

## Original Research Article

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## Chemical Composition of the Essential Oil of *Curcuma longa* and Evaluation of the Antifungal Activity on *Rhizopus stolonifer* and *Penicillium sp.* Responsible Fungi for Post-harvest Rot of *Dioscorea rotoundata* in Cameroon

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### ABSTRACT

#### Keywords

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This study focused its investigation on the use of the essential oil of a culinary and medicinal plant commonly used in Cameroon, *Curcuma longa* also called red ginger for the protection of white yam (*Dioscorea rotoundata*) during storage. The oil was obtained by hydro distillation using Clevenger apparatus with a yield of 0.26%, the chemical composition analysis revealed a predominance of oxygenated sesquiterpens (OST 63.43%) with as major compound  $\alpha$ -bisabolol (42.87%), E- $\beta$ -santalol (15.3%),  $\beta$ -cymene (8.22%). Fungi isolated from apparently healthy yam tubers were identified as *Rhizopus stolonifer* and *Penicillium sp.*, *In vitro* and *in situ* oil activity was evaluated. The oil completely inhibited the growth of *in vitro* pathogens at 1150 and 6000 ppm respectively for *Rhizopus stolonifer* and *Penicillium sp.*, the oil being more active on *Rhizopus stolonifer*. *In situ* tests were carried out only with this fungus. The essential oil of *Curcuma longa* has significantly reduced the rot caused by this pathogen, a reduction of 87.26% and 87.8% was obtained for the preventive and curative tests respectively. No significant difference in activity was observed in both tests. However, there is a significant difference ( $P \leq 0.0001$ ) in activity between the essential oil and the reference fungicide at the recommended concentration. As concerning the activity of this essence, the red ginger essential oil could be used as a biofungicide for the protection of yam tubers preserved against *R. stolonifer*, the most formidable fungus of rot white yam in Cameroon.

### Introduction

Yam creeper plant belonging to the family *Dioscoreaceae*, is one of the largest food

crops in the world specifically in the tropics and subtropics, especially in West and Central Africa (APG III, 2009). About more than 600 species, the most consumed and therefore the

most cultivated yam is *Dioscorea rotoundata* in the reason of its nutritional and organoleptic properties. Its tuber is the food base of many Cameroonians because of its rich starch, mineral salts (Calcium, Phosphorus, Iron) and because of the vitamins it contains among others thiamine, riboflavin vitamin B and C (Ngue *et al.*, 2007). Its world production is estimated at more than 60 million tons per year, with 555647 tons per year for Cameroon which ranks it 6th in the world. Nigeria ranks first, followed by Ghana, Côte d'Ivoire, Benin and Togo (Toukam *et al.*, 2015). Despite this important production, the white yam is subject to several constraints during storage including physiological and biological factors among others. Biological factors involving microorganisms (bacteria, viruses, fungi) are the most recurrent, the most important attacks are caused by fungi, the most formidable of which are *Penicillium* sp., *Aspergillus*, *Fusarium* and *Rhizopus* (Ogunleye *et al.*, 2014). The disease caused by these is known as post-harvest rot, which affects its production and affects its organoleptic and nutritional properties, thus constituting a real loss of income for the populations because this constitutes a real source of economy for most of them (Babajide *et al.*, 2006). The chemical control, which is the most used but also the most controversial method, is based on the use of synthetic fungicides such as sodium orthophenylphenate, borate, captan, thiabendazole, benomyl, and sodium hypochlorite (Foua -Bi *et al.*, 1979; Deshi *et al.*, 2014). Although repeated use of the latter has many repercussions on the health of the populations by accumulation of residues on the tuber and in the environment, the resistance of several strains is also present (Okigbo and Ogbonnaya, 2006; Deshi *et al.*, 2014). To remedy these shortcomings, many researchers have turned to new methods including biological and natural control using useful microorganisms and natural substances (Jazet *et al.*, 2009). Among the natural

substances, essential oils have proven to be the most effective because they have many biological properties, they are biodegradable, low toxicity and inexpensive (Otegwu, 2011, Jazet *et al.*, 2013, Sameza *et al.*, 2016). The Cameroonian flora is very biodiverse by plants rich in essential oils like the *Curcuma longa* commonly called "red ginger". It is an herb used for its rhizome in culinary arts as spices and condiments but more commonly as a traditional medicine for the treatment of certain ills in the locality. Several studies have approved its richness in essential oil and its biological activity as a powerful antioxidant, antimicrobial (antibacterial, antiviral and antifungal) (Gianni *et al.*, 2005, Shiyouli *et al.*, 2011, Závěská *et al.*, 2012), hence our investigation to evaluate the antifungal potential of the essential oil of the said plant on the fungi responsible for the post-harvest rot of white yam (*Dioscorea rotoundata*) in Cameroon.

## **Materials and Methods**

### **Source of plant material**

The plant was harvested in the Moungo department in the Manjo's city; yam tubers with symptoms of the disease and apparently healthy were bought at the Dakar market in the city of Douala.

### **Isolation of fungi**

Fungi have been isolated from yam tubers with symptoms of the disease, according to Koch's postulate that a fungus isolated from the laboratory can grow on its host. It consisted in taking from the margin of rots fragments of tubers which were disinfected with 70% alcohol for 1 min and then rinsed three times with sterile distilled water, the excess water was wrung out on blotting paper and the fragments were inoculated into Petri dishes containing PDA (Potatoes Dextrose

Agar) supplemented with antibiotics (penicillin 250 mg / ml and Ampicillin 250 mg / ml). The dishes were incubated at room temperature  $28 \pm 2$  ° C. The fungal strains appearing were transplanted onto new medium until pure strains were obtained. The identification of fungi focused on macroscopic and microscopic characteristics according to the identification keys of Chase *et al.*, (2002). Macroscopic features included staining, thallus texture and rapid growth of the fungus, microscopic features on vegetative and reproductive structures.

### **Pathogenicity test**

The fresh tubers of yams were washed with tap water for 20 min and then cut into a 4 cm fragment. These fragments were treated as in the case of isolation, then a 1 cm opening was made in the center of minisets using a scalpel thus allowing introducing a fungal inoculum 5 mm in diameter of a culture of 7 days, the opening was thus closed the mini fragment used for opening. The tuber fragments were incubated in plastic bins with relative humidity at room temperature ( $28 \pm 2$ °C) for 10 days. After this incubation time, infection and symptoms of tuber disease were examined. Inoculated fragments of a PDA disk only served as a control.

### **Extraction and analysis of the chemical composition of the essential oil**

Extraction of the essential oil was done by hydro distillation using a Clevenger apparatus. The excess water in the oil was dried on a column of anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ), the pure oil was stored in dark bottles at 4°C.

The essential oil obtained was analysed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS). The oil was analysed

on a Varian CP-3380 GC with flame ionization detector fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB5, film thickness 0.25  $\mu\text{m}$ ); temperature program 50°-200°C at 5°C/min, injector temperature 200°C, detector temperature 200°C, carrier gas  $\text{N}_2$ , 1 ml/min. the linear retention indices of the components were determined relative to the retention times of a series of *n*-alkanes and the percentage compositions were obtained from electronic integration measurements without taking into account relative response factors.

GC/MS analyses were performed using a Hewlett-Packard apparatus equipped with an HP1 fused silica column (30 m x 0.25 mm, film thickness 0.25  $\mu\text{m}$ ) and interfaced with a quadrupole detector (GC-quadrupole MS system, model 5970). The column temperature was programmed from 70° - 200°C at 10°C/min; injector temperature was 200°C. Helium was used as the carrier gas at a flow rate of 0.6 ml/min; the mass spectrometer was operated at 70 eV.

The identification of the constituents was assigned on the basis of comparison of their retention indices and their mass spectra with those given in the literature (Adams, 2007)

### ***In vitro* antifungal activity of EO**

The antifungal activity of EO on mycelial growth was evaluated by the agar-embedding technique (method of nutrient poisoning) as described by Lalhoun (2004). It consisted to dissolve the EO in dimethylsulfoxide (DMSO) in proportions 1/9 (v / v) to obtain 10% EO stock solutions. This was done under a laminar flow hood (LFM 8472S) and around the flame of a Bunsen burner. The stock solutions of EO / DMSO previously prepared were supplemented in the superfluous culture medium (40-50°C.) so as to obtain decreasing concentrations of 1050, 1100, 1150 and 1175

ppm and of 4500, 5000, 5500 and 6000 ppm for *R. stolonifer* and *Penicillium sp.* respectively. The whole was homogenized and poured into 90 mm Petri dishes at a rate of 10 ml per dish, then a 5 mm mycelial disc of a 1 day pre-culture for *R. stolonifer* and 3 days for *Penicillium sp.* was seeded in the center of Petri dishes. The dishes were sealed with paraffin and incubated in an inverted position at  $28 \pm 2^{\circ}\text{C}$ . Petri dishes containing the PDA and the microorganism constituted the negative control 1; whereas those, who's EO was replaced by DMSO constituted the negative control 2. Those whose EO was replaced by Bomyl (at 1000 ppm recommended concentration) served as a positive control. The white control contained only the PDA and all tests were done in triplicate. Mycelial growth was monitored by measuring growth diameters after 3 h of time for 1 day for isolate 1 and every 24 h for 7 days for isolate 2, thus allowing the percent inhibition to be calculated according to following formula:  $\%I = ((D_t - D_e) / D_t) \times 100$ . %I: Percentage of inhibition;  $D_t$  (mm): Mean diameter of mycelial growth in the negative control box; ( $D_m$ ): Mean diameter of mycelial growth in the test box. The lowest concentration of EO that inhibits any visible growth of the germ is minimal inhibitory concentration (MIC). After transplanting the explants having exhibited a total inhibition on the PDA medium not supplemented with the EO, it was possible to determine the nature of the inhibition. A regrowth considered EO solution as fungistatic and, if not, EO solution as fungicidal.

### **Evaluation of antifungal activity *in situ***

It consisted to determine the antifungal potential of the essential oil (EO) on yams with two treatments: the preventive treatment and the curative treatment according to the modified protocol of Sameza *et al.*, (2016); however, it was made on the most sensitive

pathogen. Fresh yams were treated and fragmented as described for isolation and pathogenicity testing. The preventive test consisting to spray the openings made on the tuber fragments with 1000  $\mu\text{l}$  of EO at fixed concentrations from the *in vitro* tests carried out, then to introduce 100  $\mu\text{l}$  of a suspension of spores of the susceptible pathogen calibrated at 106 spores / ml, this after 1 hour of time thus allowing the oil to be well diffused beforehand in the cells of the tubers. As for the curative test, it consisted of inoculating 100  $\mu\text{l}$  of spore suspension in tuber fragments followed by the spraying of the EO at fixed concentrations after 48 h, the time required for the fungus to colonize the cells tuber. The concentrations tested are: 2350, 4700, 7000 and 8000 ppm. The whole was incubated in plastic tubs as shown in the pathogenicity test at  $28 \pm 2^{\circ}\text{C}$  for 10 days and all tests were done in triplicate. The fragments inoculated only by the isolates constituted the negative control and those inoculated with benomyl (tested at 1000 ppm recommended concentration) positive control. The spore suspension was prepared from the 10-day fungal cultures; this by adjusting 10 ml of sterile distilled water in the medium. Calibration of the spores was done by spore counting on microscopes in the Malassez cell. The measurement of the diameter and height of each rot caused by the isolates made it possible to calculate the volume of rot according to the formula of Masher and Defago (2000):  $V_p = \pi r^2 h$  where  $V_p$ : volume of rot;  $r$ : radius of rot;  $h$ : height of rot. This made it possible to calculate the percent reduction of rot according to the formula presented in the *in vitro* tests.

### **Statistical analysis**

The data was analyzed by Stat view software version 5.0 for Windows (SAS, Institute, Inc., USA). The ordered analysis of the variance (OANVA) was used to compare mean values

between several groups ( $n \geq 2$ ). Subsequently, Fisher's Pair Least Significant Difference (PLSD) test was used to compare two by two if the OANVA result was significant. The threshold of significance was set at  $p$ -value  $< 0.05$ .

## Results and Discussion

### Extraction yield and Chemical composition of the essential oil

The essential oil of our plant is of pale yellow color, of lower density than that of water (0.92) with a yield of 0.27%. This result is lower than that of Souhila and Souhila (2014) who obtained a yield of 0.96% after hydrodistillation extraction of the powders of the same plant harvested in the region of Tlemcen (Algeria). It is the same for Li Shiyu *et al.*, (2011) who had obtained a yield of 1.5 to 5% from the dried rhizomes of this plant. They reported that the yield of fresh rhizomes ranged from 0.16% to 1.94%, interval in which ours is included. The differences in levels of essential oil of *C. longa* would be due to several factors such as geographical origin, ecological factors including climate factors (temperature and humidity), plant organ, stage of growth, collection period, the conservation of the plant material, the method of extraction and the type of treatment undergone by the organ.

After analysis of the chemical composition of the oil, 30 compounds were eluted (Table I). In general, we have a small proportion of monoterpenes (19.37%) compared to sesquiterpenes (74.50%). Likewise, the hydrocarbon monoterpenes (14.53%) are superior to the oxygenated monoterpenes (4.84%). Moreover, oxygenated sesquiterpenes (64.56%) are predominant compared to hydrocarbon sesquiterpenes (9.94%). Thus, 5 major compounds were noted:  $\alpha$ -bisabolol (42.87%), E- $\beta$ -santalol

(15.3%),  $\beta$ -cymene (8.22%), Methyl-eudesmate (6.95%) and  $\gamma$ -terpinene (4, 75%). These results have similarities but also differences with those of some authors. This is the case, for example, of the work of Husain *et al.*, (1992) who found a predominance of sesquiterpenes with germacrene, bisabolol and guanine as the major compounds. In addition, Sharma and Tripathi, (2006) during their analysis obtained atlantone, 1,8 cineole and -phellandrene as major compounds. Li Shiyu *et al.*, (2011) demonstrated a predominance of monoterpenes in the leaves of the plant and sesquiterpenes for the dried rhizomes responsible for their taste and smell. They identified 54 bisabolans, 6 germacrene, 7 guanines, 4 selinans, 3 santalanes, 2 caryophyllene, 2 elamanes, the aristolene, the bergamotanes, the carabane, the cedrane, the himachalene as a group of major compounds. Gianni *et al.*, (2005), on the other hand, obtained significant amounts of  $\alpha$  et  $\beta$ -tumerone (19.82% and 35%) and richness in monoterpenes such as  $\alpha$ -phellandrene (20.4%), 1.8-cineole (10,3%) and terpinolene (6.19%).

These differences observed in the chemical composition of the essential oil from the same species could be due to the ecological conditions undergone by the plant, to the variety of the plant, somehow to intrinsic and extrinsic factors (Massoti *et al.*, 2003, Pibiri, 2005). These differences could also show the existence of a significant number of chemotypes of this species. Indeed, chemotypes of the same botanical species make it possible to obtain EOs of different chemical compositions (Pibiri, 2005).

### Antifungal activity of the essential oil

Our oil has inhibited the different pathogens significantly. Petri dishes showed a decrease in growth diameter as the concentration of

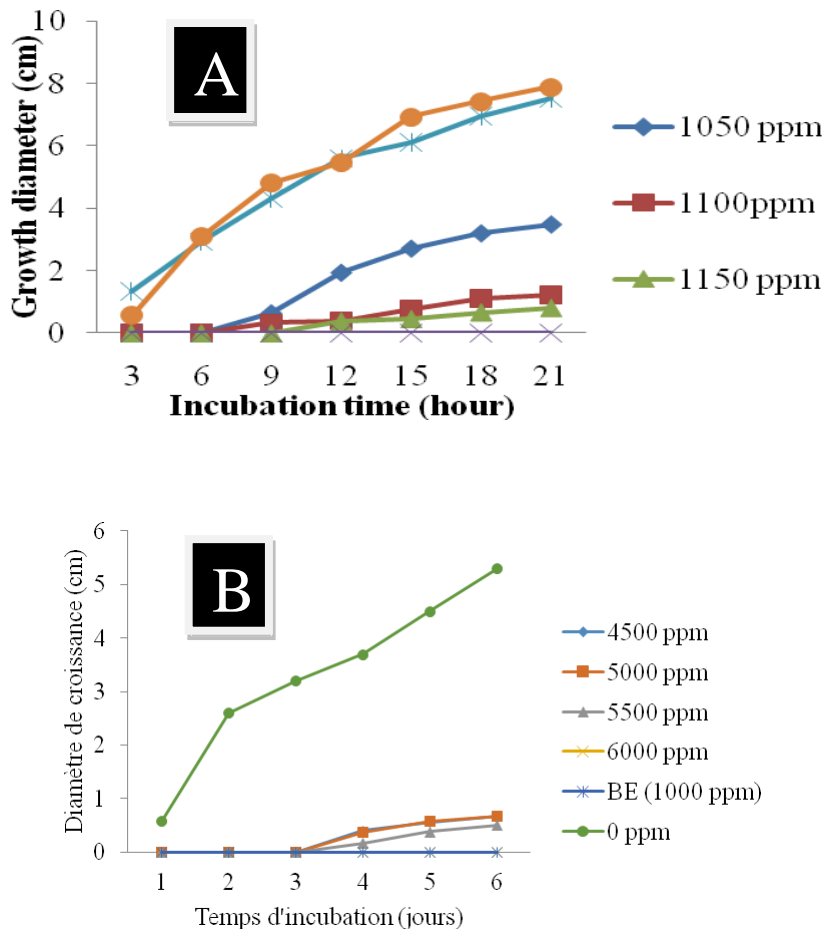
essential oil was high in both pathogens. Thus, the activity of the oil was inversely proportional to the growth diameter of the pathogens (Figure 1).

**Table.1** Chemical composition of the essential oil of *Curcuma longa*

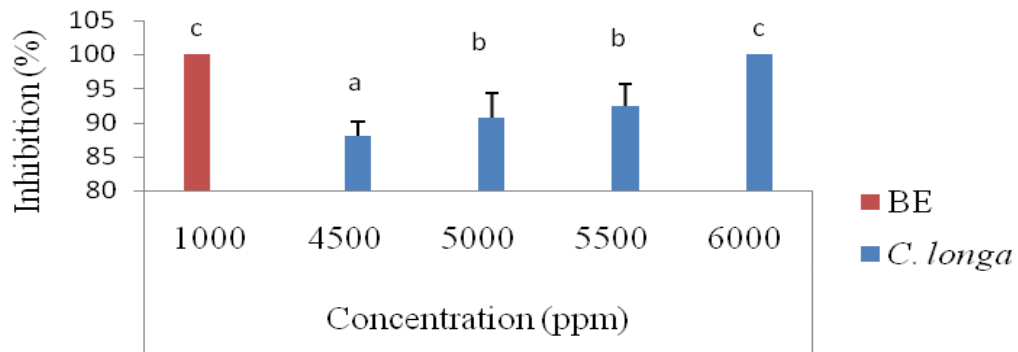
N°	KI	Chemical compounds	Percentage(%)
		<b>Monoterpenes</b>	<b>19.37</b>
		<b>Hydrocarbon monoterpenes</b>	<b>14.53</b>
1	933	$\alpha$ -pinene	0.28
2	984	$\beta$ -pinene	0.52
3	1005	D-3-carene	0.42
4	<b>1022</b>	<b>P-cymene</b>	<b>8.22</b>
5	<b>1053</b>	<b><math>\gamma</math>-terpinene</b>	<b>4.75</b>
6	1083	terpinolene	0.34
		<b>Oxygenated monoterpenes</b>	<b>4.84</b>
7	1030	1,8-cineole	2.59
8	1182	cis-pinocarveol	1.38
9	1239	carvone	0.28
10	1275	isopulphyl acetate	0.34
11	1304	terpinyl acetate	0.25
		<b>Sesquiterpenes</b>	<b>74.50</b>
		<b>Hydrocarbon Sesquiterpenes</b>	<b>9.94</b>
12	1377	$\alpha$ -copaene	0.43
13	1408	$\alpha$ -carophyllene	0.26
14	1430	$\beta$ -carophyllene	0.27
15	1469	$\alpha$ -farnesene	0.29
16	1475	$\alpha$ -humulene	0.76
17	1488	cis- $\beta$ -guaene	0.68
18	1504	$\beta$ -bisabolene	0.3
19	<b>1718</b>	<b>methyleudesmate</b>	<b>6.95</b>
		<b>Oxygenated Sesquiterpenes</b>	<b>64.56</b>
20	1561	(E)-nerolidol	0.75
21	1586	glunol	0.86
22	1630	$\alpha$ -eudesmol	1.03
23	1658	$\alpha$ -muurolol	0.53
24	<b>1685</b>	<b><math>\alpha</math>-bisabolol</b>	<b>42.87</b>
25	<b>1738</b>	<b>E-<math>\beta</math>-santalol</b>	<b>15.3</b>
26	1767	cedryle acetate	0.6
27	1783	tetradecenol	0.83
28	1786	$\alpha$ -cadinène	0.98
29	1805	farnesyl acetate	0.42
30	1853	benzyl benzoate	0.39

KI = Kovats index; NI = Not identified

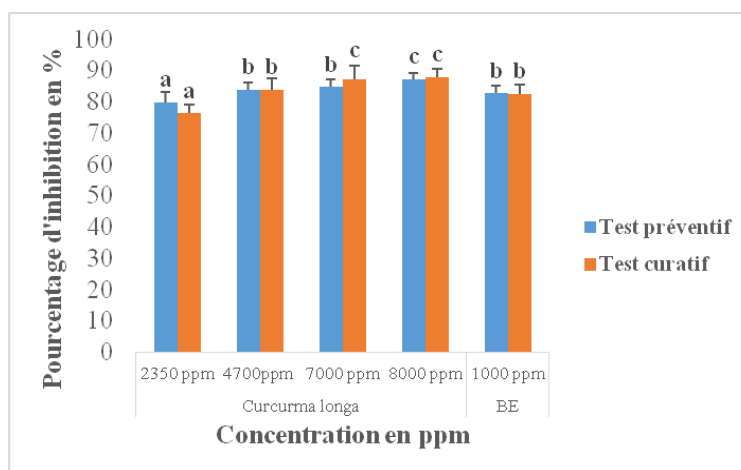
**Fig.1** Curve of evolution of the growth diameter of the two pathogens in the presence of the essential oil. A: on *R. stolonifer*; B: on *Penicillium sp.*



**Fig.2** Percent inhibition of essential oil on both pathogens based on concentrations



**b)** Percent inhibition of *C. longa* on mycelial growth of *Penicillium sp.* compared to benomyl

**Fig.3** Percentage of rot reduction caused by *R. stolonifer* based on essential oil concentrations

The coloration observed was also change during the growth of the fungi in the medium supplemented with essential oil compared to the control contained only the culture medium and the pathogens. With *Rhizopus stolonifer*, the mycelium became very transparent with no elevation of the latter. Growth remained flat; while with *Penicillium sp.*, the color has changed from greenish to yellow. However, although our oil inhibited the growth of *Penicillium sp.* at higher concentrations than *Rhizopus stolonifer*, it should be noted that inhibition diameters in *Penicillium sp.* were much lower than that of *R. stolonifer* (Figure 1). The calculation of the percentage inhibition after measuring the growth diameters allowed us to obtain total inhibition corresponding to the MIC at 1175 and 6000 ppm for *R. stolonifer* and *Penicillium sp.* respectively. After transfer of the fungal explants corresponding to the MIC in new culture medium, our oil proved to be fungicidal for both pathogens. Figure 2 illustrates the inhibition percentages as a function of EO concentration. This inhibition increases as the concentration of EO is high. Thus, it will be said that the activity of the EO is proportional to the concentration. Several studies have reported the activity of *C. longa* on pathogens of medical as well as food this would therefore justify the activity obtained

with our pathogens; this is the case of Souhila and Souhila (2014) who evaluated the activity of this oil on two plant parasitic fungi: *Boirytis cinerea* and *Fusarium oxysporum*. They obtained an inhibition diameter of 20 and 21.83 mm for the two pathogens respectively at the dose of 10 and 20  $\mu$ l of EO; However, Gurdip *et al.*, (2008) found 33.97% and 26.76% inhibition for this oil on two kinds of aflatoxin-producing fungi on food; *Aspergillus* and *Penicillium* at concentrations of 10000 ppm and 6000 ppm respectively. *R. stolonifer* being more sensitive than *Penicillium sp.*, *in vitro* activity of the oil was evaluated only on the latter. A significant reduction of rot volume was observed on tuber fragments treated with essential oil compared to the untreated control. Inhibition of 87.26% against 87.80% respectively in the preventive and curative tests was obtained at 8000 ppm (Figure 3). Fisher's post hoc doc test did not show any significant difference (P 0.0001) between both treatments performed on minisets of tubers; similar results are expected for both tests with benomyl. However, at 4700 ppm a reduction of 83.89% and 83.8% respectively was obtained for both tests, similar activity with the reference fungicide which reduced by 82.65% and 82.5% respectively. From the activity of benomyl to the recommended



concentration, there is a significant difference in action (P 0.0001) with our oil at 8000 ppm, the oil being more efficient.

The activity of the essential oil of the studied plant would be justified by its chemical composition, our oil being rich in terpene compounds (MTH and MTO). Many researchers have shown that terpenes and their oxygenated derivatives are the main components of essential oils. These compounds have a high inhibitory potential on pathogenic microbial strains like fungi (Hossain *et al.*, 2008, Sameza *et al.*, 2016). Minor constituents of essential oils especially could also be involved in antifungal activity (Bajpai *et al.*, 2010, Jazet *et al.*, 2013). Thus, the compounds corresponding to the majority of EO products are not necessarily more effective than the oils themselves. Thus, the compounds corresponding to the majority of HE products are not necessarily more effective than the oils themselves. The mechanism of action of essential oils against mold is not fully understood until now. But some authors have given several suppositions according to their observations. Most studies report their effects on cell membranes. In fact, the active compounds attack the cell wall and membrane, thereby affecting the permeability and release of intracellular constituents, also interfering with membrane function (Rasooli and Owlia 2005, Pinto *et al.*, 2006, Carmo *et al.*, 2008, Koul *et al.*, 2008, Bajpai *et al.*, 2008, Bajpai and Kang, 2010). In view of the sensitivity of *R. stolonifer* to our species compared to that of *Penicillium sp.*, it can also be deduced that the active principle of HE lies in their majority constituents and that the sensitivity of the strains to a given HE differs according to the genus and the species, as it may vary according to other criteria. This is justified by the nature of the wall of fungal strains which consists of a complex network of proteins and polycarbohydrates and which varies in composition according to the fungal

species. Disturbance of this matrix can result in a defective wall, which becomes sensitive to osmotic lysis and sensitive to antifungal agents such as essential oils among others (Yen and Chang, 2008). In most cases, the mycelium cells no longer have a cytoplasm or have a depleted cytoplasm of organelles. The color characteristic of many molds has been shown to be due to conidium pigmentation (Wiley, 2005). As a result, the color change of both pathogens after application of *C. longa* essential oil may also be due to its effect on conidium. Similarly it has been found that essential oils can cause morphological changes including insufficient sporulation, loss of pigmentation, abnormal development of conidiophores and deformation of hyphae (Rasooli and Abyaneh, 2004, Sharma and Tripathi, 2006). Reducing the amount of rot before or after applying our essential oil would have had an effect on the inhibition of enzymatic reactions caused by fungi, thus allowing starch degradation; constituent elements of yam tubers in essential molecule for their development which is glucose.

In conclusion, the rhizome of the red ginger plant has revealed richness in essential oil, with for chemical composition a predominance of OST compared to HST. This oil significantly inhibited the growth of pathogens *in vitro* and significantly reduced the rot caused by *Rhizopus stolonifer* in both tests (preventive and curative). Thus the essential oil of *Curcuma longa* could be used as a biofungicide to preserve white yam tubers from post-harvest rot in our locality.

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