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# ANTIFUNGAL ACTIVITY ON *LASIODIPLODIA THEOBROMAE* AND PHYTOCHEMICAL STUDY OF *MITRACARPUS VILLOSUS* AND *MORINGA OLEIFERA* FROM KISANGANI (D.R.CONGO)

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## ABSTRACT

*Lasiodiplodia theobromae* is one of the fungal strains that attacks cocoa crops. This strain has become a critical threat to cocoa cultivation in some regions of the word including in the Kisangani region in the Democratic Republic of Congo. This work involved the identification, extraction and *in vitro* evaluation of the antifungal activity of the phytochemical groups of *Mitracarpus villosus* and *Moringa oleifera* on the strain of *Lasiodiplodia theobromae*. The aqueous, 95% ethanolic and ethereal extracts of *M. oleifera* are more antifungal (respective percentages of inhibition PI: 85.8; 77.8 and 77.4%) compared to those of *M. villosus* (71.4; 74.2 and 80.0%). The minimum inhibition concentration, MIC of the aqueous extract of *M. oleifera* is the lowest (20 mg/mL with a PI at the MIC of 85%) followed by that of the ethanolic extract, (25 mg/mL with a PI at 77% MIC). The chemical screening revealed the very abundant presence of tannins in the two plants and abundant presence of saponins in *M. villosus*. The extraction yields of tannins in *M. villosus* and *M. oleifera* are 16.91 and 21.25%, respectively, and that of saponins in *M. villosus*, 3.83%. *M. villosus* contains two kind of tannins (gallic and catechetical) when *M. oleifera* contains condensed tannins. *M. oleifera* tannins have a PI of 69.6% while those of *M. villosus* are 45.6%; both have PI lower than that of *M. villosus* saponins, 74.4%. Determination of active molecules from the tannins of *M. oleifera* and the saponins of *M. villosus* is undergoing.

**KEYWORD:** *Lasiodiplodia theobromae*, *Mitracarpus villosus*, *Moringa oleifera*, antifungal activity, cacao.

## INTRODUCTION

Medicinal plants have been exploited since ancestral times, particularly in Africa, but their therapeutic activity remains empirically known.[1] The phytochemical composition of most of these plants remains unknown, mainly the antimicrobial and antifungal bioactive phytochemical groups.

The exploitation of wild flora for its antifungal potential constitutes one of the inevitable alternatives for the conservation of biodiversity and the sustainable and efficient management of the environment. This approach helps to limit the harmful effects of environmental pollution caused by the use of pesticides.[2] The outcome would be the formulation of bio-fungicides from plants which are biodegradable, effective, accessible and renewable.

In fact, plant extracts are known to be safe and environmentally friendly unlike synthetic chemicals. The overuse of synthetic fungicides to control plant diseases has ultimate negative effects on human and animal health as well as on the agro-ecosystem.[3]

One then turn to the use of plant active compounds to fight against microorganisms that attack other plants.[4] Thus pterygospermin, identified in the roots of *Moringa oleifera*, has been shown to be a potent antibacterial and antifungal agent.[5,6] The work carried out by Fotem revealed that weeds such as *Mitracarpus villosus* and *Agératum conyzoides* play the role of biopesticides against pests.[7]

*Lasiodiplodia theobromae* is one of the fungal strains that ravage cocoa crops. This strain has become a critical threat to cocoa cultivation not only in India,[8] Cameroon and Western Samoa, the Philippines,[9] but also in the Kisangani region in the Democratic Republic of Congo (DRC).

To our knowledge, there is no work that offers plant-based antifungal control against this strain. This is how we began a series of studies to limit or stop the ravages of cocoa crops by using plant extracts that can act on *L. theobromae*[10,11] Total concentrated extracts of medicinal plants were tested on the strain of *L. theobromae*.

Furthermore, *M. oleifera* and some species of the genus Mitracarpus are known for their antimicrobial power.[12-15] Indeed, extracts of *M. scaber* in different solvents have shown their effectiveness on the strain of *Candida tropicalis*.[12] *M. oleifera* bark petroleum ether extract has been shown to inhibit six strains of fungi and ten strains of bacteria.[6,13] And some work on *M. oleifera* leaves has shown that they could be used to treat AIDS [14] and typhoid fever.[15]

The aim of the present work is to perform a phytochemical creening to detect the major secondary metabolites of the leaves of *M. oleifera* and *M. villosus* in order to evaluate their respective antifungal activity on the strain of *L. theobromae*. This study is a contribution to support current regulations aimed at reducing dependence on synthetic fungicides.[16]

## MATERIAL AND METHODS

### 1. Study area

This work was carried out in the region of Kisangani, the capital of the Tshopo province in the DRC. The city of Kisangani is located at 0º31' North latitude, relative to the Equator (at 57Km), 25º11' East longitude relative to the Greenwich meridian and 428 m above sea level.[17,18]

### 2. Plant material and treatment

The leaves of *M. villosus* and *M. oleifera* collected in the Kisangani region were used as plant material. These were identified at the Herbarium of the Science Faculty of the University of Kisangani. After drying, crushing and sieving, 10g of each powder of the leaves were macerated for 48 hours in 50mL of solvent (water, ethanol 95% and diethyl ether). The filtrates were evaporated to obtain the dry residues. The used solutions were prepared at 100mg/mL of residues dissolved in respective extraction solvents (water, 95% ethanol and diethyl ether).

As for the determination of the minimum inhibitory concentration (MIC), five different solutions were prepared from the residues with concentrations of 12.5; 25; 50; 100 and 200mg/mL. The remaining quantity of the leaves powder was used for the extraction of the few major phytochemical groups after their identification.

### 3. Chemical screening and extraction

The identification of the few phytochemical groups contained in the two plants studied was carried out according to universal protocols.[19-23] Only the major phytochemical groups were extracted, namely saponins of *M. villosus*[24] and tannins of the two plants studied.[25,26]

### 4. Fungal strain obtention

The strain of *L. theobromae* was isolated from cocoa pods naturally affected by brown rot. These pods were harvested directly from the tree in the cocoa fields of Bengamisa (CABEN) and Yangambi respectively 37 Km on the Kisangani-Buta road and 90 km on the Kisangani-Yangambi road. The protocol for obtaining the strain was applied to Potato dextrose agar (PDA) medium.[10,11]

### 5. Evaluation of antifungal activity

The antifungal activity was evaluated by the determination of percentage of inhibition (PI) of mycelial growth or reduction of mycelial growth (MG) of plant extracts on the *L. theobromae* strain, with six replicates. 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 5mm diameter mycelial explant was placed on the other half 2.5mm from the median line[27]. Mycelial growth was measured on both sides of the midline (fungal radius, RF) every 24 hours until the Petri dish was filled. The control was carried out under the same conditions but without extract.

The calculation of PI was carried out by the formula:

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

The standard deviation has been calculated by the standard deviations shown as an error bar on the histograms.

### 6. Statistical analysis

The statistical analyses were carried out using R 3.4.0 software.

## RESULTS AND DISCUSSION

- **Extract yield**

Figure 1 below shows the total extract yields (%) of the plants studied.

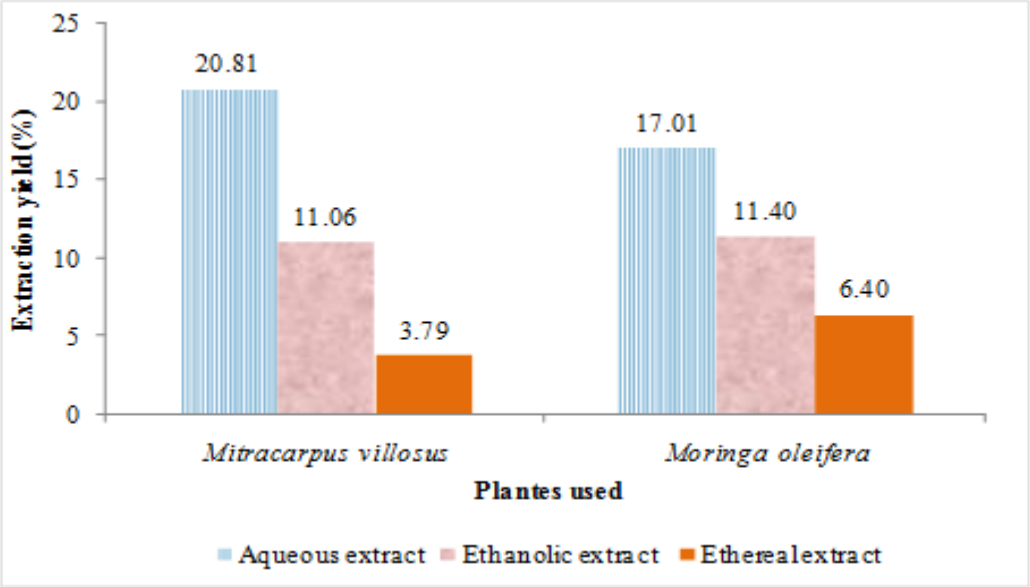


**Figure 1: Yield in total extracts.**

The yield of aqueous extraction from the dry leaves of *M.villosus* is the highest at 20.81% while the yield of ethereal extraction from the same plant is the lowest at 3.79%.

The nature of the solvent gives an indication of the threshold of the extraction yield of the compounds, i.e. the polar and nonpolar ones. The more polar the solvent, the higher the yield. This is the case for the aqueous extraction yields of *M. villosus* and *M. oleifera* of 20.81 and 17.01% respectively, compared to 3.79 and 6.40% for ethereal extraction. And this is in accordance with the "like dissolves like" principle. Since water is a universal solvent, it dissolves most of the plant compounds, mainly the polar compounds.[28]

- **Inhibition percentage of solvents**

Solvent were evaluated alone in order to be used as negative standards. Figure 2 below illustrates the PI of distilled water, 95% ethanol and diethyl ether on the *L. theobromae* strain.

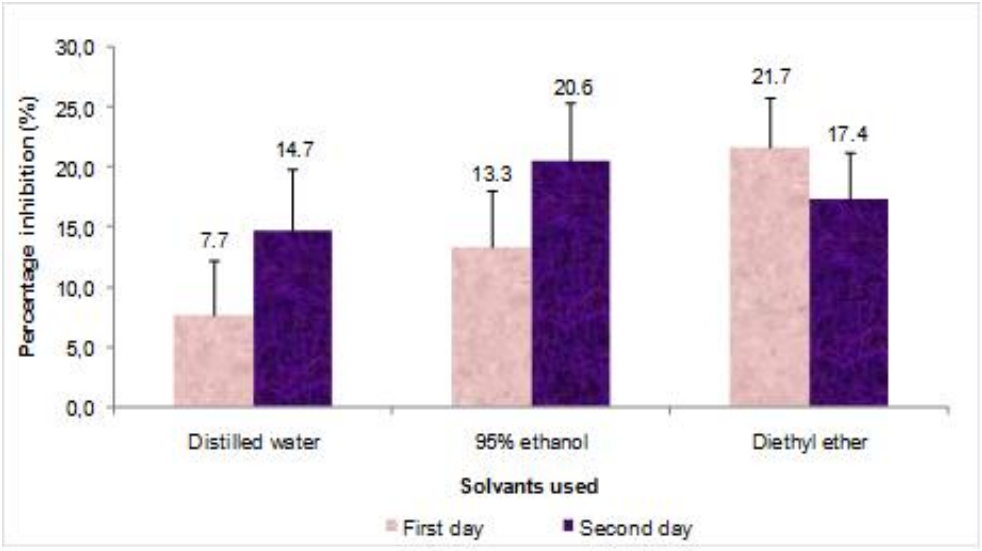


**Figure 2: Percentage inhibition of distilled water, 95% ethanol and diethyl ether on *L. theobromae* strain.**

From this figure, it appears that diethyl ether has a high PI after one day of incubation, i.e. 21.7%, whereas ethanol has a rate of 20.6% after two days of incubation. On the other hand, water has the lowest PI.

Ethanol 95%, reputed to be a generic antiseptic, is not effective against the *L. theobromae* strain. It gives a PI of

only 20.6% after two days of contact. This preliminary test finds can give the contribution of solvents on the total antifungal activity of plant extracts.

- **Inhibition percentage of the extracts**

The PI of the different dry extracts of the plants studied on the *L. theobromae* strain are illustrated in the figure 3.

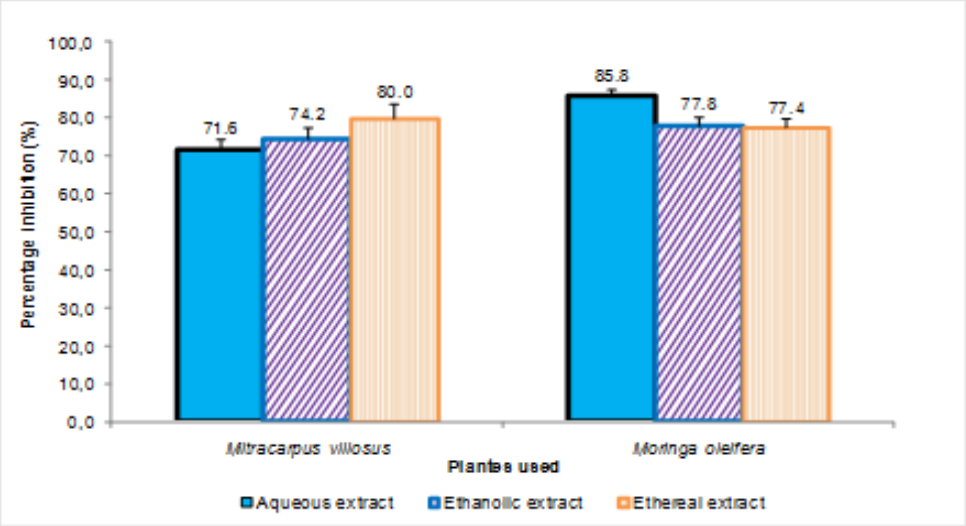


**Figure 3: Inhibition percentage of aqueous, ethanolic and ethereal extracts (100mg dry residue per mL of solvent) of dry leaf powders on *L. theobromae* strain after two days incubation.**

The aqueous extract of *M. oleifera* has a higher PI of 85.8% followed by the ethereal extract of *M. villosus* of 80.0% after two days of incubation. The PI of the aqueous extract of *M. villosus* was lower at 71.6%.

The PI of the dried residue from the three solvent extracts of *M. oleifera* are higher than that of solvent extracts from the fresh plant of the same plant specie as found in our previous work.[10] This indicates that, in addition to the nature of the substances dissolved in different solvents, the antifungal activity is concentration-depend.

These results indicate that the PI values of the three crud extracts of *M. villosus* and *M. oleifera* are significantly different (P=.001).

- **Minimum inhibitory concentration**

The MIC of the aqueous and ethanolic extracts of *M. villosus* and *M. oleifera* obtained by interpolation on the plotted curves of PIs versus concentrations are shown in Table 1.

**Table 1: Concentrations and percentages of inhibition characteristic of aqueous and ethanol extracts of *M. villosus* and *M. oleifera* on *L. theobromae* strain.**

| Plants | Extract | $IC_{50}$ (mg/mL) | MIC (mg/mL) | PI (%) to MIC | $IC_{75}$ (mg/mL) | Ratio MICol/MICaq |
|---|---|---|---|---|---|---|
| *Mitracarpus villosus* | Aqueous | 9.0 | 30 | 74 | 135 | 1.67 |
| | Ethanolic | 9.5 | 50 | 72 | 70 | |
| *Moringa oleifera* | Aqueous | 7.5 | 20 | 85 | 12 | 1.25 |
| | Ethanolic | 8.5 | 25 | 77 | 20 | |

Legend MIC: Minimal Inhibitory Concentration; IC50: Concentration for fifty percent inhibition; IC75: Concentration for seventy-five percent inhibition; MICaq: Minimal Inhibitory Concentration of the aqueous extract; MICol: Minimal Inhibitory Concentration of the ethanolic extract.

This table shows that the aqueous and ethanolic extracts of *M. oleifera* have low MIC values of 20 and 25mg/mL and CI75 values of 12 and 20mg/mL respectively.

The antifungal activity of *M. oleifera* on the *L. theobromae* strain is characterized by significantly high PIs and MIC of 85 and 77% respectively for its aqueous and ethanolic extracts. According to BOUAZZA's categorization, the very high PI values of *M. oleifera* extracts make it a highly active plant[27] and this confirms our previous results.[10]

Although the aqueous extract of *M. villosus* is more active with a low MIC, nevertheless its MIC75 remains higher (135mg/mL). Thus the aqueous extract of *M. villosus* could only effectively inhibit the growth of the *L. theobromae* strain at high concentrations, that is not the same for ethanolic extract.

BAGRE's work on the antifungal activity of *Cryptococcus neoformans* revealed that the aqueous and ethanolic extracts of *Morinda morindoides* have IC50 of 14.3 and 6.3mg/mL respectively.[29] YAPI also showed in his work that the 70% ethanolic extract of *Eclipta prostrata* has antifungal activity on *Candida albicans* with a MIC of 25MG/ML, on *Trichophyton mentagrophytes* with a MIC of 6.25mg/mL and on *Cryptococcus neoformans* with a MIC of 25mg/mL.[21]

The ratios calculated between the MIC of the aqueous and ethanolic extracts (1.67 for *M. villosus* and 1.25 for *M. oleifera*) stipulate that the aqueous extracts would be more active. As a result, the latter would be rich in active ingredients against the *L. theobormae* strain. Moreover, these active ingredients would be of similar nature due to their solubility in water.

- **Identification of phytochemical groups**

The different phytochemical groups listed in Table 2 have been identified in *M. villosus* and *M. oleifera*.

**Table 2: Phytochemical groups identified in *M. villosus* and *M. oleifera*.**

| **Phytochemical groups** | ***Mitracarpus villosus*** | ***Moringa oleifera*** |
|---|---|---|
| Alkaloids | - | + |
| Anthocyanins | - | - |
| Flavonoids | + | - |
| Saponins | ++ | + |
| Sterols and Terpenes | ++ | ++ |
| Quinones | - | - |
| Tannins | +++ | +++ |

Legend - : Absence + : Trace presence ++ : Abundant presence +++ : Very abundant presence

These results show that in *M. villosus* and in *M. oleifera*, tannins were found to be very abundant followed by sterols and terpenes. Saponins were only abundant in *M. villosus*. Whereas quinones and anthocyanins are absent in both plants.

The two studied plants differ from each other in the abundance of saponins, flavonoids and alkaloids. This difference in compositions of secondary metabolites could explain the difference in biological activity.

The identified tannins were subjected to different characterization tests, the obtained results show that *M. villosus* contains the gallic or ellagic tannins and the catechic tannins while *M oleifera* contains the condensed tannins. AISSOU have also found condensed tannins in *M. oleifera*.[30]

Tannins are known for their fungicide activity. Indeed gallic and catechic tannins found in *Mallotus oppositifolius* were active when tested on *Fusarium* sp. and *Phytophthora* sp. [31]. The same results were found for tannins of *Mitracarpus scaber* and *Psorospermum guineense* used in the traditional treatment of dermatosis in Mali.[21]

- **Phytochemical group contents**

Figure 4 below illustrates the aqueous extraction yield of tannins and saponins of *M. villosus* and *M. oleifera*

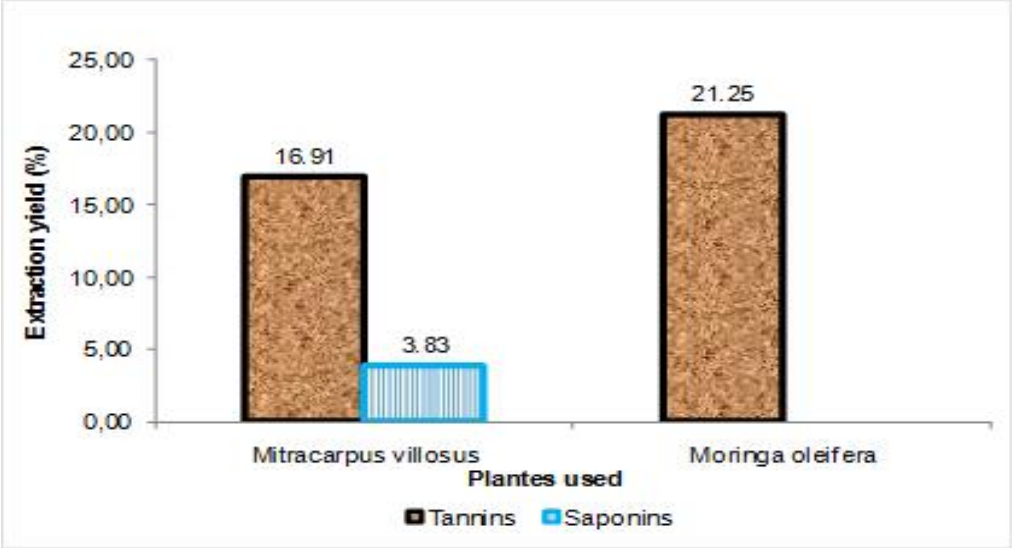


**Figure 4: Extraction yields tannins and saponins of *M. villosus* and *M. oleifera*.**

From this figure, it can be seen that *M. oleifera* has a high tannins content of 21.25%, compared to that of *M. villosus* (16.91%). This can be due to water solubility of that polyphenolic group.

Saponins are in small quantity in *M. villosus* (3.83) while in *M. oleifera* the quantity is almost zero. This although the qualitative analysis showed a weak presence in this last plant specie.

- **Percentage of inhibition of Tannins and saponins**

The PI tannins and saponins extracted from the studied plants are given in Figure 5.

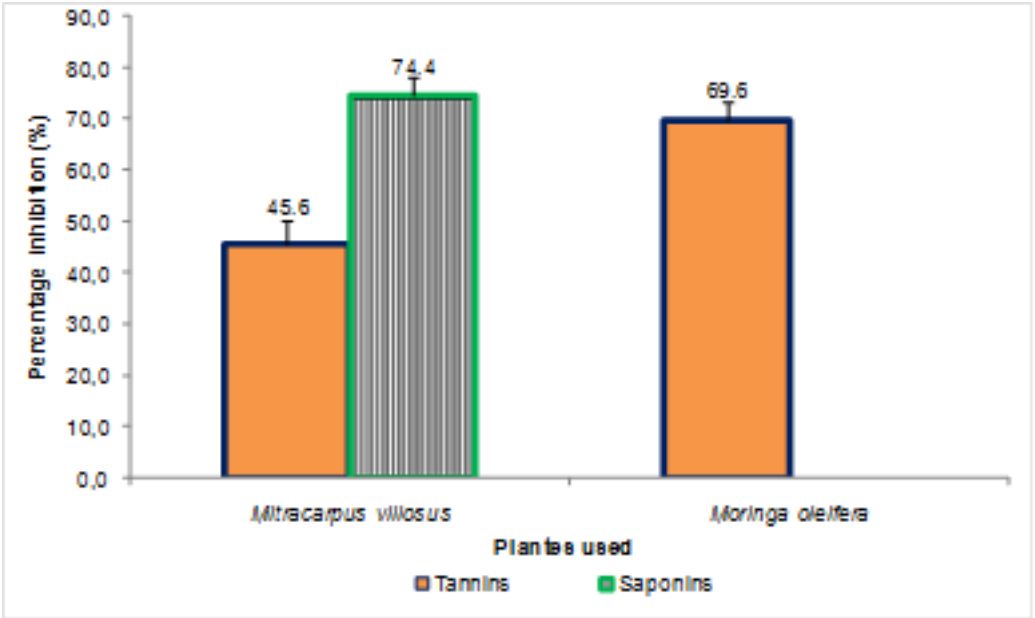


**Figure 5: Percentages inhibition of tannins and saponins (100mg/mL) of *M.villosus* and tannins of *M. oleifera* on *L. theobromae* strain after two days of incubation.**

It can be seen from figure 5 that the saponins of *M.* villosus have a higher pi of 74.4%, followed by the tannins of *M. oleifera* at 69.6%. The PI saponins of *M.* villosus is almost the same as that of the ethanolic extract of the plant (fig.3) indicating that saponins would be the main active compounds of the ethanolic extract of *M.* villosus.

Saponins are known for their antimicrobial and antifungal properties.[24,32,33] Indeed, these secondary metabolites induce a loss of structural integrity and an increase in ionic permeability of fungal cell membranes.[32,34,35]

The tannins of *M. oleifera* show lower PI (69.6%) than those of all crud extracts of this plant (Fig.3). This indicates that the high antifungal of *M. oleifera* leaves could be due to the synergetic effect of the constituents.

The Figure 5 also shows that tannins of *M. oleifera* are more active than those of *M. villosus*. The antifungal activity of these tannins could be related to their nature. *M. oleifera* contains condensed tannins when *M. villosus* contains the gallic and the catechic tannins as already stated above. Tannins are known to be antimicrobial and antifungal.[33,36,37]

- **Maximum growth time**

Table 3 shows the maximum growth times (MGT) of *L. theobromae* against the different substrates tested.

**Table 3: Maximum growth time (in days) of *L. theobromae* strain against extraction solvents, antibiotics (positive controls) and phytochemical groups of *M. villosus* and *M. oleifera*.**

| Substances tested | Mycelial growth time (day) | Substances tested | Mycelial growth time (day) |
|---|---|---|---|
| Negative control (fungal strain incubated alone) | 2 | Gentamycin | 4 |
| Distilled water | 3 | Butanol | 3 |
| Ethanol 95% | 3 | Tannins of *Mitracarpus villosus* | 3 |
| Diethyl ether | 3 | Tanins of *Moringa oleifera* | 5 |
| Ampicillin | 4 | Saponins of *Mitracarpus villosus* | 4 |

The table 3 shows that the MGT of the *L. theobromae* strain against the tannin extract of *M. oleifera* is higher, 5 days, followed by the *M. villosus* saponin extract, 4 days.

The Mycelial growth time of the *L. theobromae* strain in the negative control and solvents are only of 2 and 3 days. When compared to the extracts of the phytochemical groups, the MGT is 5 days for the tannins of *M. oleifera* and 4 days for the saponins of *M. villosus*. This indicates the capacity of these compounds to slow down the growth of fungal strains. Indeed, The MGT depends to the fungistatic character of the used substance.[21] Tannins of *M. oleifera* have a higher fungistatic capacity than gentamycin used as positive control. The total extracts of the same plants in terms of MCT.[10] It would be interesting to make a fractionation of this group in order to determine active molecules.

## 4. CONCLUSION

The objective of this study was the identification and extraction of the main phytochemical groups of *Mitracarpus villosus* and *Moringa oleifera*, in order to evaluate *in vitro* their antifungal activity on the strain of *Lasiodiplodia theobromae*. It was shown that tannins and saponins as well as sterols and terpenes are present in the two plants in different abundances. Gallic and catechic tannins were detected in *M. villosus* and condensed in *M. oleifera*. The inhibition percentages on the *L. theobromae* strain after two days of incubation is 69.6% for tannins in *M. oleifera* and 74.4% for saponins in *M. villosus*. Future work should focus on determining the active molecules from the tannins of *M. oleifera* and the saponins of *M. villosus*.

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