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In vitro evaluation of antifungal activity of *Aloe vera, Moringa oleifera* and *Newbouldia laevis* on the Strain of *Lasiodiplodia theobromae* in Region of Kisangani / DR CONGO

Kwembe JTK[1], Mbula JP[1], Onautshu O[2*], Mpiana PT[3], Haesaert G[4]

[1]Department of Chemistry, Faculty of Sciences, University of Kisangani, B.P. 2012 Kisangani, DR Congo
[2]Department of Biotechnological Sciences, Faculty of Sciences, University of Kisangani, B.P. 2012 Kisangani, DR Congo
[3]Department of Chemistry, Faculty of Sciences, University of Kinshasa, B.P. 190 Kinshasa XI, DR Congo
[4]Department of Plants and Cultures, Faculty of Biosciences Engineering, University of Ghent, Ghent 9000, Belgium

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***Corresponding author:** Didy Onautshu Odimba

Abstract

This work is part of the fight against *in vitro* the growth of *Lasiodiplodia theobromae* with some medicinal plants including *Aloe vera*, *Moringa oleifera* and *Newbouldia laevis*. This fungus causes brown cocoa rot in the Kisangani region. The antifungal activity of the crude, aqueous, ethanolic and ethereal extracts of fresh and dried leaves of the plants studied were evaluated *in vitro* by six repetitions on Potato dextrose agar medium. After two days of incubation, the raw extract of the fresh leaves of *M. oleifera* inhibited the growth of *L. theobromae* by up to 74.1% followed by that of *A. vera* (29.6%) and *N. laevis* (14.7%). The aqueous, ethanolic and ethereal extracts of the fresh leaves of *M. oleifera* revealed respective inhibition percentages of 43.0; 53.3 and 71.1. As for the extracts of the dry leaves, *A. vera* was active with its ethereal extract (58.1%) and ethanolic extract (56.7%). Notwithstanding, *M. oleifera* remains the very active plant with an excellent inhibition rate of 74.1% having considerably slowed down the maximum growth time of *L. theobromae* by reducing it from two days for the control to six days for the raw extract. Although the plant extracts studied have a fungistatic effect, their purification by extraction of phytochemical groups in our next studies, could reveal their respective fungicidal properties.

Keywords: Activity, antifungal, *Aloe vera*, *Lasiodiplodia theobromae*, *Moringa oleifera*, *Newbouldia laevis*, Kisangani.

INTRODUCTION

Agriculture, one of the main sources of income in the world and particularly in sub-Saharan Africa [1], faces several constraints including that caused by parasites thus reducing the yield of the harvest [2]. *Theobroma cacao* is planted in several botanical homes around the world, particularly in the tropical zone for its beans used in the manufacture of chocolate and the extraction of vegetable fats known as butters [3]. However, around the 1980, cocoa orchards displayed an unusual dieback disease, observed in all cocoa producing areas of Cameroon, affecting 100% of cocoa trees in certain plantations [4].

Lasiodiplodia theobromae (syn. *Botryodiplodia theobromae*), a common endophyte and an opportunistic pathogen on more than 500 tree species in tropical and subtropical regions [5], was first reported on cocoa in Cameroon in 1895 [6, 7]. This fungus is the basis of the decline of the cocoa tree and is often isolated from twigs, bark, vascular tissue and the affected cocoa pod [4]. *L. theobromae* is becoming an increasingly dangerous threat to cocoa cultivation not only in India [8], Cameroon and Western Samoa, the Philippines [9], but also in the region of Kisangani at Democratic Republic of Congo.

Traditionally, to fight against certain phytopathogenic germs, farmers most often use chemical synthesis products as pesticide. Unfortunately the use of these is not without danger for the health of farmers and consumers [10]. In addition, the new regulations discourage the use of synthetic fungicides for fear of the appearance of new resistant pathogenic germs [11, 12] and thus these rules limit the potential risks for human health as well as environmental pollution [13, 14].

Certain approaches are in the making based on biological control by the isolation and screening of microbial antagonists as biological agents acting against *L. theobromae* [9]. But in the region of Kisangani, no initiative has been reported so far to limit or stop the invasion of cocoa fields by *L. theobromae* by using plants as a means of control, so this work serves as a light in this field.

Several studies have shown that plants have an antimicrobial and / or antifungal effect on certain phytopathogenic or zoopathogenic germs. Natural bioactive products can be extracted from plant species with significant antifungal activity, including *Aloe vera* [15], *Moringa oleifera* [16] and *Newbouldia laevis* [17].

Thus, this investigation consisted to evaluate *in vitro* the antifungal activity of the extracts of these plants on the strain of *L. theobromae* responsible for brown cocoa rot. This work is part of the objective of meeting the current trend which consists of the formulation of biofungicides from plants which naturally are biodegradable, effective, and accessible to all budgets and in addition are reputed as renewable resources.

MATERIALS AND METHODS

Study environment

This work was carried out in the Kisangani region, capital of the Tshopo province in the Democratic Republic of Congo (Figure 1). The city of Kisangani is located at 0º31 'north latitude, from the Equator (57 km), 25º11' east longitude from the Greenwich meridian, and 428 meters above sea level [18, 19].

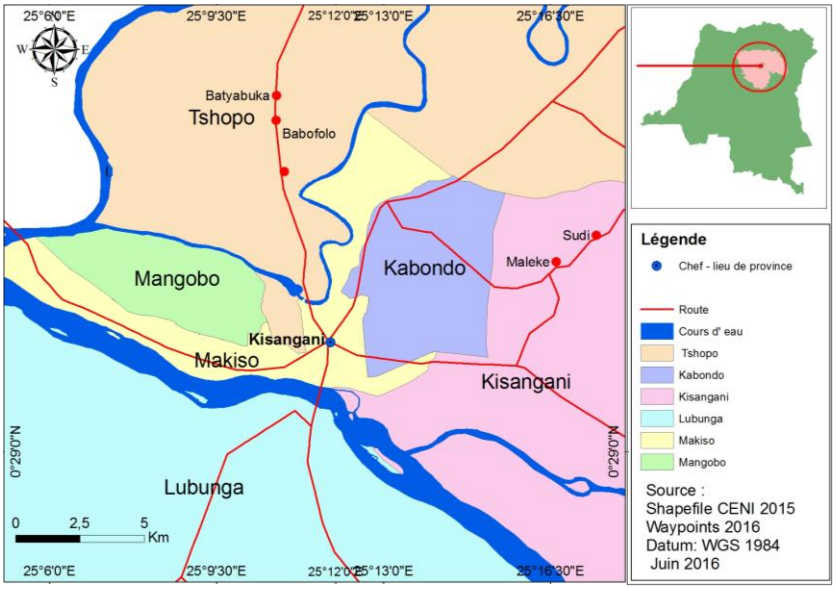

Fig-1: Map of the Kisangani region

Plants and treatment

The leaves of *A. vera*, *M. oleifera* and *N. laevis* were harvested in the region of Kisangani to serve as plant material after being identified by the Herbarium service of the Faculty of Sciences of the University of Kisangani. Three different batches were made up for each plant in order to obtain three different types of extracts: raw extract from fresh pressed leaves; extract of fresh macerated leaves and extract of dry macerated leaves.

For this, 10 g of plant materials (fresh leaves or dry leaf powder) were macerated for 48 hours in 50 ml of solvent consisting of distilled water for the aqueous extract, 95% ethanol for the ethanolic extract and diethyl ether for the ethereal extract. The macerated filtrates were concentrated in an oven at 40 ° C, and at a rate of 2 ml of the concentrate from 10 ml of the macerated filtrate.

Obtaining the fungal strain

The *L. theobromae* strain was isolated from cocoa pod naturally affected by brown rot. These pods were harvested directly from the tree in the cocoa plantations of Bengamisa (CABEN) and Yangambi respectively 37 km on the Kisangani-Buta road axis and 90 km on the Kisangani-Yangambi road axis. After washing the cocoa pod, a rotten piece was removed, washed with 5% bleach and rinsed in sterile distilled water to finally be seeded on potato dextrose agar (PDA) at 25 ° C in the dark for 5 days. Transplanting was carried out on PDA at 25 ± 2 ° C under permanent white light. To prevent bacteria from growing, 100µL of Ampicillin (50mg / ml) and 100µL of Chloramphenicol (50mg / ml) were added beforehand to each 100ml of PDA at 45°C before solidification.

Assessment of antifungal activity

The antifungal activity was evaluated on the basis of percentage inhibition (PI) of mycelial growth or reduction of mycelial growth (RCM) of plant extracts on the strain of *L. theobromae*, with six repetitions. 12ml of PDA was poured into each 90mm diameter Petri dish. A line was drawn in advance on the median of the Petri dish, one half to apply the extracts and the mycelial explant of 5mm in diameter, was placed on the other half at 2.5mm from the median line [20]. Mycelial growth was measured on either side of the midline (Fungal Ray, R.F) each after 24 hours until the Petri dish was filled. The control was carried out in the same conditions but without extract.

The PI calculation was performed by this formula:

$$PI = \frac{(F.R\ control - R.F.Extract)}{R.F.control} X\ 100$$

The R 3.4.0 software was used to compare the means of the PI by performing the one-factor variance analysis test. The standard deviation was calculated by the standard deviations shown in the error bar on the histograms.

RESULTS

Inhibition percentage

Extraction solvents

The sensitivity of the fungal strain to extraction solvents was assessed in the sense of evaluating their antifungal activities as illustrated in figure 2 below.

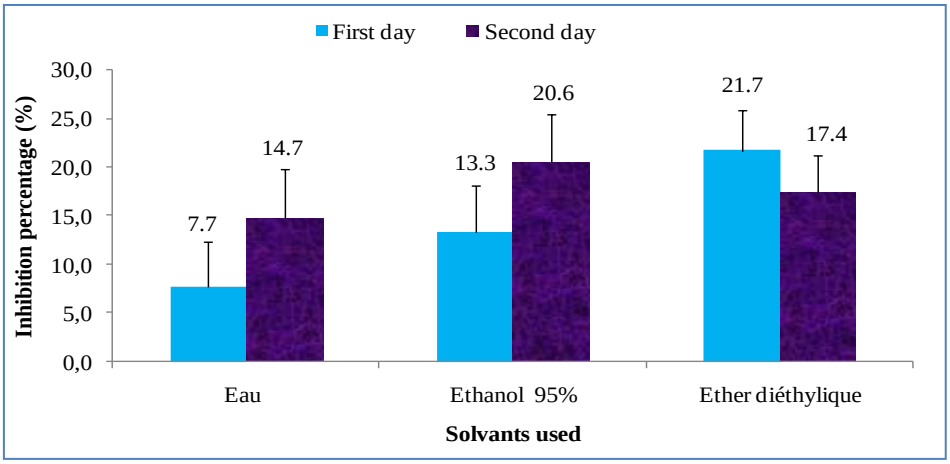

Fig-2: Percentage inhibition of water, 95% ethanol and diethyl ether on the strain of *L. theobromae*

Diethyl ether exhibited a high inhibition rate just after one day of incubation (21.7%) while ethanol achieved a rate of 20.6% after two days of observation. As for water, a low PI of 14.7% was revealed after two days of incubation.

Extracts of fresh leaves

Figure 3 below illustrates the antifungal activity of the fresh leaves of the plants used, after two days of incubation.

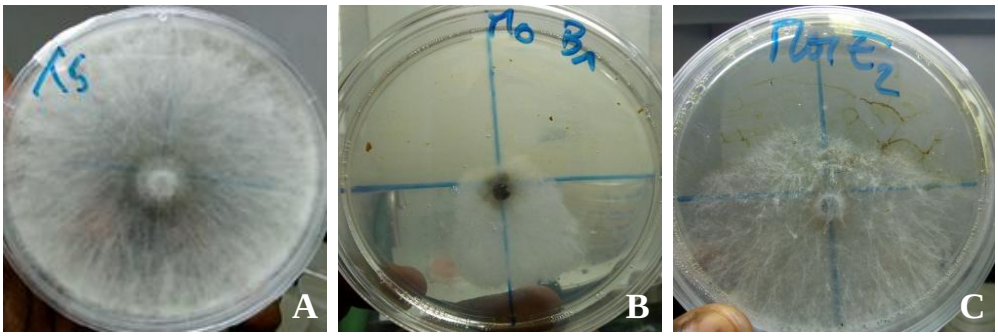

Fig-3: A: Control strain of *L. theobromae*, B: Raw extract of fresh leaves of *M. oleifera* and C: Ethereal extract of fresh leaves of *M. oleifera* against the strain of *L. theobromae* after 2 days of incubation

It should be noted from Fig. 3 that the Petri dishes are only half filled with the strain of L. theobromae unlike that of the control, which is completely filled after two days of incubation.

Raw extracts

Figure 4 below illustrates the PI of the raw extracts of the fresh leaves studied on the strain of *L. theobromae*.

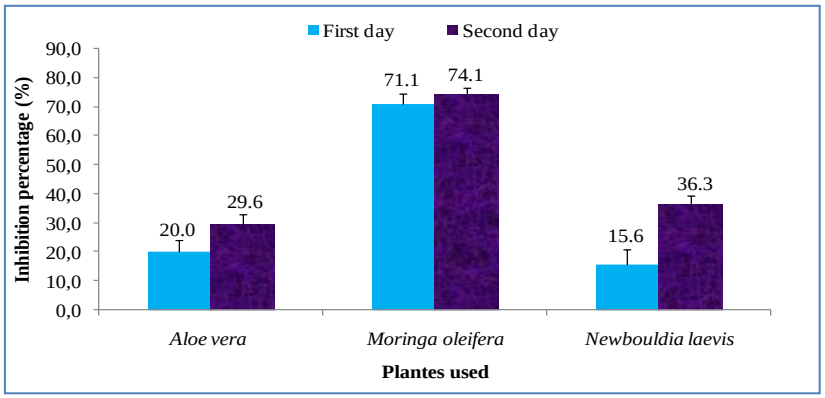

Fig-4: Percentage inhibition of crude extracts from fresh leaves of *A. vera*, *M. oleifera* and *N. laevis* on the *L. theobromae* strain

It appears from this figure that the raw extract of fresh leaves of *M. oleifera* exhibited a high PI during the two days of incubation, respectively 71.1 and 74.1% while the raw extract of fresh leaves of *N. laevis* posted a weak PI after one day, which is 15.6%. As for the raw extract of *A. vera*, a weak PI of 29.6% was released after two days of incubation.

Macerated extracts

The fresh leaves macerated in aqueous, ethanolic and ethereal extracts revealed the PI (after two days of incubation) on the *L. theobromae* strain as illustrated in figure 5 below.

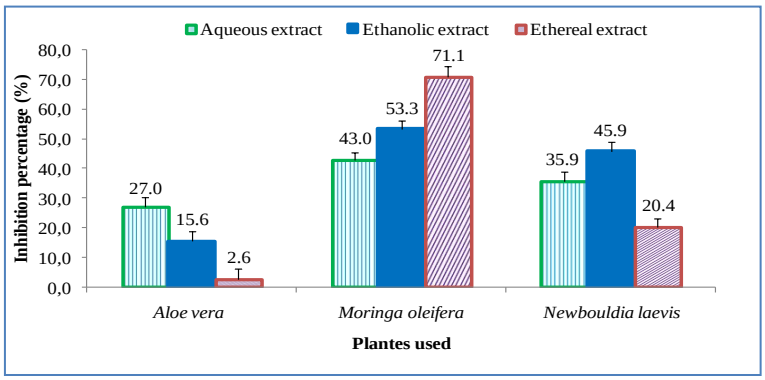

Fig-5: Percentage inhibition of extracts of fresh leaves of *A. vera*, *M. oleifera* and *N. laevis* on the *L. theobromae* strain after two days of incubation

The observation in figure 5 above shows that the aqueous, ethanolic and ethereal extracts of *M. oleifera* display higher PI, 43.0; 53.3 and 71.1% on the *L. theobromae* strain after two days of evaluation of antifungal activity compared to those of other plants.

Extracts of dry leaves

The inhibition percentages of aqueous, ethanolic and ethereal extracts of dry leaves of *A. vera*, *M. oleifera* and *N. laevis* on the *L. theobromae* strain are illustrated in the following figure 6.

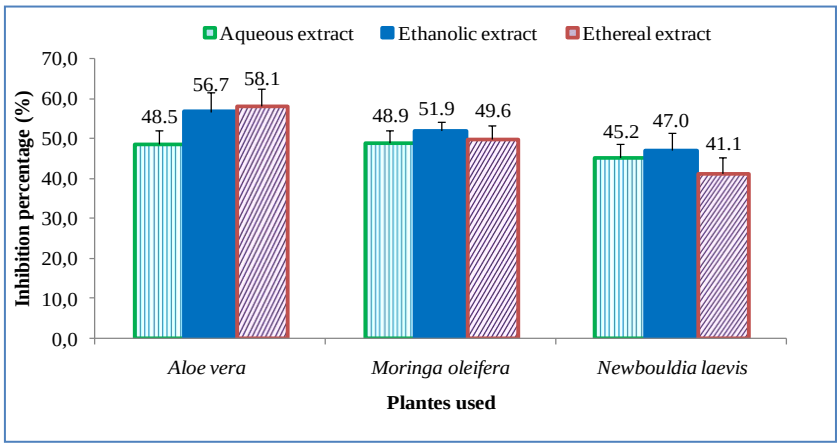

Fig-6: Percentage inhibition of extracts of dry leaves of *A. vera*, *M. oleifera* and *N. laevis* on the *L. theobromae* strain after two days of incubation

In light of figure 6, it appears that after two days of incubation, the ethereal extract of *A. vera* presented a high PI, 58.1% followed by the ethanolic extract of the same plant, or 56.7% on the *L. theobromae* strain. In addition, all the extracts from *N. laevis* did not present significant enough PI.

Maximum growth time

Tables 1 and 2 show respectively the maximum growth time MGT (in days) of *L. theobromae* against the various extraction solvents and extracts from the plants used.

Table-1: Maximum growth time of the *L.theobromae* strain against extraction solvents

Extraction solvent	Mycelial growth time (day)
Control	2
Distilled water	3
Ethanol 95%	3
Diethyl ether	3

Table-2: Maximum growth time of the *L. theobromae* strain against the extracts of the plants studied

Plants used	Mycelial growth time (day)						
	Raw extract	Aqueous extract		Ethanolic extract		Ethereal extract	
	Fresh leaves	Fresh leaves	Dry leaves	Fresh leaves	Dry leaves	Fresh leaves	Dry leaves
Aloe vera	3	3	5	3	4	3	4
Moringa oleifera	6	4	4	5	4	5	4
Newbouldia laevis	3	3	5	4	4	3	4

It appears from Table 1 that the TCM of *L. theobromae* is only 2 days for the control and 3 days for the solvents, moreover, for the extracts of the plants, the growth time varied from 3 to 6 days

Water content

The fresh leaves of the plants studied were subjected to the drying test and revealed the water contents illustrated in figure 7 below.

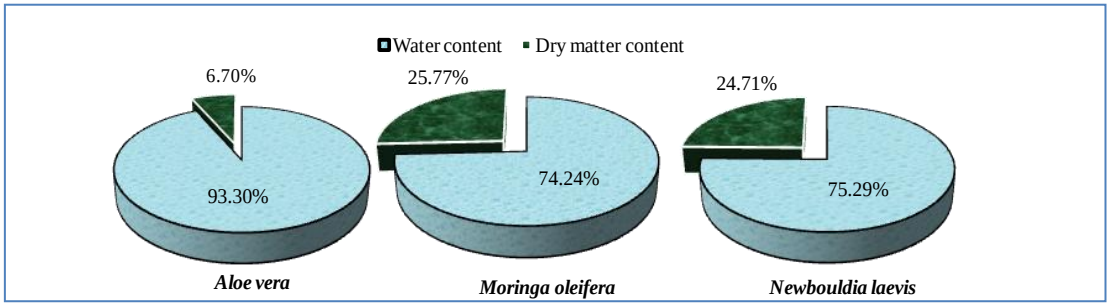

Fig-7: Water content of fresh leaves of *A. vera*, *M. oleifera* and *N. laevis*

It is evident that the fresh leaves of *A. vera* have a high water content of 93.3% however those of *M. oleifera* and *N. laevis* have relatively low and similar contents, respectively 74.2 and 75.3%.

Total extract content

The extraction yields after complete evaporation of the solvents from the maceration of the dry leaves are illustrated in the following figure 8.

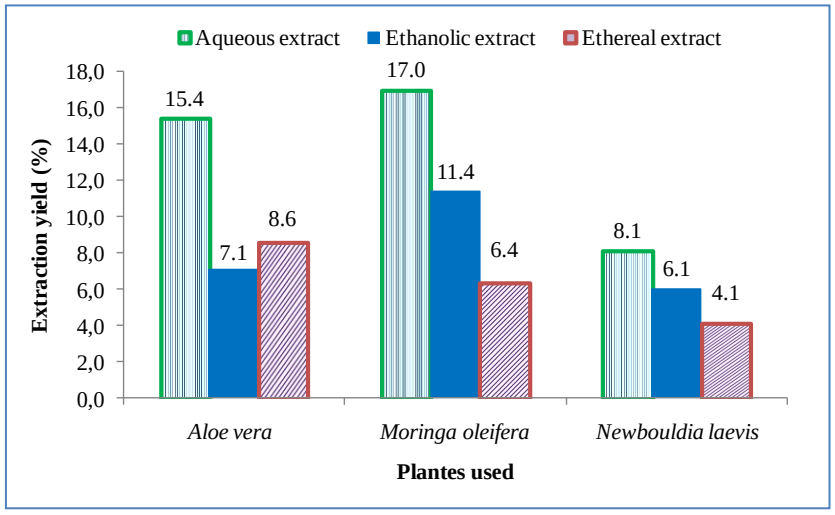

Fig-8: Yield of total extracts of *A. vera*, *M. oleifera* and *N. laevis*

With regard to this figure, it follows that the extraction yield of M. oleifera by water is higher at 17.0% followed by that of A. vera is 15.4% while that of extraction of *N. laevis* by ether is the lowest at 4.1%.

DISCUSSION

The *L. theobromae* strain is expected to achieve maximum growth *in vitro* by completely occupying the surface of the culture medium (Fig. 2). The growth rate of the control increases more quickly by occupying 33.33 and 100% of the surface respectively after one and two days of incubation.

Faced with extraction solvents, the strain is not inhibited (Fig. 2), hence the maximum growth time is only one day longer than that of the control (Tab.1). It should be stated that water, ethanol 95% and diethyl ether have no (considerable) antifungal activity on *L. theobromae* since they all have PI values below 25 % [20].

Although the *L. theobromae* strain exhibited low sensitivity on water, ethanol 95% and diethyl ether, their PI are significantly different (p value = 0.00335). Thus, it should be noted that diethyl ether has a high PI (21.7%) after one day of incubation, but this value decreases significantly on the second day (17.4%). However, ethanol displays growing PI, going from 13.3% after one day to 21.1% after two days of incubation. These two readings (which oppose each other) made on diethyl ether and 95% ethanol would be justified by the fast or slow volatility of each solvent over time.

In figure 2, the PI of water and ethanol have an ascending pace from the first to the second day, which defines the second day as the right time to compare the PI of the different extracts with each other.

Furthermore, in the presence of plant extracts, the *L. theobromae* strain is considerably slowed down (Fig. 3) and this prolongs the MGT which goes from 2 days for the control to 3 or 6 days (Tab. 3) depending on the type of extract and nature of plant tested.

The PI of crude extracts are generally high compared to that of the control and are significantly very different from each other (p value = $2.e^{-16}$).

Extracts from fresh leaves of *M. oleifera* have significant antifungal activities against the strain of *L. theobromae* (Fig. 5) compared to extracts from fresh leaves of *A. vera* and *N. laevis*. *M. oleifera* ethereal extract has the highest PI at 71.1%. The antifungal molecules contained in this extract could be the basis of this more pronounced activity.

Even though all the extracts from the dry leaves are all antifungal (Fig. 6), the strain of *L. theobromae* is more sensitive first to ethereal (58.1%) and ethanolic (56.7%) extracts of *A. vera* followed by ethanolic (51.9%) and ethereal (49.6%) extract from *M. oleifera*. This observation could be explained by two facts:

The high water content of *A. vera* 93.3% (Fig. 7) would justify the low inhibition rate of its crude extract, however that of *M. oleifera* (74.2%), did not significantly influence the inhibition rate of the extract ethanolic because the PI of the ethanolic extract of the fresh leaves is 53.3% while that of the dry leaves is 51.9% (p value = 0.9221) but has led to a significant drop in PI of the ethereal extract of fresh leaves: 71.1 to 49.6% for the same extract but dry leaves (Fig 5 and 6) of *M. oleifera*.

The high yields of total extracts (Fig 8) from *A. vera* and *M. oleifera*, thus increased the concentration of vegetative matter in the extracts tested.

Furthermore, the water content of *M. oleifera* (74.2%) is not too different from that found by Broin, ie 75% [21]. The extraction yield depends on the nature of the plant and the solvent used. The yields of aqueous extract of all the plants studied are high compared to those of ethanolic and ethereal extracts but are all low to that of aqueous extract of the leaves of *Cupressus lusitanica* (21.20%) found by Tsopmbeng in Cameroon [22]. As for *N. laevis*, the yield of methanolic extract obtained by Minaflinou in Benin is 14% [23] and that of ethanolic extract by Hounzanbge, 12.8% [24], are all very high compared to the yield of the ethanolic extract in our study, ie 6.1%. These differences would be justified by the habitat and the geographic study area.

The PI of the raw extract of *M. oleifera* (74.1%) remains the highest with a MGT of 6 days compared to the PI of the extracts of the same plant macerated in various solvents (in fresh or dry state). This more pronounced activity would find its justification in the natural composition of the plant, which once macerated; *M. oleifera* partially loses its antifungal compounds such as essential oils. This plant would have molecules acting in symbiosis against this fungus *L. theobromae*.

In addition to the classification of degree (PI) of antifungal activity of plants [20]: very active plant with the PI between 75 and 100%; active plant whose PI vacillates between 50 and 75%, moderately active plant with the PI between 25 and 50% and finally little or non-active plant whose PI is less than 25%; in our study, the ethanolic extract of *A. vera* (56.7%), the ethereal extract from the same plant (58.1%) and the ethanolic extract from the dry leaves of *M. oleifera* (51.9%) and fresh leaves from the same plant (53.3%) classify *A. vera* and *M. oleifera* among the "active plants".

Furthermore, according to the classification criteria established by Abdellatif [25] as taken up by Hajji [26], *M. oleifera* in the present study could be classified among the plants with "excellent activity" taking into account PI of its crude extracts and ethereal fresh leaves of 74.1 and 71.1% respectively.

There were very significant differences between the PI of ethereal extracts of dried leaves (p value = 0.0001), but significant for those of ethanolic extracts (p value = 0.0129 to 95%), however, there is no significant difference between the PI of the aqueous extracts (p value = 0.2400). On the basis of these observations, it should be stated that the extraction solvent plays a very preponderant role in the selective extraction of the antifungal compounds from plants. The latter each have specific properties, since they are not of the same kind and above all their profiles as secondary metabolites would also be very different. Thus the antifungal activity on the strain of *L. theobromae* is due to the less polar molecules for the leaves of *A. vera* and *M. oleifera*.

Thus taking into account the diversity of chemical molecules that these plants can contain and their antifungal properties, a more in-depth study is to be considered in our next investigations in order to define the qualitative and quantitative compositions of these medicinal plants.

CONCLUSION

This study was focused on the *in vitro* evaluation of the antifungal activity of extracts of *Aloe vera*, *Moringa oleifera* and *Newbouldia laevis* on the *Lasiodiplodia theobromae* strain. It was found that all the dry leaves of the plants studied showed interesting PI, firstly *M. oleifera* with a higher inhibition power (74.1% for the raw extract of dry leaves) followed by *A. vera* (58.1% for the ethereal extract of dry leaves). But the leaves of *N. laevis* are less antifungal. The solvents used for extraction, although they do not have an antifungal supply, but play a key role in the extraction of plant-specific secondary metabolites depending on whether they are polar or non-polar. These plants being naturally different could also have different compositions in terms of active principles which they contain.

In view of the results obtained, determining the composition of phytochemical groups and identifying the active principle responsible for this antifungal activity will be the subject of our future investigations.

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