



Antifungal Activity on the Strain of *Lasiodiplodia theobromae* and Phytochemical Study of *Ageratum conyzoides* and *Newbouldia laevis* from the Kisangani Region / DR Congo

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Authors' contributions

This work was carried out in collaboration among all authors. Author JTKK wrote the first draft of the manuscript, together with author JPM, they designed the study and wrote the protocol. Authors OO, PTM and GH managed the analyses of the study and directed the bibliographical research. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To extract, identify and evaluate *in vitro* the antifungal activity of the phytochemical groups of *Ageratum conyzoides* and *Newbouldia laevis* on the strain of *Lasiodiplodia theobromae*.

Study Design: Exploitation of medicinal plants to combat the growth of *L. theobromae*, responsible for the decline of cocoa cultivation.

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Location and Duration of Studies: Faculty of Sciences, University of Kisangani, between April 2017 and February 2018.

Methodology: The crude extracts of the dry leaves of *A. conyzoides* and *N. laevis* were tested (at 100 mg/mL). Potato dextrose agar was used as the culture medium. After chemical screening, abundant phytochemical groups were isolated and tested.

Results: The aqueous, 95% ethanolic and ethereal crude extracts of *A. conyzoides* are more antifungal (respective percentages of inhibition PI: 80.74; 84.10 and 85.64%) than those of *N. laevis* (63.28; 72.64 and 75.23%). The minimum inhibitory concentration (MIC) of the aqueous crude extract of *A. conyzoides* is lower (25 mg/mL) than that of the ethanolic extract (50 mg/mL). Tannins are very abundant in *A. conyzoides* and in *N. laevis*. Saponins, sterols and terpenes are abundant in both plants. The extraction yields of tannins and saponins are respectively 20.67 and 2.43% in *A. conyzoides* and 10.47 and 2.38% in *N. laevis*. *A. conyzoides* contains the gallic tannins while *N. laevis*, the condensates and catechics. The saponins and tannins of *A. conyzoides* are more antifungal (respective PI: 84.40 and 54.44%) than those of *N. laevis* (PI: 75.56 and 32.96%).

Discussion: The saponins of *A. conyzoides* and *N. laevis* are more active on the strain of *L. theobromae* than the tannins. Saponins are surfactants that can destabilize membrane structure of microorganisms including fungi.

Conclusion: The saponins of the two plants have shown a very interesting antifungal power on the strain of *L. theobromae*. The identification of their active molecules is ongoing.

Keywords: Antifungal; *Lasiodiplodia theobromae*; *Ageratum conyzoides*; *Newbouldia laevis*; phytochemical.

1. INTRODUCTION

The cocoa cultivation is often threatened by fungi, which significantly reduce the crop yield. Around the 1980s, cocoa orchards were damaged by brown pod rot in Cameroon, affecting 100% of cocoa trees in some plantations. After several investigations, *Lasiodiplodia theobromae* (syn. *Botryodiplodia theobromae*) a common endophyte and opportunistic pathogen was identified to be responsible [1]. This fungus has been isolated in the tropics and subtropics, including in Cameroon, India, Western Samoa and Philippines [2-6]. *L. theobromae* has also been found in the Kisangani region in the Democratic Republic of the Congo (DRC) [7].

It is therefore essential to find the means to fight against this phytopathogen. The use of pesticides has harmful consequences on the ecosystem [8]. This can cause resistance due to genetic mutations. Hence the current attraction towards biofungicides [9]. This is because plant extracts are known for their antimicrobial and/or antifungal effects on certain phytopathogenic or zoopathogenic germs. This is particularly the case of *Ageratum conyzoides* and *Newbouldia laevis* [10-15]. Besides, plants are known to be efficient, non-polluting and accessible to everyone. The use of plant extract fungicides

complies with new environmental regulations that discourage the use of synthetic fungicides [16,17].

To our knowledge, there is no study on the antifungal activity of these two plants on the strain of *L. theobromae*. Therefore our research team began a series of studies on the inhibitory effect of plant extracts on this fungal strain [7,12,18].

The objective of the present study is to identify the phytochemical groups contained in *A. conyzoides* and *N. laevis*, to determine the active principle responsible for the antifungal activity on the strain of *L. theobromae*.

2. MATERIALS AND METHODS

2.1 Study Area

This work was carried out in the region of Kisangani, the capital of the Province of Tshopo in the DRC. This city is 428 meters above sea level and is located at 0°31'North latitude and 25°11' East longitude [19,20].

2.2 Plant Material

The plant material consists of the leaves of *A. conyzoides* and *N. laevis* collected in the

Kisangani region. After their identification at the Herbarium service of the Sciences Faculty of the University of Kisangani, the leaves were dried, crushed and sieved. Ten grams of powder were macerated for 48 hours in 50mL of solvent (water, ethanol and diethyl ether). The filtrates were evaporated to obtain the dry residue, used to prepare various extract solutions. For the minimum inhibitory concentration (MIC), the concentrations of 12.5; 25; 50; 100 and 200 mg/mL were used.

2.3 Chemical Screening and Extraction

Universal protocols [21-25] were used for the identification of phytochemical groups on leaves powder. Only the major groups were extracted, particularly saponins and tannins [26-28].

2.4 Fungal Strain

Brown rot cocoa pods were used to isolate the strain of *L. theobromae*. The Potato dextrose agar (PDA) medium was used according to well-known protocol [7,12].

2.5 Antifungal Activity

The antifungal activity was determined by evaluating the percent of inhibition (PI) of mycelial growth of extracts from the plants on the strain of *L. theobromae*, with six repeats. 12 mL

of PDA were poured into each 90 mm diameter Petri dish. A midline was drawn on each Petri dish. On one side the extract was applied and on the other, the 5mm diameter mycelial implant was placed at 2.5mm from the midline [29]. Mycelial growth was measured on either side of the midline (Fungal radius, FR) every 24 hours until the Petri dish was filled. The negative control consisted of PDA on which only the mycelial implant was placed.

The calculation of PI was performed by the formula:

$$PI = \frac{(FR \text{ of negative control} - FR \text{ Extract})}{FR \text{ of negative control}} \times 100$$

The standard deviation was calculated by the standard deviations, represented by error bars on the histograms.

2.6 Statistical Analysis

Statistical analyzes were performed using R 3.4.0 software.

3. RESULTS AND DISCUSSION

3.1 Total Extract Yields

Fig. 1 gives the yields of total extracts of the studied plants.

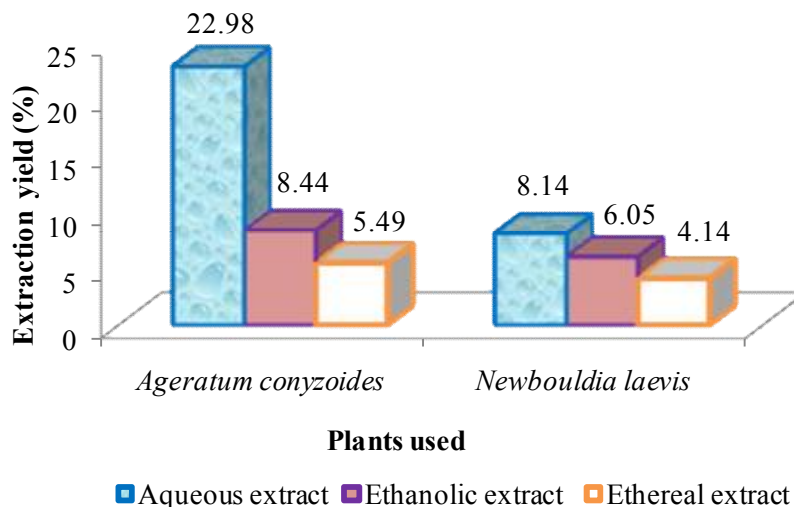


Fig. 1. Yield in total extracts

The aqueous extract from the leaves of *A. conyzoides* gives the highest yield, 22.98%, while the ethereal extract from the leaves of *N. laevis* has the lowest yield, at 4.14%.

The yield of total extracts for both plants decreases from water to diethyl ether via ethanol. This is because water, due to its high polarity, extracts more of the polar compounds. Many of the constituents of these two plants would therefore be polar [18,30].

The low yields observed for the total extracts of *N. laevis* compared to those of *A. conyzoides* would be due to the type of leaves of *N. laevis*, which naturally are made of several ribs. Compared to our previous work, the aqueous extract of *A. conyzoides* (22.98%) gives a higher yield than those of *Mitracarpus villosus* (20.81%) and *Moringa oleifera* (17.01%) [18]. On the other hand, the aqueous extract of *N. laevis* (8.14%) has a low yield.

3.2 Percentage of Inhibition of Total Extracts

The PIs of the various total extracts of the plants studied on the strain of *L. theobromae* after two days of incubation are given in Fig. 2.

The aqueous, ethanolic and ethereal extracts of *A. conyzoides* have higher PIs, respectively 80.74, 84.10, and 85.64% compared to those of *N. laevis*. The total extracts prepared at 100 mg/mL of these two plants have PIs much higher than those found in our previous work. This difference is due to the preparation method of the extracts. In fact, in our previous work solutions were of lower concentration [7,12].

All the PIs of the total extracts (aqueous, ethanolic, and ethereal) of *A. conyzoides* (respectively 80.74; 84.10 and 85.64%) are all higher than those of *M. villosus* (71.6; 74.2 and 80.0%) and *M. oleifera* (77.8% for the ethanolic extract and 77.4% for the ethereal extract) obtained in our previous work [18].

Considering the classification of plants with antifungal activity [29], *A. conyzoides*, *M. villosus* and *M. oleifera* are all very active against the strain of *L. theobromae*. Their PI of the total extracts are greater than 70%. However, there are significant differences between the types of extracts from these plants. The means of the PIs of the three total extracts of *A. conyzoides* are significantly different from each other ($P = 0.026$). It is the same for those of *N. laevis* ($P = 1.89 \times 10^{-6}$).

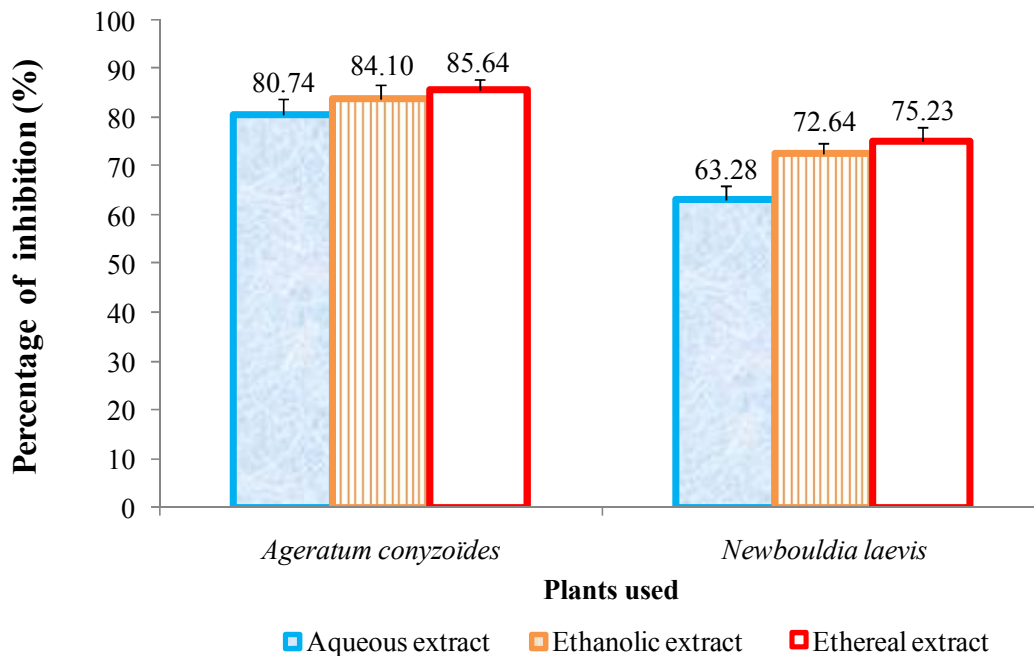


Fig. 2. Percentage of inhibition of aqueous, ethanolic, and ethereal extracts (100 mg / mL) of powders from dry leaves on the strain of *L. theobromae* after two days of incubation

3.3 Minimum Inhibitory Concentration

As *A. conyzoides* have shown higher PI, the MIC, IC₅₀, IC₇₅, PI to MIC, and the ratio of its total extracts were determined and are given in Table 1.

The MIC of the aqueous extract is two times lower (25 mg / mL) than that of the ethanolic extract (50 mg / mL). This trend is confirmed by the values of IC₅₀ and IC₇₅.

These results show that the aqueous extract is more interesting than the ethanolic extract considering its low MIC value.

The MIC of the aqueous extract (25 mg / mL) of *A. conyzoides* is the same as that of the ethanolic extracts of *M. oleifera* on *L. theobromae* [18] and of *Ecliptaprostrata* on *Candida albicans* and on *Cryptococcus neoformans* [23]. However, this value is lower than those of the aqueous (30mg / mL) and ethanolic (50 mg / mL) extract of *M. villosus* on *L. theobromae* [18].

Morinda morindoides showed antifungal activity on *Cryptococcus neoformans*, with IC₅₀ of 14.3 and 6.3 mg/mL for these aqueous and ethanolic extracts [31]. According to Saraka [32], the aqueous and ethanolic extracts of *Mallotus oppositifolius* have respective MICs of 100 and 25 mg / mL on *Fusarium* sp., whereas on *Phytophthora* sp., they are respectively 100 and 50 mg/ mL. The rate of inhibition of a fungal strain would therefore depend on the nature of the plant, the concentration of the substrate and the target fungal species.

3.4 Phytochemical Groups

A. conyzoides contains tannins in very high abundance, saponins, sterols, and terpenes in abundance as well as flavonoids in trace amounts. While *N. laevis* contains saponins, tannins, sterols, and terpenes. Alkaloids,

anthocyanins, quinones are absent in both plants.

These two plants display a similar phytochemical profile. However, the contents of tannins and flavonoids are different.

The phytochemical composition of *A. conyzoides* appears to be like that of *M. villosus* [18]. These results are similar to those found by other researchers for the leaves of *A. conyzoides* [33,34] and *N. laevis* [35-37].

The characterization of the tannins shows that *A. conyzoides* contains gallic tannins and *N. laevis*, condensed and catechetical tannins.

Due to the abundance of tannins and saponins in the two plants studied and the antifungal activity of these secondary metabolites [18,38,39], it is useful to extract these two phytochemical groups and evaluate their antifungal activities on the strain of *L. theobromae*.

3.5 Extraction Yield of Tannins and Saponins

Fig. 3 gives the extraction yield of tannins and saponins of *A. conyzoides* and *N. laevis*

From this figure the tannins have a higher yield than the saponins in the two plants. However, their yield in *A. conyzoides* (20.67%) is higher than that of *N. laevis* (10.47%), thus confirming the qualitative result obtained from chemical screening. This content in *A. conyzoides* is also higher than that of *M. villosus* (16.91%), but remains slightly lower than that of *M. oleifera* (21.25%) as indicated in our previous work [18].

The yield of saponins in both plants is relatively low (2.43% in *A. conyzoides* and 2.38% in *N. laevis*). In general, the extraction yield for different *N. laevis* substrates is lower than for other plants [7,12].

Table 1. Minimum inhibitory concentrations and percentages of inhibition characteristic of aqueous and ethanolic extracts of *A. conyzoides* on the strain of *L. theobromae*

Plants	Extract	MIC (mg/mL)	IC ₅₀ (mg/mL)	IC ₇₅ (mg/ml)	PI (%) to MIC	Ratio MICol/MICaq
<i>Ageratum conyzoides</i>	Aqueous	25	8.5	23	76	2.0
	Ethanolic	50	9.0	25	84	

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

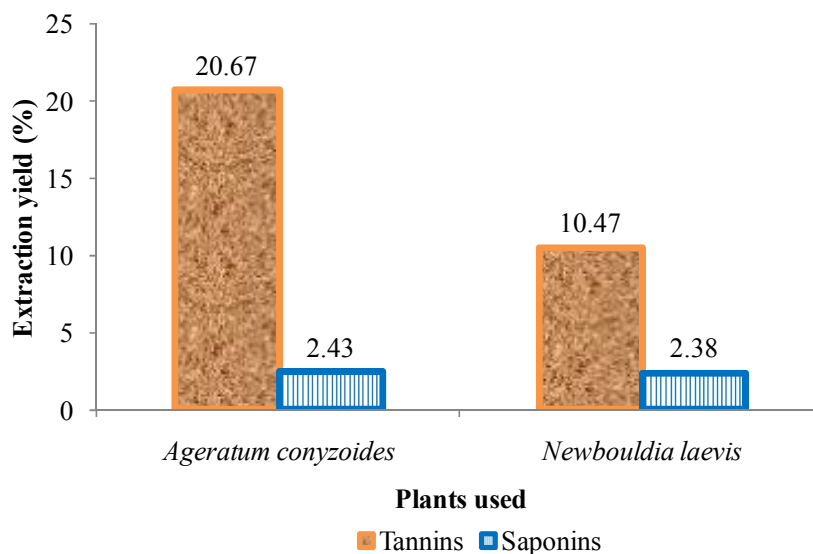


Fig. 3. Extraction yield of tannins and saponins from *A. conyzoides* and *N. laevis*

3.6 Inhibition of *L. theobromae* Growth by Tannins and Saponins

Fig. 4 gives for illustration the *in vitro* inhibition of the fungal strain by the saponins of *A. conyzoides* with the dissolving solvent of saponins, butanol.

It can be seen from this figure that five days after incubation, the strain of *L. theobromae* in the presence of saponins of *A. conyzoides* has not reached maximum growth, whereas after only three days, in the presence of butanol alone, the strain has reached maximum growth. This indicates considerable antifungal activity of the saponins of *A. conyzoides*. Similar

behavior was observed for the tannins and saponins of two plants studied.

The PIs of tannins and saponins in plants are given in Fig. 5.

This figure indicates that saponins have higher PIs (84.40% for *A. conyzoides* and 75.56% for *N. laevis*) than those of tannins (54.44 and 32.96% for *A. conyzoides* and *N. laevis* respectively).

Indeed, saponins are surfactants that can destabilize membrane structure of microorganisms including fungi. They can interact with sterols, proteins and phospholipids of cell membranes of fungi leading to loss of their integrity [39].

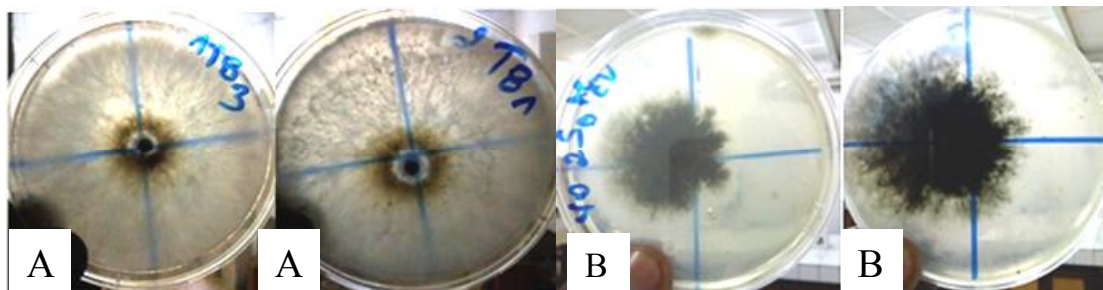


Fig. 4. Inhibition of *L. theobromae* growth

L. theobromae strain in the presence of butanol after 3 days of incubation (A) and in the presence of the extract of saponins of *A. conyzoides*, 5 days after incubation (B).

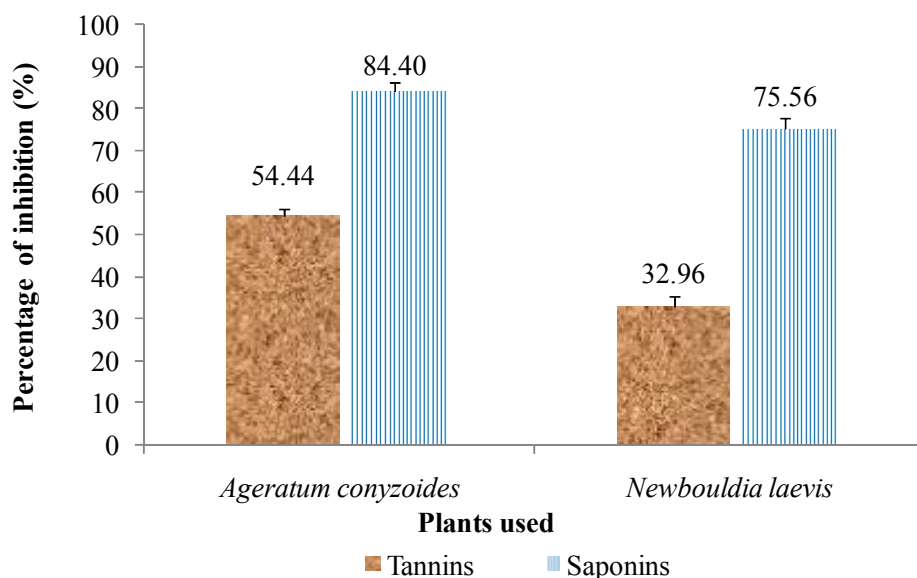


Fig. 5. Percentage of inhibition of tannins and saponins (100 mg / mL) of *A. conyzoides* and *N. laevis* on the strain of *L. theobromae* after two days of incubation

Also, the PIs of tannins and saponins are higher for *A. conyzoides* than those for *N. laevis*. This confirms the results of the PIs of the crude extracts (fig 2). This would justify the more frequent use of *A. conyzoides* in traditional medicine against fungi compared to *N. laevis* [40,41]. According to the classification of plants with antifungal activity [29], taking into account the PI values of the tannins and saponins found in *A. conyzoides*, the latter is among the plants very active on the strain of *L. theobromae* [12]. The tannins of *A. conyzoides* are moderately active while those of *N. laevis* are less active.

Compared to the PI of aqueous crude extracts of the two plants, the tannins gave low values. This indicates that the hydrophilic compounds of the two plants act in synergy. On the other hand, as for saponins, their PIs are slightly higher than those of ethanolic crude extracts, indicating that

saponins are the main group responsible for the antifungal activity of these plants.

However, considering the PI of the ethereal extracts (fig 2), the apolar substances (essential oils, sterols and terpenes) identified in the two plants would also have interesting antifungal activity.

Compared to previous results [18], the antifungal activity of saponins from *A. conyzoides* (PI: 84.40%) is much higher than that of saponins from *M. villosus* (PI: 74.4%) and tannins of *M. oleifera* (69.6%).

3.7 Maximum Growth Time

The maximum growth times (MGT, in days) of the strain of *L. theobromae* in the presence of the different substances tested are given in Table 2.

Table 2. Maximum growth time of the *L. theobromae* strain in the presence of extraction solvents, antibiotics (positive controls), and phytochemical groups of plants

Test substances	Mycelial growth time (day)	Test substances	Mycelial growth time (day)
Negative control	2	Butanol	3
Distilled water	3	Saponins of <i>Ageratum conyzoides</i>	10
Ethanol 95%	3	Saponins of <i>Newbouldia laevis</i>	5
Diethyl ether	3	Tannins of <i>Ageratum conyzoides</i>	4
Ampicillin	4	Tannins of <i>Newbouldia laevis</i>	3
Gentamycin	4		

This table shows that the MGT of *L. theobromae* is only two days for the control and three days for the solvents. In the presence of saponins, the value of MGT reaches 10 days for *A. conyzoides* and five days for *N. laevis*.

These results confirm the high antifungal activity of saponins. Indeed, when the PI is significantly high, the fungal strain is slowed down, thus leading to an increase in MGT.

The saponins of both plants are therefore more active than the tannins. This confirms the results of the evaluation of the activity of these two phytochemicals on the strain of *L. theobromae* (fig 5). Furthermore, the MGT values of these two secondary metabolites are higher for *A. conyzoides* than for *N. laevis*. The antifungal supply (higher) on the strain of *L. theobromae*, would therefore be linked to the nature of the plant species.

The values of PI and MGT obtained (Fig. 5 and Table 2) show that the saponins and tannins of two plants are more active than the total aqueous, ethanolic and ethereal extracts on the strain of *L. theobromae* [7,12]. Thus the phytochemical groups are more active than the total extracts. The total extracts contain, apart from the saponins and tannins, other less active chemical groups that can interfere with the active ingredients.

4. CONCLUSION

The objective of this work was to, extract identify, and evaluate the antifungal activity of the secondary metabolites of the leaves of *Ageratum conyzoides* and *Newbouldia laevis* on the strain of *Lasiodiplodia theobromae*. It was found that saponins, sterols, and terpenes are abundant in both plants, however tannins are very abundant in *A. conyzoides* and abundant in *N. laevis*. *A. conyzoides* contains gallic tannins while *N. laevis*, condensates and catechics. Saponins were found to be more antifungal (Percentage of inhibition PI: 84.40% for *A. conyzoides* and 75.56% for *N. laevis*) than tannins (PI: 54.44 and 32.96% respectively) on the strain of *L. theobromae* after two days of incubation. The identification of the saponins molecules is ongoing

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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