfish (*Clarias gariepinus*).

## MATERIALS AND METHODS

### Collection of materials

The dried seeds of *M. myristica*, were obtained from Oba market in Edo State and were identified and authenticated in the Department of Plant Biology and Biotechnology, University of Benin, Benin City. The seeds were then ground into fine powder and stored in an air tight plastic container and placed at room temperature (28 ± 2°C) for 24 h before use.

### Extraction of the essential oils

Ground dried powder (3000 g) was weighed into distillation flask fitted with a condenser. Steam was supplied to the flask through a steam generator at constant flow for about 45 to 50 min. The essential oil was condensed and collected by a separating funnel. The oil was separated by gravity and dried using anhydrous sodium sulphate, filtered and stored at 4°C for 24 h before being analysed (Hussain et al., 2008).

### Gas chromatography-mass spectrometry (GC-MS) analysis

The essential oil was analysed using a QP2010 Plus Shimadzu, Japan-gas chromatography-mass spectroscopy (GC-MS) at the National Research Institute for Chemical Technology (NARICT), Zaria, Nigeria. A fused silica capillary column HP5-MS (30 m × 0.32 mm, film thickness of 0.25 µm) was used. The carrier gas used was Helium at a flow rate of 36.2 cm/s and at a constant pressure of 90 kPa. The samples were injected by splitting using a split ratio of 41.6. The column flow rate was 0.99 mL/min. The column oven initial temperature was 60°C held for 1 min. The temperature was then slowly increased at 10°C $min^{-1}$ to 180°C and held for 3 min. This was then increased at 12°C $min^{-1}$ to 280°C which was finally held for 2 min, while maintaining the injection temperature at 250°C.

### Identification of constituents

The identification of components was based on comparison of their mass spectra and their retention index with those present in the National Institute for Standard Technology computer data bank (Adams, 2001).

### Antimicrobial assay of essential oils

#### *Collection of bacterial and fungal isolates from smoke dried fish samples*

The micro-organisms used for the antimicrobial activity were obtained from smoke dried fish samples purchased from Oba Market, Benin City, Edo State. Fish samples were kept in plastic baskets and stored on open bench under laboratory conditions for a period of four (4) weeks. Ten grams of smoke dried fish samples were weighed each and homogenized in 90.0 ml of sterile distilled water to prepare a stock solution. 1.0 ml of the stock solution (homogenate) was transferred into a sterile test tube containing 9.0 ml of sterilized distilled water. This process was repeated for other sterilized test tubes so that at the end, dilution of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ were obtained. A liquid solution of 0.1 ml of each dilution was plated in a plate count agar (PCA) using pour plate technique. The PCA were treated with Fulcin (50 mg/20 ml NA) to discourage fungal growth. The plates were incubated aerobically at 37°C for 24 h. While for fungi a liquid of $10^{-5}$ dilutions was dropped upon sterile Petri dishes in triplicates and 0.2 ml of anti-bacterial mixture which comprised Penicillin (100 ml 5%) and Streptomycin (100 ml 7.5%) was added to discourage bacterial growth. Potato dextrose was poured into various dishes and allowed to solidify before incubating aerobically at 28 ± 2°C for 72 h (Emoghene, 1996).

#### *Identification of bacterial and fungal isolates from smoke dried fish*

All isolates were identified by standard microbiological and biochemical which includes: Gram stain, sugar fermentation, methyl red test, catalase, oxidase, coagulase, mannitol, Voges-proskaur

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test, indole test, citrate test, urease test and motility test (Cowen and Steel, 2003). While fungal isolates were identified on the basis of cultural and morphological characteristics as described by Oyeleke and Okusanmi (2008) (Tables 2 and 3). The organisms were then subcultured and preserved at -20°C in sterile McCartney's bottles containing either nutrient broth and 15% sterile glycerol (bacteria) or potato dextrose broth containing 15% sterile glycerol (fungi).

### *Extraction DNA from bacteria isolates*

Genomic DNA was gotten from the bacteria isolated from smoke-dried fish sample using Sigma Aldrich DNA extraction kit. A single discrete band was observed when resolved on Agarose Gel. The PCR amplicon was purified by column purification in order to remove contaminants. DNA sequencing was carried out with PCR amplicon. The 16S rDNA sequence was used to carry out BLAST with the nrs database of NCBI genbank database (Table 4).

### Polymerase chain reaction (PCR)

16S rDNA region was amplified by PCR from bacterial genomic DNA using PCR universal primers: 16S Forward Primer: 5'-AGAGTTTGATCMTGG -3'; 16S Reverse Primer: 5'-ACCTTGTTACGACTT-3'. PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. 3 µl of PCR product was loaded with 3 µl bromophenol blue (Loading Dye) in 1.5% agarose gel. The gel was ran at constant voltage of 100 V and current of 45 A for a period of 30 min till the bromophenol blue has travelled 6 cm from the wells. After amplification, the expected PCR product was verified by gel-electrophoresis.

### Purification and DNA sequencing of samples

Amplified PCR product was purified using column purification as per manufacturer's guidelines, and further used for sequencing reaction. The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on Genetic Analyzer.

### Sequence analysis of 16S rDNA

Each nucleic acid sequence was edited manually to correct falsely identified bases and trimmed to remove unreadable sequence at the 3 'and 5' ends (considering peak and quality values for each base) using the sequence analysis tools. The edited sequences were then used for similarity searches using Basic Local Alignment Search Tool (BLAST) program in the NCBI GenBank which is a DNA database for identifying the bacterial strains.

### Antibacterial screening of essential oils

The antimicrobial activity of plant essential oils was studied using agar well diffusion method (Okeke et al., 2001). Each bacterial isolate preserved in 15% glycerol broth was sub-cultured on freshly prepared Nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, UK) plate and incubated at 37°C for 24 h. A bacterial colony was picked up with a sterile wire loop, and emulsified in 5 ml Mueller Hinton broth (Oxoid Ltd., Basingstoke, Hampshire, UK). The turbidity of the resulting mixtures was adjusted with sterile saline solution to 0.5 Mcfarland standard ($1.5 \times 10^8$ cells·mL$^{-1}$). Fresh Mueller Hinton agar plates were labelled and seeded with the test bacterial suspension using a sterile swab steak. These plates were allowed to incubate for 15 min thereafter agar punched out with sterile cork borer to create wells of 4 mm diameter. The different concentrations of the essential oils extracts (150, 100, 50, 25, 12.5 and 6.25 mg/ml) were diluted with DMSO (0.5% v/v). 100 µl dilute essential oils of the different concentrations were dispensed into each agar well in a plate; plates were allowed to stand for 1 h and then incubated at 37°C for 24 h. A 0.5% w/v suspension of ciprofloxacin (Fidson Healthcare Ltd., Nigeria) was used as positive control while a sterile solvent was used as negative control. This experiment was performed in triplicate plates for each of the isolate per dissolution. The diameter (mm) of zone of inhibition was recorded for the different concentrations of essential oil.

### Antifungal screening of essential oil

The fungal isolates were sub cultured on potato dextrose agar at (28 ± 2°C) for 3 to 5 days. Equidistant wells were bored radially on sterile potato dextrose agar plates using a 4 mm diameter sterile cork borer. 100 µl dilute essential oils of the different concentrations were poured into the wells as described earlier. A 4 mm diameter agar mycelial disc of the fungi was then placed at the centre of the plate. 0.05% w/v Nystatin suspension (Mutual Pharmaceutical Company, Inc., Philadelphia, PA, USA) was used as positive control while the different dissolution solvent, was used as negative control. Triplicate plates were used in each treatment. Plates were incubated at 28 ± 2°C for 3 days. The zone of inhibition was recorded to the nearest mm.

### Determination of minimum inhibitory concentrations (MICs)

The standard agar dilution technique with doubling dilution was used to determine the MICs of the extracts (Oboh et al., 2007). Different concentrations of the oil was prepared in DSMO and then diluted to attain a decreasing concentration of 150, 100, 50, 25, 12.5 and 6.25 mg/ml, respectively. Each dilution was introduced into nutrient agar plates and potato dextrose agar plates already seeded with each test organism. All test plates were incubated at 37°C for 24 h for bacteria and 28 ± 2°C for 72 h for fungi. The minimum inhibitory concentration (MIC) of the extracts for each test organism was regarded as the agar plate with the lowest concentrations without growth.

### Determination of minimum bactericidal/fungicidal concentrations (MBCs/MFCs)

The MBC/MFC of the plant extracts was obtained by sub culturing portions of the agar from plates that showed no growth in the tests for determination of MICs. These agar portions were transferred respectively into plates containing freshly prepared nutrient agar and potato dextrose agar. These plates were incubated at 37°C for 24 h for bacteria while fungi plates were incubated at 28±-2°C for 3 to 5 days while growth were observed. No growth at the end of incubation period was considered as total cell death. The minimum concentration of the plant extracts that produces total cell death is taken as the MBC/MFC (Ajaiyeoba et al., 2003).

### Statistical analysis

All data obtained in this study were represented as mean ± standard error (SE) of triplicates values. The data were then subjected to a one-way analysis of variance laid in a completely randomized design replicated thrice. Duncan Multiple Range Test

at 95% confidence level was used to separate significant.

## RESULTS AND DISCUSSION

### Chemical composition of essential oil of African nutmeg (*M. myristica*)

The detected components, chemical formula and their relative percentages of the essential oil of *M. myristica*, are shown in Table 1. Twenty-five components were characterized which represents 100% of the oil. This value was higher than that obtained by Owokotemo and Ekundayo (2012) who identified 22 compounds in the seed oil and 20 compounds in the stem bark oil of *M. myristica.* The oil consisted of 70.9% monoterpene hydrocarbons. The major monoterpene hydrocarbons constituents were limonene (17.6%), α-phellandrene (16.3%), α-pinene (12.2%), β-myrcene (11.2%), 3-Thujene (7.3%), and β-Linalool (7.3%). This is in agreement with Koudou et al. (2007) who identified alpha-phellandrene (34.4%) and p-cymene (22.2%) as major constituents of the oil. Several studies regarding the constituents of the essential oils of *M. myristica* essential oils obtained in Nigeria have exhibited a few contrasts. Owolabi et al. (2009) reported geranial (40.1%), neral (29.74%) and myrcene (11.3%) as the major components of the oil while Onyenekwe et al. (1993) reported alpha-phellandrane (50.4%), alpha-pinene (5.5%), myrcene (4.35%) and germacrene-D-4-ol (9.0%) as the most abundant compounds of essential oil of *M. myristica*. The disparity in occurrence and concentration of compound may perhaps be due to the origin of the plant material (Lawrence et al., 1988), genetic factors, culture and environmental conditions (Charles and Simon, 1990). Some of the compounds identified in this study like, α-limonene, are used to promote weight reduction, prevent and treat cancer and bronchitis (Crowell, 1999). In foods, beverages, and chewing gum, limonene is used as a flavoring agent. In addition, in pharmaceuticals, limonene is added to help medicinal ointments and creams penetrate the skin (Tsuda et al., 2004). α-Phellandrene is a flavouring agent used for food products such as yoghurt and baked foods. While α-pinene has been widely used as a food flavoring ingredient (Limberger et al., 2007; Rivas et al., 2012) and has anti-microbial properties (Gomes-Carneiro et al., 2005).

### Antimicrobial activity of African nutmeg (*M. myristica*) essential oil

The antibacterial activity of African nutmeg (*M. myristica*) essential oil against bacterial test microbes is shown in Table 5. No activity was recorded at 6.25, 12.5 and 25 mg/ml of the essential oil against all test microbes. There was also no activity observed for *Corynebacterium pseudodiphtheriticum* DSM 44287 and *Pseudomonas aeruginosa* PB112 165 at 50 mg/ml, the other organisms were inhibited with zones ranging from 1.33 mm for *Staphylococcus saprophyticus* ATCC 15305 to 9.33 mm for *P. aeruginosa* AAU2. At 100 mg/ml, all bacterial test organisms were inhibited with *Bacillus subtilis* KCTC 1028 having the lowest zone of inhibition (1.67 mm) and *Staphylococcus epidermidis* PM221 with the highest inhibition zone (12.33 mm). No activity was recorded for *P. aeruginosa* PB112 165 at this concentration. *P. aeruginosa* PB112 165 had the lowest inhibition zone (11.67 mm) while *P. aeruginosa* AAU2 had the highest zone of inhibition (23.33 mm) at 150 mg/ml. There were significant differences ($p<0.05$) between all concentrations and Ciprofloxacin (positive control) except 150 mg/ml for *Staphylococcus aureus* NCTC 8325 and *S. epidermidis* PM221. However, no significant differences ($p>0.05$) was recorded at concentrations below 100 mg/ml for *C. pseudodiphtheriticum* DSM 44287 and *P. aeruginosa* PB112 165, 50 mg/ml for *S. aureus* NCTC 8325, *S. epidermidis* PM221, *Micrococcus luteus* NCTC 2665, *B. subtilis* 6051-HGW, *Staphylococcus saprophyticus* ATCC 15305 and *B. subtilis* KCTC 1028, 25 mg/ml for *P. aeruginosa* AAU2 and DSMO (negative control). The essential oil inhibited the growth of the bacterial strains used in this study at different rates depending on the concentration and the type of tested organisms. These results are in agreement with Mohamed et al. (2016), Mardafkan et al. (2015) and Paster et al. (1990), who concluded that Gram positive and Gram negative organisms were both susceptible to the oil. The expressed antimicrobial activity was concentration dependent as higher concentrations of the respective oils elicited a corresponding maximal antimicrobial activity. This trend is consistent with earlier reports by Idu et al. (2014) and Karigar et al. (2010) which revealed concentration dependent antimicrobial activity of seed oil extracts of *Khaya senegalensis* and *Leucaena leucocephala*, respectively. The response of the positive control was significantly ($P>0.05$) greater than the essential oil at its maximum concentration of 150 mg/mL. Only *P. aeruginosa* AAU2 displayed inhibitory zones that were greater than zones elicited by exposure to ciprofloxacin. However, *S. epidermidis* PM221 elaborated a maximal mean inhibitory zone upon exposure to *M. myristica* essential oil that was identical to that elicited by the same culture exposed to the control antibiotic. The antifungal activity of African nutmeg (*M. myristica*) essential oil against test organisms is shown in Table 6. No activity was recorded at lower concentrations except for *Rhizopus* species (3.33 mm) at 50 mg/ml. Zone of inhibition was observed and recorded at 100 and 150 mg/ml with *Mucor* and *Tricoderma* species having the lowest zone of inhibition (3.67 and 9 mm), respectively. The highest zones for both concentrations were observed against *Rhizopus* spp. (9.00 mm) and *Aspergillus flavus* (13 mm), respectively. There were

**Table 1.** Chemical composition of essential oil of African Nutmeg (*M. myristica*).

| S/N | Compound | Chemical formula | RT | Area (%) |
|---|---|---|---|---|
| 1 | 3-Thujene | $C_{10}H_{16}$ | 5.258 | 7.3 |
| 2 | α-pinene | $C_{10}H_{16}$ | 5.400 | 12.2 |
| 3 | Camphene | $C_{10}H_{16}$ | 5.625 | 1.0 |
| 4 | β-pinene | $C_{10}H_{16}$ | 6.025 | 3.5 |
| 5 | β-myrcene | $C_{10}H_{16}$ | 6.142 | 11.2 |
| 6 | α-phellandrene | $C_{10}H_{16}$ | 6.492 | 16.3 |
| 7 | Limonene | $C_{10}H_{16}$ | 6.825 | 17.6 |
| 8 | γ-terpinene | $C_{10}H_{16}$ | 7.208 | 0.8 |
| 9 | Terpinolene | $C_{10}H_{16}$ | 7.650 | 1.0 |
| 10 | beta.-Linalool | $C_{10}H_{18}O$ | 7.850 | 7.3 |
| 11 | Terpinenol, cis-β | $C_{10}H_{18}O$ | 8.267 | 0.6 |
| 12 | p-menth-1-en-8-ol | $C_{10}H_{18}O$ | 9.342 | 1.2 |
| 13 | Cis-Sabinol | $C_{10}H_{16}O$ | 9.550 | 5.7 |
| 14 | 13-Oxabicyclo[10.1.0]tridecane | $C_{12}H_{22}O$ | 10.150 | 0.8 |
| 15 | Copaene | $C_{15}H_{24}$ | 11.825 | 0.8 |
| 16 | β-cis-Caryophyllene | $C_{15}H_{24}$ | 12.475 | 1.7 |
| 17 | β-farnesene | $C_{15}H_{24}$ | 12.692 | 1.5 |
| 18 | -Caryophyllene | $C_{15}H_{24}$ | 12.933 | 0.6 |
| 19 | α-amorphene | $C_{15}H_{24}$ | 13.158 | 0.8 |
| 20 | trans-.α-Bergamotene | $C_{15}H_{24}$ | 13.292 | 1.8 |
| 21 | α- Bisabolene | $C_{15}H_{24}$ | 13.475 | 1.8 |
| 22 | Aromadendrene | $C_{15}H_{24}$ | 13.71 | 1.8 |
| 23 | δ-Cadinene, (+)- | $C_{15}H_{24}$ | 13.783 | 2.2 |
| 24 | Germacrene D-4-ol | $C_{15}H_{26}O$ | 14.758 | 0.7 |
| 25 | tau.-Muurolol | $C_{15}H_{26}O$ | 15.950 | 0.2 |
| **Total identified (%)** | | | | **100** |
| Monoterpene hydrocarbons | | | | 70.9 |
| Oxygenated monoterpenes | | | | 15.6 |
| Monoterpenoids | | | | 85.5 |
| Sesquiterpene hydrocarbons | | | | 13.0 |
| Oxygenated sesquiterpenes | | | | 0.9 |
| Sesquiterpenoids | | | | 13.9 |

significant differences ($P<0.05$) between the highest concentration of 150 mg/ml for all organisms and the Nystasin (positive control) except for *Aspergillus niger* and *A. flavus*. No significant differences ($P>0.05$) were recorded at concentrations below 100 mg/ml for *A. niger, A. flavus, Aspergillus* species and *Mucor* spp. and 50 mg/ml for *Rhizopus* spp. and DMSO (negative control). *A. flavus*, *Aspergillus* spp. and *Rhizopus* spp. elicited mean inhibitory zones greater than the zones elaborated by the same respective isolates exposed to Nystatin. The antifungal activity of the tested oil varied. This may be as a result of a wide variety of secondary metabolites in the plants (Fleischer, 2003). The antifungal potency of the oil is indicated as a decrease in the radial mycelia growth with increase in the concentration of essential oil tested.

### Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the essential oils against bacterial isolates

The minimum inhibitory concentration and minimum bactericidal concentration of the essential oils against bacterial isolates are shown in Table 7. The MIC and MBC of *M. myristica* varied from 150 to 600 mg/ml against the test organisms. The oil inhibited *Bacillus*

**Table 2.** Biochemical characterization of bacterial isolates from Catfish (*Clarias gariepinus*) at 4 weeks storage.

| S/N | Colonial morphology | Gram Reaction | Oxidase | Catalase | Citrate | Urea | Methyl Red | MRVP Gas | Voges Proskauser | Indole | Coagulase | Motility | Dextrose | Lactose | Maltose | Fructose | Sucrose | Galactose | Mannitol | Bacterial identity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | LRFC | GPR | + | + | + | - | - | - | - | - | - | + | - | - | - | - | - | - | A+ | *Bacillus sp.* |
| 2 | GDRR | GPR | + | + | + | - | - | + | + | + | + | - | - | + | - | + | +A | + | - | *Cornyebacterium* spp. |
| 3 | GFRC | GNB | - | + | - | - | - | - | - | + | - | + | + | + | + | + | + | - | - | *P. aureginosa* |
| 4 | B/GFRC | GPC | - | + | - | + | - | + | + | - | - | - | - | + | + | + | + | - | - | *S. epidermidis* |
| 5 | SFRC | GPC | + | + | - | - | - | - | + | - | - | - | - | - | - | - | A+ | - | A+ | *Micrococcus* spp. |
| 6 | LRFC | GPR | + | + | + | - | - | - | - | - | - | + | - | - | - | - | - | - | + | *B. subtilis* |
| 7 | PROCC | GPC | - | + | - | + | - | - | + | - | + | - | - | - | + | + | + | - | + | *S. aureus* |
| 8 | PROCC | GPC | - | + | - | + | - | - | + | - | - | - | - | - | + | + | + | - | + | *S. saprophyticus* |
| 9 | LRFC | GPR | + | + | + | - | - | - | - | - | - | + | - | - | - | - | - | - | + | *B. subtilis* |
| 10 | GFRC | GNB | - | + | - | - | - | - | - | + | - | + | + | + | + | + | + | - | - | *P. aureginosa* |
| 11 | PROCC | GPC | - | + | - | + | - | - | + | - | + | - | - | - | + | + | + | - | + | *S. aureus* |

A: Acid production, G: gas production, GPC: Gram positive cocci, GNB: Gram negative bacillus, GNR: Gram negative rods, SFRC: small flat round colonies, PROCC: pink raise, opaque, circular cocci, GFRC: green flat round colonies, B/GFRC: blue/green flat round colonies, LRFC: large round flat colonies, GDRR: golden diplocci raised round.

**Table 3.** Biochemical characterization of fungal isolates from Catfish (*Clarias gariepinus*) at 4 weeks storage.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1 | Growth form | Black, woolly with profuse growth | Greenish, woolly with profuse growth | Yellowish, woolly with profuse growth | White extensive woolly cottony with coenocytic hyphae | Whitish, luxuriant with profuse growth fluffy | Greenish patches or cushion luxuriant growth |
| 2 | Colour of reverse plate | Black | Creamy | Creamy | Whitish | Creamy | Green |
| | **Microscopy** | | | | | | |
| 3 | Hyphae | Septate | Septate | Septate | Non-septate (young) Septate (old) | Non-septate | Septate |
| 4 | Conidiophores | Non-seplate terminating in globose swelling | Non-septate terminating in clavate swelling | Non-seplate, terminating in globose swelling | Non-septate, long erect usually unbranch single from coenocytic hyphae | Non-septate, upright terminating in globose swelling | Hyaline, upright much branched |
| 5 | Conidia | Present one-celled globose in dry basipetal chain | Present, globose in dry basipetal chains | Present, one-celled globose in dry basipetal chain | Present, hyaline one-celled, globose non-motile | Present, one-celled globose in dry basi[eta; chain | Hyaline, one-celled ovoid borne in small terminal clusters |
| 6 | Stolen | Absent | Absent | Absent | Absent, presence of coenocytic hyphae | Present | Absent |
| 7 | Rhizoid | Absent | Absent | Absent | Absent | Present, multi-branched short rooted | Absent |
| 8 | Spore colour | Black | Greenish | Creamy | Whitish | Dark | Greenish |

**Table 3.** Contd.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 9 | Spore attachment | Bear sterigmata at the apex with conidia attached at the tip | Radiate from the entire surface at the tip | Bear phialides at the apex with conidia at the tip | Tip of sporangiophore in the sporangia | Consist of terminal swelling of multinucleated hyphal branches with conidia at the tip | Phialids single with small terminal cluster at tip |
| 10 | Tentative Identity | *Aspergillus niger* | *Aspergillus flavus* | *Aspergillus* spp. | *Mucor* spp. | *Rhizopus* spp. | *Trichoderma* spp. |

**Table 4.** DNA Sequence Blast for isolates obtained from spoilt catfish (*Clarias gariepinus*).

| Isolate code | Sequence blast | Ascension no. | Sequence identity | Query coverage length | Score bits (%) |
|---|---|---|---|---|---|
| 1 | HG519617.1 *Bacillus licheniformis* DSM 13<br>GGGGACGTTACGGATTCGACAGGGACGGATCGAGCTTGAGCTGCGAGCCGAGAGGCGATCTCGTAAAAACGCACCTAAAT<br>ATAACTGGCAAATCTAACCAGAACTTAGCACTAGCTGCCTAATTAGCGCAGCGAGCTCTTGCCCGCATCGCCTATGTGCCG<br>GTTAAGAGCCCATAACGAAGTAGGCTACGCTTGCGCCCCCGTCTGAGGGCGCAGGAAGAGACTCATCAGACTAGCTCTCC<br>TAGGGCCCGCCCGCAGGCACGAAGATGAGCGAAACTAAATATGCAGGGCTACGCTCGTAGACGCTGAAGCAATCGACGTT<br>TCTGGACGTGGGTTCGACTCCCACCGTCTCCAT | HG519617.1 | *B. licheniformis* DSM 13 | 399 | 319/366 (87) |
| 2 | TTTATGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGAAAGGCCTCTT<br>CGGAGGTACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTCTGGGATAAGCCTGGGAAACTGG<br>GTCTAATACCGGATAGGACCATGCTTTAGTGTGTGGTGGAAAGTTTTTTCGGTGTAGGATGAGCCCGCGGCCTATCAGC<br>TTGTTGGTGGGGTAATGGCCTACCAAGGCGGCGACGGGTAGCCGGACTGAGAGGTTGGTCGGCCACATTGGGACTGAGA<br>TACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTG<br>GGGGATGACGGCCTTCGGGTTGTAAACTCCTTTCGCCAGGGACGAAGCGTTTTGTGACGGTACCTGGAGAAGAAGCACCG<br>GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGG<br>TGGTTTGTCACGTCGTCTGTGAAATTCCACAGCTTAACTGTGGGCGTGCAGGCGATACGGGCTGACTTGAGTACTGTAGGG<br>GTAACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTTACTGGGCAG<br>TTACTGACGCTGAGGAGCGAAAGCATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGGTGGGCGC<br>TAGGTGTAGGGACCTTTTGGGGTTTCTGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCT<br>AAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACC<br>TGGGCTTGACATACACTGGATCGCTGCAGAGATGTAGTTTCCCTTGTGGCTAGTGTACAGGTGGTGCATGGCTGTCGTCAG<br>CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTATGTTGCCAGCACGTTATGGTGGGGACT<br>CATGAGAGACTGCCGGGGTTAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCAC<br>ACATGCTACAATGGCCGGTACAATGCGCAGCGAGCCTGTGAGGGTGAGCTAATCGCTTAAAGCTGGTCTTAGTTCGGATTG<br>GGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAATGCTGCGGTGAATACGTTCCCGGGC<br>CTTGTACACACCGCCCGTCACGTCATGAAAGTTGGTAACACCCGAAGCCGGTGGCTCAAACTCGTTTAGGGAGCCGTCGA<br>AGGTGGGATCGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGG<br>AGTTTTTA | JIAH01000003.1 | *C. pseudodiphtheriticum* DSM 44287 | 1085 | 1265/1585 (80) |

*licheniformis* DSM 13. *C. pseudodiphtheriticum* DSM 44287, *S. aureus* NCTC 8325 and *S. saprophyticus* ATCC 15305 were inhibited at 300 mg/ml. MIC/MBC of 600 mg/ml inhibited growth of *B. subtilis* KCTC 1028 and *P. aeruginosa* PB112 165. While *P. aeruginosa* AAU2, *subtilis* 6051-HGW were inhibited at the lowest concentration of 150 mg/ml.

The lowest MIC/MBC negative bacteria negates earlier reports that they are more resistant or less susceptible to antimicrobial activities because of their capacity form biofilm (Marwah et al., 2007;

**Table 4.** Contd.

| Isolate code | Sequence blast | Ascension no. | *Sequence identity* | Query coverage length | Score bits (%) |
|---|---|---|---|---|---|
| 3 | AGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTG GGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTAT CAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGAT GATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAG CCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTA ATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCA AGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTG GGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATAT AGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGG ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACG CGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGA GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGC CTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGC GCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGG GATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCG CGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTA GTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTG CTCCAGAAGTAGCTAGTCTAACC | JQ904623.1 | *P. aeruginosa* strain AAU2 | 2551 | 1383/1384 (99) |
| 4 | CGAAGAATTATTAGAATTAGTTGAAATGGAAGTTCGTGACTTATTAAGCGAATATGACTTCCCAGGTGACGATGTACCTGTA ATCGCTGGTTCTGCATTAAAAGCATTAGAAGGCGATGCTGAATACGAACAAAAAATCTTAGACTTAATGCAAGCAGTTGATG ATTACATTCCAACTCCAGAACGTGATTCTGACAAACCATTCATGATGCCAGTTGAGGACGTATTCTCAATCACTGGTCGTGG TACTGTTGCTACAGGCCGTGTTGAACGTGGTCAAATCAAAGTTGGTGAAGAAGTTGAAATCATCGGTATGCACGAAACTTCT AAAACAACTGTTACTGGTGTAGAAATGTTCCGTAAATTATTAGACTACGCTGAAGCTGGTGACAACATCGGTGCTTTATTAC GTGGTGTTGCACGTGAAGACGTACAACGTGGTCAAGTATTAGCTGCTCCTGGTTCTATTACACCACACACAAAATTCAAAGC TGAAGTATACGTATTATCTAAAGATGAAGGTGGACGTCACACTCCATTCTTCACTAACTATCGCCCACAATTCTATTTCCGTA CTACTGACGTAACTGGTGTTGTAAACTTACCAGAAGGTACAGAAATGGT | NZ_HG813242.1 | *S. epidermidis* PM221 | 1153 | 624/624 (100) |
| 5 | GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGG TGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGA ACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATA CCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGACCATTCCACGGTTTCCGCGCCGCAGCTAACGCATTA AGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCAT GCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTTCCCGATCGCCGTAGAGATACGATTTCCCCTT TGGGGCGGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA CCCTCGTTCCATGTTGCCAGCACGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGAGGAC GACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAG GTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAA TCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTCGGTAACAC CCGAAGCCGGTGGCCTAACC | NC_012803.1 | *M. luteus* NCTC 2665 | 721 | 738/906 (81) |
| 6 | TTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGA TGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATAC CGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAG CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT GAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGG TACCTAACCAGAAAGCC | NC_020507.1 | *B. subtilis* strain 6051-HGW | 894 | 493/497 (99) |

**Table 4.** Contd.

| Isolate code | Sequence blast | Ascension no. | *Sequence identity* | Query coverage length | Score bits (%) |
|---|---|---|---|---|---|
| 7 | GGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACGGACGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGG TGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGAACCGCATGG TTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCA ACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGG AATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGA AGAACATATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCC | NC_007795.1 | *S. aureus* NCTC 8325 | 767 | 460/483 (95) |
| 8 | GGCTTTCTGATTAGGTACCGTCAAGACGTGCACAGTTACTTACACGTTTGTTCTTCCCTAATAACAGAGTTTTACGAGCCGAAACCCT TCATCACTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGT GTCTCAGTTCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGTATCGTCGCCTTGGTAAGCCGTTACCTTACCAACTAGCTAATA CGGCGCGGGTCCATCTATAAGTGATAGCAAAACCATCTTTCACTTTAGAACCATGCGGTTCCAAATGTTATCCGGTATTAGCTCCGG TTTCCCGAAGTTATCCCAGTCTTATAGGTAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACGTCAAAGGAGCAAGCTCCTTA TCTGTTCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCATCC | NC_007350.1 | *S. saprophyticus* ATCC 15305 | 845 | - |
| 9 | TTTGTATGCAATGAATGATTTAGGCATGACAAGTGACAAGCCTTATAAAAAATCCGCGCGTATCGTTGGAGAAGTTATCGGGAAATAC CACCCGCACGGTGATTCAGCGGTATATGAATCCATGGTCAGAATGGCTCAGGATTTCAACTACCGTTATATGCTCGTTGACGGTCAC GGAAACTTCGGTTCTGTTGACGGAGACTCAGCGGCGGCCATGCGTTATACAGAAGCACGAATGTCTAAAATCTCAATGGAGATTCTT CGCGACATCACAAAAGACACAATCGATTACCAGGATAACTATGACGGGTCAGAAAGAGAACCTGTCGTTATGCCTTCAAGGTTCCCG AATCTGCTCGTGAACGGTGCTGCCGGCATTGCGGTAGGTATGGCAACAAACATTCCTCCGCACCAGCTGGGAGAAATCATTGACGG TGTACTTGCTGTTAGTGAGAATCCGGACATTACAATTCCAGAGCTTATGGAAGTCATTCCAGGGCCTGATTTCCCGACCGCGGGTCA AATCTTGGGACGCAGCGGTATCCGGAAAGCATACGAATCAGGCCGAGGCTCTATCACGATCCGGGCAAAAGCTGAGATCGAACAA ACATCTTCGGGTAAAGAAAGAATTATCGTTACAGAGTTACCTTACCAAGTAAATAAGGCGAAATTAATTGAGAAAATTGCTGATCTCG TAAGGGACAAAAAGATAGAGGGTATCACAGATCTGCGTGATGAGTCAGATCGTACAGGTATGAGAATTGTCATTGAAATCAGACGCG ATGCCAATGCGAATGTTATCTTAAACAATCTGTACAAACAAACTGCTCTACAAAC | NZ_CP011115.1 | *B. subtilis* KCTC 1028 | 1519 | 832/837 (99) |
| 10 | AGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGAT AACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCT AGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGA ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGT GTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAAC AGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTA GAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGAT ACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGT TGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGA ACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT TAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGG AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCC AAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTA GTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAG AAGTAGCTAGTCTAACC | JN996498.1 | *P. aeruginosa* strain PB112 16S | 1446 | 1383/1384 (99) |
| 11 | GGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACGGACGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGG TGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGAACCGCATGG TTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCA ACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGG AATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGA AGAACATATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCC | NC_007795.1 | *S. aureus* NCTC 8325 | 767 | 460/483 (95) |

**Table 5.** Zone of inhibition (mm) for antibacterial activity of African Nutmeg (*M. myristica*) essential oil by the disc diffusion method.

| Bacterial isolate | Zone of inhibition (mm) | | | | | | Positive control (Ciprofloxacin) | Negative control (DMSO) |
|---|---|---|---|---|---|---|---|---|
| | 150 mg/mL | 100 mg/mL | 50 mg/mL | 25.0 mg/mL | 12.5 mg/mL | 6.25 mg/ mL | | |
| **Gram positive** | | | | | | | | |
| *B. lichenformis* DSM 13 | 13.00$^{b}$±0.58 | 8.33$^{c}$±0.88 | 3.33$^{d}$±0.33 | 0.00$^{e}$±0.00 | 0.00$^{e}$±0.00 | 0.00$^{e}$±0.00 | 25.00$^{a}$±0.40 | 0.00 $^{e}$±0.00 |
| *C. pseudodiphtheriticum* DSM 44287 | 12.67$^{b}$±1.200 | 5.00$^{c}$±0.58 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 21.67$^{a}$±0.11 | 0.00$^{d}$±0.00 |
| *S. aureus* NCTC 8325 | 16.33$^{a}$±0.88 | 9.33$^{b}$±0.88 | 2.67$^{c}$±1.76 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 17.67$^{a}$±0.30 | 0.00$^{d}$±0.00 |
| *S. epidermidis* PM221 | 19.00$^{a}$±0.00 | 12.33$^{b}$±0.88 | 2.67$^{c}$±1.45 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 19.00$^{a}$±0.20 | 0.00$^{d}$±0.00 |
| *M. luteus* NCTC 2665 | 16.67$^{b}$±0.088 | 8.00$^{c}$±0.58 | 4.33$^{d}$±0.33 | 0.00$^{e}$±0.00 | 0.00$^{e}$±0.00 | 0.00$^{e}$±0.00 | 27.33$^{a}$±0.41 | 0.00$^{e}$±0.00 |
| *B. subtilis* 6051-HGW | 16.00$^{b}$±1.53 | 9.00$^{c}$±2.08 | 3.33$^{d}$±0.88 | 0.00$^{e}$±0.00 | 0.00$^{e}$±0.00 | 0.00$^{e}$±0.00 | 25.0$^{a}$±0.20 | 0.00$^{e}$±0.00 |
| *S. saprophyticus* ATCC 15305 | 16.00$^{b}$±0.00 | 9.00$^{c}$±0.88 | 1.33$^{d}$±0.88 | 0.00$^{d}$± 0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 19.67$^{a}$±0.30 | 0.00$^{d}$±0.00 |
| *B. subtilis* KCTC 1028 | 8.33$^{b}$±0.88 | 1.67$^{c}$±0.88 | 1.33$^{c}$±0.88 | 0.00$^{c}$±0.00 | 0.00$^{c}$±0.00 | 0.00±0.00 | 21.67$^{a}$±0.11 | 0.00$^{c}$±0.00 |
| **Gram negative** | | | | | | | | |
| *P. aeruginosa* AAU2 | 23.33$^{a}$±0.88 | 20.00$^{b}$±0.58 | 9.33$^{d}$±0.88 | 3.67$^{e}$±0.88 | 0.00$^{f}$±0.00 | 0.00$^{f}$±0.00 | 17.00$^{c}$±0.20 | 0.00$^{c}$±0.00 |
| *P. aeruginosa* PB112 165 | 11.67$^{b}$±1.20 | 5.00$^{c}$±0.58 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 17.67$^{a}$±0.50 | 0.00$^{d}$±0.00 |

*****Values are expressed as mean ± standard error of mean; values are exclude diameter of agar well (4 mm). *Means in the same row with same superscripts are not significantly different (P>0.05) from the positive control.

**Table 6.** Zone of inhibition (mm) for antifungal activity of Nutmeg (*Monodora myristica*) essential oil by the disc diffusion method.

| Fungal isolate | Zone of inhibition (mm) | | | | | | Positive control (Nystasin) | Negative control (DMSO) |
|---|---|---|---|---|---|---|---|---|
| | 150 mg/mL | 100 mg/mL | 50 mg/mL | 25.0 mg/mL | 12.5 mg/mL | 6.25 mg/mL | | |
| *A. niger* | 10.00$^{a}$±1.15 | 5.33$^{b}$±1.33 | 0.00$^{c}$±0.00 | 0.00$^{c}$±0.00 | 0.00$^{c}$±0.00 | 0.00$^{c}$±0.00 | 11.00$^{a}$±0.20 | 0.00$^{c}$±0.00 |
| *A. flavus* | 13.00$^{a}$±0.58 | 7.33$^{b}$±1.20 | 0.00$^{c}$±0.00 | 0.00$^{c}$±0.00 | 0.00$^{c}$±0.00 | 0.00$^{c}$±0.00 | 12.00$^{a}$±0.20 | 0.00$^{c}$±0.00 |
| *Aspergillus* spp. | 10.00$^{a}$±0.58 | 5.00$^{c}$±1.15 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 8.00$^{b}$±0.20 | 0.00$^{d}$±0.00 |
| *Mucor* spp. | 10.33$^{b}$±0.88 | 3.67$^{c}$±1.20 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 13.67$^{a}$±0.30 | 0.00$^{d}$±0.00 |
| *Rhizopus* spp. | 12.33$^{a}$±0.88 | 9.00$^{c}$±0.58 | 3.33$^{d}$±0.88 | 0.00$^{e}$±0.00 | 0.00$^{e}$±0.00 | 0.00$^{e}$±0.00 | 10.33$^{b}$±0.11 | 0.00$^{e}$±0.00 |
| *Trichoderma* spp. | 9.00$^{b}$±0.58 | 4.67$^{c}$±0.88 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 0.00$^{d}$±0.00 | 13.33$^{a}$±0.11 | 0.00$^{d}$±0.00 |

*****Values are expressed as mean ± standard error of mean; values are exclude diameter of agar well (4 mm). *Means in the same row with same superscripts are not significantly different (P>0.05) from the positive control.

Wannissorn et al., 2009; Bouhdid et al., 2009; Park and Lee, 2011; Gabriel et al., 2013). However, the variation observed between the two strains of *P. aeruginosa* AAU2 and *P. aeruginosa* PB112 165 at MIC and MBC of 150 and 600 mg/ml, respectively may be as a result of the cell wall composition of the organisms and their physiological properties. The MIC and MFC of the essential oils against the fungal isolates are shown in Table 8. *M. myristica*, MIC and MFC ranged between 300 and 600 mg/ml. *A. flavus* and *Rhizopus* spp. were inhibited minimally at 300

**Table 7.** Minimum inhibitory concentration (mg/ml) and minimum bactericidal concentration (mg/ml) of the bacterial isolates exposed to varying concentrations of *M. myristica* essential oil.

| Isolate | MIC | MBC |
|---|---|---|
| **Gram positive** | | |
| *B. lichenformis* DSM 13 | 300 | 300 |
| *C. pseudodiphtheriticum* DSM 44287 | 300 | 300 |
| *S. epidermidis* PM221 | 150 | 150 |
| *M. luteus* NCTC 2665 | 150 | 150 |
| *B. subtilis* 6051-HGW | 150 | 150 |
| *S. aureus* NCTC 8325 | 300 | 300 |
| *S. saprophyticus* ATCC 15305 | 300 | 300 |
| *B. subtilis* KCTC 1028 | 600 | 600 |
| **Gram negative** | | |
| *P. aeruginosa* AAU2 | 150 | 150 |
| *P. aeruginosa* PB112 165 | 600 | 600 |

**Table 8.** Minimum inhibitory concentration (mg/ml) and minimum fungicidal concentration (mg/ml) of the fungal isolates exposed to varying concentrations of *M. myristica* essential oil.

| Isolate | MIC | MFC |
|---|---|---|
| *Aspergillus flavus* | 300 | 300 |
| *Aspergillus niger* | 600 | 600 |
| *Aspergillus* spp. | 600 | 600 |
| *Mucor* spp. | 600 | 600 |
| *Rhizopus* spp. | 300 | 300 |
| *Trichoderma* spp. | 600 | 600 |

mg/ml. All other fungal isolates were inhibited at 600 mg/ml. The higher MIC/MFC values among fugal isolates suggest that active compounds in *M. myristica* were less effective against *A. niger, Aspergillus* spp., *Mucor* spp. and *Trichoderma* spp.

## Conclusion

In this study, *M. myristica* essential oil contained twenty-five components which were mainly monoterpene hydrocarbons constituents consisting of limonene (17.6%), α-phellandrene (16.3%), α-pinene (12.2%), β-myrcene (11.2%), 3-Thujene and β-Linalool (7.3%). The oil showed a lesser antimicrobial activity than the positive control (Ciprofloxacin), but showed antifungal activity comparable to Nystatin at maximum concentration of 150 mg/mL. However, the oil had the greatest bacteriocidal activity at MIC/MBC of 150 mg/mL against *S. epidermidis* PM221, *M. luteus* NCTC 2665, *B. subtilis* 6051-HGW and *P. aeruginosa* AAU2 and the greatest MIC/MFC of 300 mg/mL against *A. flavus* and *Rhizopus* spp. In view of the aforementioned, the expressed antimicrobial activity is a demonstration of the efficacy of the oil against fish spoilage organism and use in the fishery industry.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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