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Nonlinear regression application in *Aspergillus flavus* radial mycelial growth inhibition by essential oils of *Piper guineense* Schum and Thonn., *Zingiber officinale* roscoe and *Monodora myristica* (Gaertn) Dunal

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Abstract
The objective pursued by the present study is to contribute to application of nonlinear regression in Aspergillus flavus radial mycelial growth inhibition by essential oils extracted from three plants: *Piper guineense*, *Zingiber officinale* and *Monodora myristica*. The techniques of hydrodistillation, poisoning and microdilution followed by seeding in an agar medium free of essential oil were used.
The analysis of the dynamics of the diameters obtained under the effect of three essential oils in increasing concentrations was carried out by the nlm () function of the R software. It made it possible to establish the sigmoidal curves characterized by three growth simulation parameters: Asym, the asymptotic diameter, Xmid, the time at Asym/2 and Scal, the spread over time. It also made it possible to simulate growth beyond the incubation time.
Partial inhibition is marked by the non-zero values of these simulated parameters and total inhibition by their zero value. These zero values correspond to the minimum inhibitory concentration. The essential oil of *Piper guineense* exerts a fungistatic power with partial inhibition over the entire range of concentrations studied. The essential oil of *Zingiber officinale* exerts a fungicidal power with partial inhibition at concentrations less than 0.25% and total inhibition at concentrations greater than 0.5% and the essential oil of *Monodora myristica* exerts a fungistatic power with growth stimulation at concentrations less than 0.1%, partial inhibition between 0.25 and 1.5% and total inhibition above 1.5%.

Keywords: Antifungal activity, Nonlinear regression, essential oils, *Piper guineense*, *Zingiber officinale*, *Monodora myristica*; *Aspergillus flavus*

Introduction
Molds growth inhibition by essential oils interests several researchers [1-3]. These hopeful studies show the effectiveness of most essential oils extracted from aromatic plants in inhibiting many molds growth because of diversity chemical substances that constitute them [4-7]. Some authors believe that they can be used as biopesticides [8]. Molds are reputed to be dangerous agents of various organic substrates degradation but also considered as useful allies [9]. By their harmful effects, they are capable to damaging plants in full cultivation [1, 10], they are responsible for post-harvest losses [11-12]; they contaminate and spoil food by producing secondary metabolites called mycotoxins. However, they are also used in food industry and environment.
To demonstrate this antifungal activity, the most widely technique used is the poisoning method or dilution in agar method which makes the possibility to establish antifungal spectrum by calculating the inhibition percentage of mycelial growth after the incubation time [13-14]. With this poisoning method, the minimum inhibitory concentration can be estimated [9]. Micro- and macro-dilution methods followed by inoculation in agar medium free of essential oil are also used to determine the minimum inhibitory concentration and the minimum fungicide concentration [14].
Dongmo *et al.* [13] proposed *in vitro* inhibition curves of the mycelial growth of *Phaeoramularia angolensis* by essential oils extracted from the fruits of six species of the *Citrus* genus. After analysis by Duncan's multiple comparison method at the 5% threshold, they discriminated the diameters formed by the molds during 50 days of incubation.

Sokamté *et al.* [9] used the poisoning method with *Xylopia aethiopica* essential oil diluted in DMSO and monitored the antifungal index of this essential oil every 48 hours on certain strains of *Aspergillus* and *Fusarium oxysporum*.
Vawazola *et al.* [14] used the poisoning method which consisted of adding undiluted essential oils, respectively of *Cyperus articulatus* and *Xylopia aethiopica*, to Sabouraud Dextrose Agar medium at different concentrations. He monitored daily the percentage of inhibition of mycelial growth or the antifungal spectrum (index) of the *Aspergillus flavus* strain.
For the aforementioned studies, results are presented as histograms showing the percentage inhibition of mycelial growth by essential oils over the incubation time.
The present study offers another way of presenting the dynamics of inhibition of the growth of the fungal strain *Aspergillus flavus*, by the essential oils of *Piper guineense*, *Zingiber officinale* and *Monodora myristica*.
This new presentation in a nonlinear sigmoid regression curve adjusted by the least squares method using the nlm() function of the R software, of the diameters measured on different mycelia formed by *A. flavus* under the effect of a poisoning on Sabouraud Dextrose agar in Petri disks at respective increasing concentrations of three essential oils throughout the incubation period.
Nonlinear regression was used by Lukombo *et al.* [14] and Kabongo *et al.* [15] to simulate growth and leaf area index, which express the physiological and phenotypic processes of maize varieties in sub-Sahelian regions of Africa.
Microbial growth, being a physiological process that depends on nutrients and inhibitory substrates present in the non-renewed culture medium, is best represented by a sigmoid curve that can be simulated by the SSlogis function implemented in the R software.
The aim of the present study is to apply the nonlinear regression in the radial mycelial growth inhibition of *Aspergillus flavus* by three essential oils: *Piper guineense*, *Zingiber officinale* and *Monodora myristica*.

Materials and Methods

Materials

Plant materials: The plant materials consist of three plants organs: *Piper guineense* Schum. and Thonn. (Seed), *Zingiber officinale* Roscoe (Rhizome) and *Monodora myristica* (Gaertn) Dunal (Seed).

Fungal strain: Fungal isolate culture of *Aspergillus flavus* was come from collection of the Microbiology Laboratory of the Agronomy Faculty of Kinshasa University.

Methods

Preparation of plant material: The preparation consisted of the sorting, cutting and grinding of the plant material.

Determination of the water and organic volatile component content and the dry matter content: The water content and organic volatile component (WOVC) expressed in percentage, is determined by baking at 105±1 ° C according to French standard NF V 03-909 quoted by Vawazola *et al.* [17]. The dry matter content of the sample is obtained from the % WOVC value: % Dry Matter = 100 - % WOVC.

Extraction of essential oils by hydro distillation: An amount of 100-350 g of a mash of each plant was subjected to hydro distillation for 2-3 hours. The essential oils obtained were dried by using sodium sulfate and subsequently weighed they were finally kept in brown bottles kept in dark.

Determination of the essential oil yield: The essential oil yield was calculated using the followed equation.

$$YEO = \frac{MEO}{MDP} \times 100$$

Where YEO: Essential oil yield (%); MEO: Mass of essential oil (g) and MDP: Mass of the dry plant material (g).

Agar dilution method: Antifungal activity of the two essential oils was determined by the agar dilution method proposed by Udomslip *et al.* [11] and Vawazola *et al.* [14]. Precise quantities of the two essential oils were previously deposited in sterile and empty Petri plates. Precise volumes of the Sabouraud 4% agar, in supercooled form, were cast in these different Petri plates with the aim of obtaining 15 ml of homogenized mixtures concentrated respectively in 0, 0.1, 0.25, 0.5, 1, 1.5, 2.5 and 3.5% (v/v) of each essential oil. After solidification of the mixture, a loop of the *A. flavus* strain (4-5 mm diameter) from 3-5 days old culture was placed in the center of all Petri plates. The experiment was carried out in 3 repetitions. All cultures were incubated at room temperature (27±2 °C) for 7 days. The colony diameter was measured.

Data process

Effects of increasing essential oils concentrations on mycelial growth of *Aspergillus flavus:* The effect of three essential oils on radial mycelial growth of *A. flavus* has been quantitatively assessed by three independent parameters: (1) the size or the upper or asymptotic limit (Asym), (2) the rate or time needed for growth to take place (xmid) and (3) the shape or extent of growth spread over time (1/scal); this is the slope at the inflection point.

It can be well modeled by the logistic curve
Diameter = Asym/(1+exp((xmid)/scal)).

The function is implemented in R software by the SSlogis function [18]. The nonlinear model parameters estimations are determined by least squares and by nls function [19]. On each curve, the situation observed until the 7th day was given. A simulation of what would happen until the 10th day was also given.

Broth macro dilution method followed by inoculation in agar medium free of essential oil: The minimal inhibitory concentration (MIC) and the minimal fungicide concentration (MFC) were determined by serial broth macro dilution method followed by inoculation in agar medium free of essential oil proposed by Mann and Markham [4], Bouzouita *et al.* [2] and Vawazola *et al.* [14]. Different preparations of 3 mL volume were performed in different test tubes with Czapek broth modified by addition of 0.15% agar, inoculum (120 µL of 10^3-10^4 spores/mL) and pure essential oil in order to obtain concentrated preparations at 0, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 1, 1.5, 2.5, and 3.5% (v/v) essential oil. After homogenization, the different test tubes were then incubated at room temperature for 7 days. And then, an inoculating loop of each preparation was inoculated by streaking respectively at the center of a sterile Petri plate in free-oil Sabouraud's CAF 4% agar medium in 3 repetitions. The plates inoculated were subsequently incubated at room temperature for 7 days.

Types of power exerted by each essential oil

The types of power exerted by each essential oil on *Aspergillus flavus* is determined by calculating the ratio MFC/MIC. The power exerted is fungistatic when this ratio is greater than 4 whereas it is fungicidal when this ratio is less than or equal to 4 [20, 14].

Results

Yield extraction of the three essential oils by hydro distillation: Extraction with hydro distillation made from valuable organs of three plants organs led to the production of essential oil with varying yields. Table 1 gives, for each plant, the organ used and the average yield of essential oil obtained.

Table 1: Name and Organ used of three plants and Average yield of extraction (%)

Plant Name	Organ used	Essential oils yield (%)
Piper guineense	Seeds	4.9378±2.5468
Zingiber officinale	Rhizomes	1.3064±0.0289
Monodora myristica	Seeds	1.5721±0.1509

Effects of increasing essential oils concentrations on mycelial growth of *Aspergillus flavus:* Figures 1, 2 and 3 show growth inhibition curves of *A. flavus* under effect of respective increasing concentrations of *Piper guineense*, *Z. officinale* and *Monodora myristica* essential oils and Table 2, 3 and 4 illustrate three simulated parameters evolution of each essential oil.

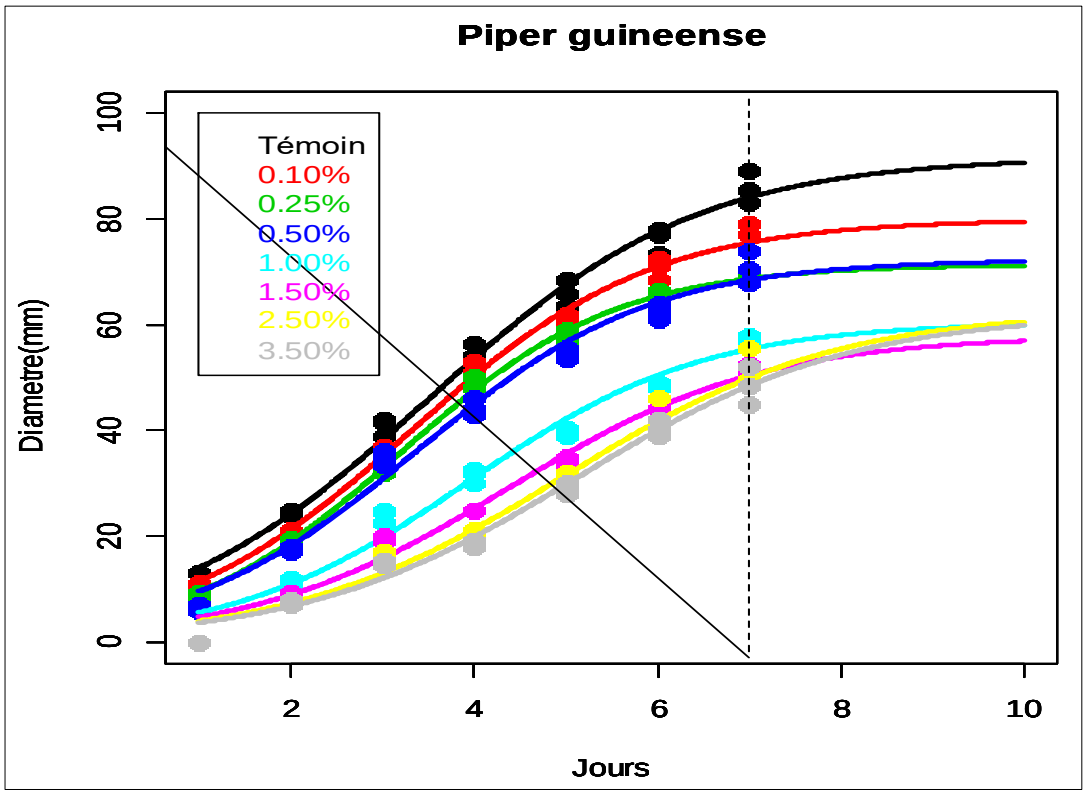

Fig 1: Effects of increasing concentrations of *Piper guineense* essential oil on *A. flavus* radial mycelial growth

Table 2: Simulated parameters evolution of mycelial growth in *A. flavus* under effect of *P. Guineense* essential oil

Concentration (% v/v) of *P.guineense* essential oil	Simulated parameters		
	Asym (mm)	Xmid (jour)	Scal (%)
0	91.70±3.11***	3.50±0.14***	4.46±0.10***
0.1	79.92±0.18***	3.32±0.09***	1.30±0.07***
0.25	71.37±0.81***	3.19±0.04***	1.17±0.04***
0.5	72.30±2.424***	3.38±0.13***	1.26±0.10***
1	60.41±3.60***	3.93±0.23***	1.28±0.15***
1.5	57.86±3.99***	4.36±0.26***	1.39±0.15***
2.5	62.63±7.52***	4.98±0.44***	1.48±0.21***
3.5	62.04±6.25***	5.12±0.37***	1.48±0.17***

Legend: *** = Very hyghly significant

Table 3: Simulated parameters evolution of mycelial growth in *A. flavus* under effect *Z. officinale* essential oil

Concentration (% v/v) of *Z. officinale* essential oil	Simulated parameters		
	Asym (mm)	Xmid (day)	Scal (%)
0	91.70±3.11***	3.50±0.14***	4.46±0.10***
0.1	93.36±3.69***	4.83±0.14***	1.38±0.07***
0.25	97.98±22.55***	7.21±0.59***	1.39±0.13***

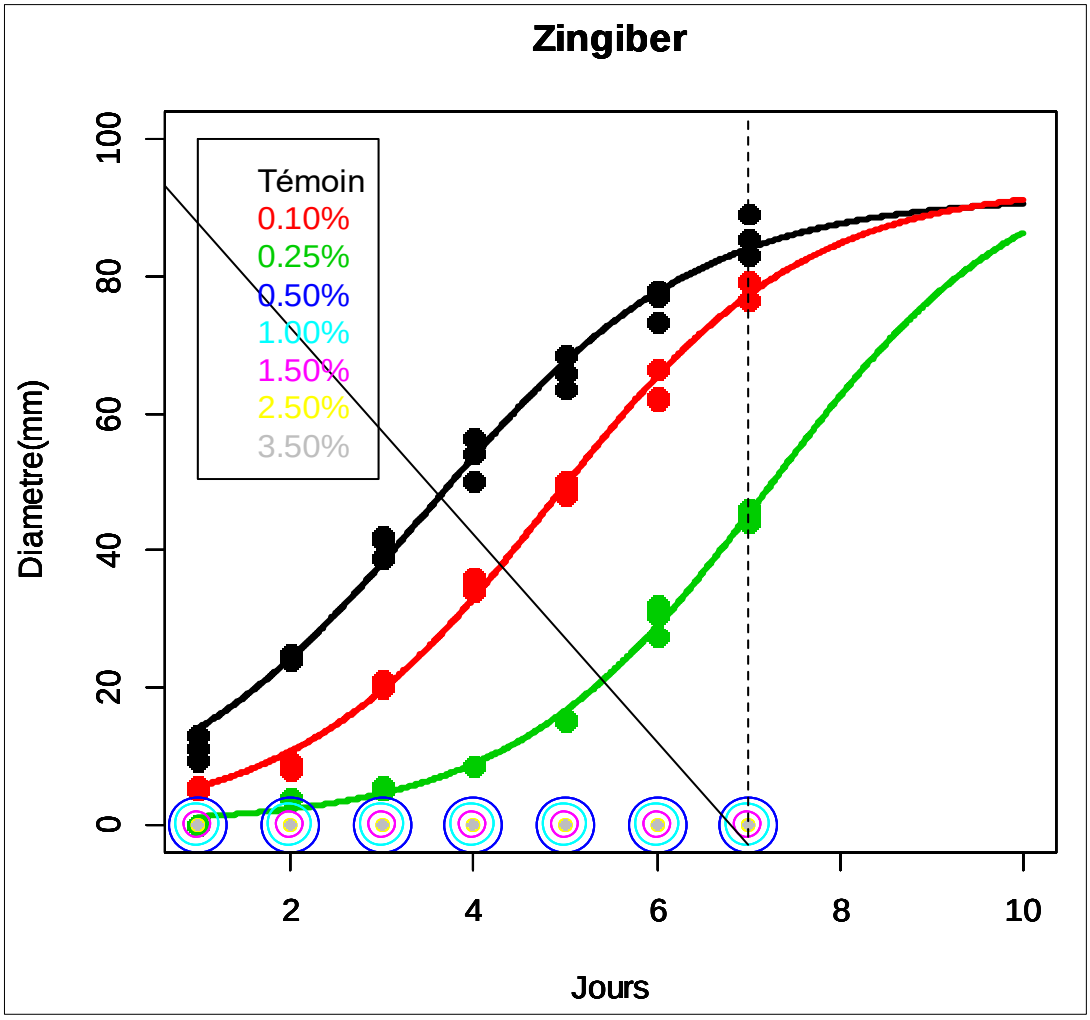

Fig 2: Effects of increasing concentrations of *Zingiber officinale* essential oil on *A. flavus* radial mycelial growth

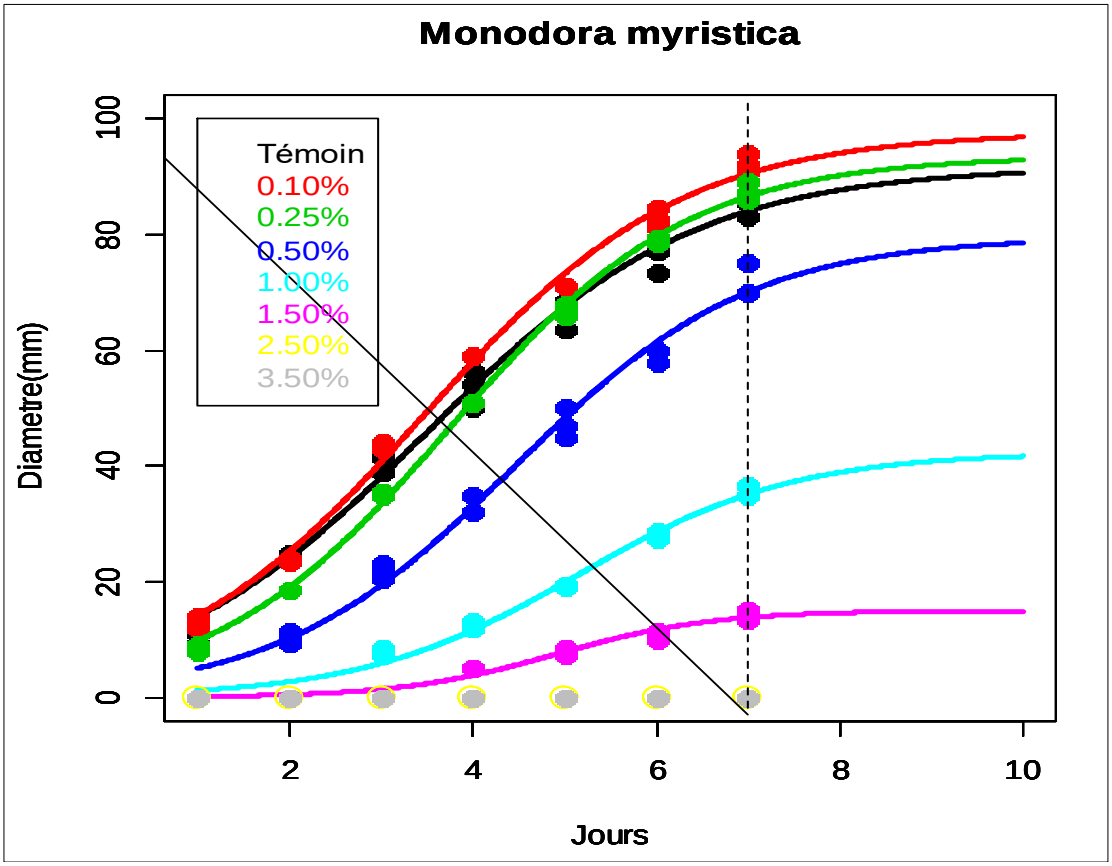

Fig 3: Effects of increasing concentration of *Monodora myristica* essential oil on *A. flavus* radial mycelial growth

Table 4: Simulated parameters evolution of mycelial growth in *A. flavus* under effect of increasing concentrations of *Monodora myristica* essential oil

Concentration (% v/v) of *M. myristica* essential oil	Simulated parameters		
	Asym (mm)	Xmid (days)	Scal (%)
0	91.70±3.11***	3.50±0.14***	4.46±0.10***
0.1	97.69±2.14***	3.47±0.09***	1.39±0.07***
0.25	93.55±1.29***	3.76±0.05***	1.29±0.04***
0.5	79.56±4.19***	4.45±0.19***	1.28±0.11***
1	42.33±3.13***	5.14±0.23***	1.18±0.12***
1.5	15.00±1.07***	4.91±0.19***	0.85±0.13***

Minimal inhibitory concentration (MIC), Minimal fungicide concentration (MFC) and essential oils Power on *A. flavus*

Minimal inhibitory concentration (MIC), minimal fungicide concentration (MFC), MFC/MIC ratio and power exerted by each essential oil on *A. flavus* are summarized in Table 5 below.

Table 5: MIC, MFC, Ratio MFC/MIC and Essential oils Power on *A. flavus*

Essential oils	MIC (%)	MFC (%)	Ratio MFC /MIC	Essential oils Power
Piper guineense	0.5	>3.5	>7.5	Fongistatic
Zingiber officinale	0.25	0.5	2	Fongicidal
Monodora myristica	1.5	> 3.5	> 4	Fongistatic

Discussion

The extraction yield of these essential oils varies from one species to another. The essential oil extraction yield depends on several factors, including cultivation practices, harvesting season and stages, storage conditions and extraction technique. This explains the disparity in the extraction yield values calculated for each species.

The biological activity of an essential oil is to be related to its chemical composition, the functional groups of major compounds (alcohols, phenols, terpene and ketone compounds) and the possible synergistic effects between the components. Thus, the nature of chemical structures that constitute it, but also their proportions, play a determining role. However, it is likely that minority compounds act synergistically. In this way, essential oil value depends on its "Totum", i.e. all of its components and not only on its majority compounds [21].

One of the explanations for low antifungal activity or absence of antifungal activity of essential oils is due to the more or less advanced biodegradation of their components. Indeed, some fungal strains can consume terpenes as a carbon source, degrade or transform them [22-23].

Piper guineense essential oil exerts a weak inhibition of *A. flavus* mycelial growth over the entire range of concentrations in the present study (Figure 1) and does not contain good antifungal actives capable of disrupting mold growth. Tankam and Ito [24] and Morufu *et al.* [25] reported the presence of linalool and dimethoxytoluene as major compounds in this essential oil. Jirovetz *et al.* [26] reported that β-caryophyllene is the majority compound and linalool cited among the minority compounds.

The growth inhibition decreases with incubation time regardless of level concentrations. This may be due to the lack of antifungal active ingredients and the ability of the mold to assimilate essential oil constituents. At concentrations below 0.25%, Asym, Xmid and Scal values (Table 2), being lower than controls and decreasing with increasing essential oil concentration, indicate partial inhibition (weak antifungal activity of *Piper guineense*'s essential oil), early spore germination and resumption of cell division. At concentrations above 0.25%, Asym decreases, Xmid and Scal increase. Although the concentration increases, the simulation shows that inhibition remains partial and decreases with the incubation time, probably due to the volatile nature of the antifungal active ingredients, with a weak influence on the resumption of cell division.

Figure 2 above shows that antifungal activity of *Zingiber officinale* essential oil on *A. flavus* growth manifested by partial inhibition at concentrations less than or equal to 0.25% and total inhibition at concentrations greater than 0.5%. This partial inhibition decreases with incubation time probably due to the volatile nature of essential oil antifungal active ingredients. The *Z. officinale* essential oil CCM profile established by Vawazola *et al.* [17] confirms the spot presence in R_f eugenol, which is renowned for its antimicrobial effects. Hydrocarbon sesquiterpenes (zingiberene, camphene, α-farnesene, β-sesquiphellandrene) constitute the majority fraction [27]. The CG/MS analysis of *Zingiber officinale* essential oil carried out by El-Baroty *et al.* [28] reported the absence of eugenol and the presence, in high quantity, of hydrocarbon sesquiterpenes (β- sesquiphelladrene, cis-caryophyllene, zingiberene, α-farnesene, α and β-bisabolene), in minor quantities, monoterpenes (mainly limolene) and, in trace amounts, oxygenated compounds such as octanol, linalool, geraniol, geranial, α-terpinen-4-ol, 1,8-cineole and others. Azam *et al.* [29] cite sesquiterpenes as the main compounds alongside monoterpenes. Meliani *et al.* [30] report that *Z. officinale* essential oil has as major constituents citral at 30.8% and zingiberene at 17.07%. Dhanik *et al.* [31] quote hydrocarbon and oxygenated monoterpenes as cineol, borneol, geraniol, limonene, linalool, zingiberol.

Analysis of the simulated parameters in Table 3 (Asym and Xmid greater than controls) shows that the *A. flavus* growth resumption (or spore germination) is delayed at concentrations less than or equal to 0.25%; but beyond the incubation time, growth would be stimulated such that the maximum diameter of the mold under treatment would be greater than that of the control. In this situation, *Z. officinale* essential oil would be considered as a growth factor. The absence of the simulated parameters coincides with the minimal inhibitory concentration which temporarily or totally prevents spores' germination or cell division resumption.

According to Figure 3 and Table 4 above, *Monodora myristica* essential oil effect on *A. flavus* mycelial growth is characterized by: (1) Stimulation of *A. flavus* growth at a concentration of 0.1%: Asym at this concentration is greater than control, Xmid and Scal smaller than control. The *Monodora myristica* essential oil constituents behave as growth factors for *A. flavus* growth. Gabriel *et al.* [32] noted stimulating effects of essential oils on microbial growth. (2) At 0.25%, growth inhibition is low (Xmid increases and Scal decreases), this inhibition disappears with incubation time

after four days, probably due to the volatile nature of some antifungal active ingredients present in this essential oil; beyond 4th day of incubation, growth is stimulated until it exceeds the control. (3) Between 0.5 and 1.5% (Xmid increases and Scal decreases), inhibition is partial and decreases with incubation time, probably due to the volatile nature of the antifungal active ingredients of this essential oil. Indeed, according to Lamaty *et al.* [33] and Bakarnga-Via *et al.* [34], *M. myristica* essential oil consists mainly of hydrocarbon and oxygenated monoterpenes which are volatile. Cimanga *et al.* [35] reported the presence of cineol, β-pinene, α-terpineol, p-cymene, myrcene and limonene, as abundant compounds in the oil of *Monodora myristica*. (4) From 2.5% and above (Xmid increases and Scal smaller), spores germination cell division resumption delayed, *A. flavus growth* of is totally inhibited during incubation period.

The absence of the simulated parameters coincides with the minimal inhibitory concentration which temporarily or totally prevents spores' germination or cell division resumption. Essential oil action on *A. flavus* can be inhibitory (fungistatic) or lethal (fungicide). The fungistatic power, responsible for reversible cell damage, is exerted by *Piper guineense* and *Monodora myristica* essential oils; on the other hand, the fungicidal power, accompanied by irreversible cell damage, is exerted by *Zingiber officinale* essential oil (Table 5). The fungicidal power would result from the irreversible damage caused by phytochemical compounds of these essential oils on the cell membrane causing its permeability [36-38] (Cox *et al.*, 2000; Pawar and Thaker, 2006; Dutta *et al.*, 2007) and by weakening enzymatic processes involved in energy production and in structural components synthesis. In fungi, the mechanism of essential oils phenolic compounds action is mainly based on fungal enzymes inhibition containing the thiol group (SH) in their active site [39].

Conclusion

The effects of three essential oils increasing concentrations under study manifest themselves differently on three simulated parameters that characterize the different growth curves. Total inhibition is characterized by absence of growth curves (absence of simulated parameters). Partial inhibition is characterized by a sigmoidal curve whose three simulated parameters determine essential oils effectiveness on *A. flavus* strain under study. For Asym and Scal values lower than control and Xmid higher than control, greater is essential oil antifungal activity.

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