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Antifungal Potential of Acetone and Ethyl Acetate Extracts of *Thevetia peruviana* on Development of *Phytophthora colocasiae*, Causal Agent of Late Blight of Taro (*Colocasia esculenta* (L.) Schott) from Three Agro-Ecological Zones of Cameroon

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Authors' contributions

This work was carried out in collaboration among all authors. Author CSE designed the study, wrote the protocol and wrote the first draft of the manuscript. Author ZA designed the study and reviewed all drafts of the manuscript. Authors WNTK, HB and DNM managed the experimental process and identified the fungal strains. Authors PZN, WNTK and DTD performed the statistical analysis and reviewed all the drafts of the manuscript. Authors MT, AKN, SBM and AH managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was aimed to evaluate the antifungal activities of acetone and ethyl acetate extracts of *Thevetia peruviana* seeds on the *in vitro* growth of the fungus.
Study Design: A randomized sample block design containing four treatments (T- = Negative control; T2= Ethyl acetate extract; T3= Acetone extract; T+=Callomil Plus) with three repetitions was used. Plant extracts were used at three concentrations: C1: 12.5 µl/ml; C2: 25 µl/ml and C3: 50 µl/ml; the chemical fungicide at the dose of 12.5 µL/ml.
Place and Duration of Study: The study was conducted in the University of Yaoundé 1, Faculty of Sciences, Department of Plant Biology, Laboratory of Phytopathology and Crop Protection, and in the Institute of Agricultural Research for Development (IARD) of Yaoundé, Laboratory of Phytopathology, during the year 2019-2020.
Methodology: acetone and ethyl acetate extracts of *T. peruviana* were prepared and used at concentrations of 12.5, 25 and 50 µl/ml. *P. colocasiae* was isolated from infected taro leaf cultivars "Macumba or Ibo coco" located in three different regions: west, Littoral and Centre. The various explants were were put in V8 agar medium and maintained in pure culture. Mycelial fragments of *P. colocasiae* of about 0.8 cm in diameter were cut and placed in sterile Petri dishes containing Potato Dextrose Agar (PDA) medium supplemented with different concentrations of plant extracts and incubated at 23±1°C for seven days for the evaluation of the radial growth.
Results: The results obtained showed that the acetone and ethyl acetate extracts have completely inhibited the growth of the strain of West at 25 µ/ml while total inhibition of the pathogen was not obtained with strain of Centre region. The lowest inhibition was obtained with the strain of Littoral region: 93.88 % for acetone extract and 90.78 % for ethyl acetate extract compare to 100 % for west and Centre region at highest concentration.
Conclusion: The acetone and ethyl acetate extracts at the concentration of 25 µ/ml totally inhibited the *in vitro* radial growth of some strains of *P. colocasiae*. These extracts, which are effective against P. colocasiae, may substitute fungicides in the fight against taro leaf blight.

Keywords: Extracts of Thevetia peruviana; antifungal potential; Phytophthora colocasiae; taro.

1. INTRODUCTION

"Cocoyams (*Colocasia esculenta*) are well adapted food crops across many agro-ecological zones of sub-Saharan Africa. They rank third in importance, after cassava and yam, among the root and tuber crops cultivated and consumed in many West and Central Africa countries. Cocoyams are nutritionally superior to both cassava and yam in the possession of higher protein, mineral and vitamin contents as well as easily digestible starch" [1]. "Africa in the last three decades has consistently accounted for an increasing percentage of global cocoyam production, which currently stands at about 10 million tonnes per annum" [1,2]. "Cocoyam is therefore undoubtedly an important food crop in sub-Saharan Africa (SSA), particularly in Nigeria, Ghana and Cameroon. Global production is estimated at about 10.64 million tonnes on a cultivated area of 1.67 million hectares" [1,2,3]. "In addition, 77% of global taro production comes from sub-Saharan Africa" [1]. "However, the increasing production in the region has depended largely on farming more land rather than increasing crop yields. This is contrary to the projections of FAO that the 70% growth in global agricultural production needed to feed an additional 2.3 billion people by 2050 must be achieved by increasing yields and cropping intensity on existing farmlands, rather than by increasing the amount of land brought under agricultural production" [1,2]. This could be due to the enemies of this crop such as diseases that hinder its production. One of the most important is late blight caused by *Phytophthora colocasiae* [4,5]. It was first described in Java by Marian Raciborski in 1900 [6]. The disease mainly affects the leaves of the taro (Fig. 1B), and can completely destroy susceptible cultivars in less than 10 days and cause yield losses in the range of 50 to 100% [4,5,6]. This loss of yield has a remarkable impact on farmers' incomes as well as on the food security of human populations. pH 7 and temperature of 27°C are the optimal conditions for the pathogen to grow in the field [7,8,9].

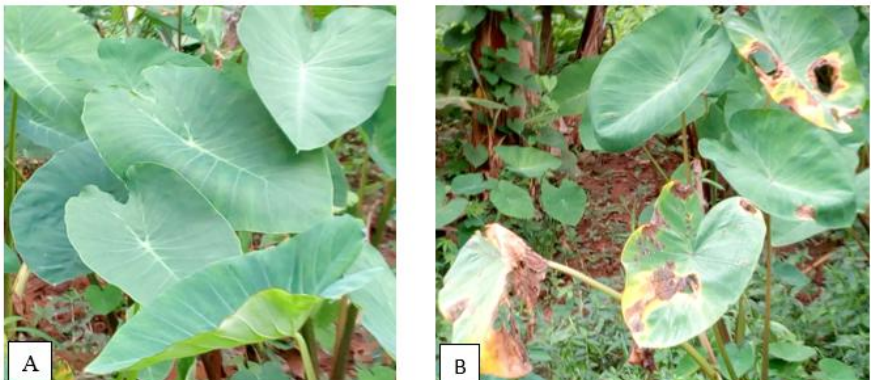

Fig. 1. (A): *Colocasia esculenta* plants. (B): Attacked plants showing symptoms of taro late blight on the upper leaf surface

Control strategies for this pathogen are most often focused on the use of metalaxyl-based chemical fungicides [10,11]; but due to the problems of residues in groundwater [12], the development of resistance in the target organism and the danger to man and the environment, alternative control methods are increasingly being considered. Currently, considerable efforts are directed towards the exploration of plant extracts with pesticide potential as alternative or complementary sources to synthetic pesticides. Plant extracts have the advantage of being not only available to farmers, but also non-toxic and easily biodegradable and therefore healthy for the environment [13,14]. Several studies have shown the antifungal effects of plant extracts on *Phytophthora infestans*, the causative agent of late blight in potatoes, tomatoes and black nightshades [15,16,17], but no information is available on the effect of seed extracts of plants such as *Thevetia peruviana* on *P. colocasiae* in Cameroon. The seeds, leaves, fruits and roots of the Yellow Oleander (*Thevetia peruviana*) are considered potential sources of biological compounds active as insecticides [18,19,20] fungicides [20,21,22] virucides [23,24] and bactericides [25]. Thus, the present work aims to evaluate the antifungal potential of acetone and ethyl acetate extracts of *Thevetia peruviana* seeds on the *in vitro* development of *P. colocasiae* from three agro-ecological zones of Cameroon.

2. MATERIALS AND METHODS

2.1 Plant and Chemical Material

The plant material consists of the kernels of *Thevetia peruviana* collected in the city of Yaounde where the tree serves as a house fence; and leaves of *Colocasia esculenta* collected in peasant plantations located in the locality of: Bafang in the Department of Haut-Nkam in West (OU123), Penja in the department of Moungo in Littoral (LT122) and Yaoundé in the department of Mfoundi in Central Cameroon (CE111); and taken to the lab. The chemical material is a product with the trade name Callomil Plus 72 WP consisting of 12% metalaxyl and 60% copper oxide; and organic solvents such as acetone and ethyl acetate.

2.2 Methods

2.2.1 Preparation of extracts of *Thevetia peruviana* seeds

The plant of *Thevetia peruviana* has been identified according to the botanical systematics key of the species with reference to the recent version of the International Code of Botanical Nomenclature [26,27]. The mature *T. peruviana* fruits were picked, the stones extracted from the fruits were crushed and the resulting kernels were dried at room temperature for 3 to 4 weeks in the laboratory and then crushed using a manual mill to obtain a paste. The organic extract was prepared by maceration of 1 kg of paste in 5 L of solvent for 48 hours and then filtered. The resulting filtrate was concentrated at 60 °C using a rotary evaporator and the solvent extract obtained was stored in the refrigerator at 4 °C until use. Doses of extracts of 12.5; 25 and 50 µl/ml were obtained following a progression geometry of reason 2 [28] from a stock solution of 500 µl/ml.

The extraction yield of each extract was calculated using the formula cited by [29,30]:

$$Rd\% = \frac{\text{Mass of extract}}{\text{Mass of powder}} X100$$

2.2.2 Isolation and purification of *Phythopthora colocasiae*

"The infected leaves of the harvested taro variety "Macumba" were cut into fragments of about 2 cm^2 at the growth front of the pathogen and disinfected superficially in a solution of 5% sodium hypochlorite for 2 minutes. After three rinses with sterilized distilled water (EDS), the fragments were dried on hydrophilic paper and then deposited at the rate of four fragments in a petri dish poured in the gelled V8 culture medium supplemented with a solution of antibiotics composed of penicillin (250 mg / l), ampicillin (250 mg / l) and nystatin (20 mg / l)" [31,32,33]. "After three days of incubation in the laboratory at a temperature of 23±1°C, colonies of the pathogen, visible around the fragments, were taken and transplanted into new petri dishes containing the PDA culture medium. This process was repeated several times until pure morphological cultures of the mycelium (not septate) and fruiting (sporangia) as described" by [34] and [35] were obtained (Fig. 2).

The isolates obtained are characterized according to morphological criteria such as pathogenicity and growth rate [36].

2.2.3 *In vitro* evaluation of the antifungal activity of the crude extracts

The in vitro evaluation of the antifungal activity of the extracts was done at concentrations of 12.5; 25 and 50 µl/ml for the two extracts from the stock solutions of 500 µl/ml for each. A synthetic fungicide (Callomil Plus 72 WP) was used as a positive control by taking from a 50g sachet, 1g of powder per 5 ml of distilled water. Mycelial explants of *P. colocasiae* about 8 mm in diameter were taken with a cookie cutter from a pure fruiting culture seven days old and placed in the center of the petri dish containing the media enriched with the different extracts or chemical fungicide. A negative control not supplemented with extract was developed. Each treatment was repeated 3 times. Incubation was carried out at 23±1°C under a photoperiod of 12/12 for one week. A daily measurement of the radial growth diameter of each cultured explant was taken and continued until the mycelium filled the control dishes. The radial (D) growth of the pathogen was assessed by measuring two perpendicular diameters traced to the back of the petri dish. The average of the two perpendicular measurements removed from the diameter of the explant represents the measure of the radial growth of the fungus. It is obtained by the formula described by [37].

$$\mathrm{D} = \frac{\mathrm{D1} + \mathrm{D2}}{2} - \mathrm{D0}$$

Where: D0 is the diameter of the explant; D1 and D2 are the culture diameters measured in both perpendicular directions (Fig. 3).

The percentage of inhibition (I%) due to each extract is evaluated in relation to the mycelial growth in the control boxes according to the formula developed by [37].

$$I\% = \frac{\mathrm{Dto\ mm} - \mathrm{Dxi\ mm}}{Dto\ mm}\ X100$$

With I (%): Percentage of inhibition; Dto is the average diameter of the control batch and Dxi is the average diameter of the batches in the presence of the extract.

2.2.4 Correlation between concentration and inhibition percentages

"Correlation tests were performed to determine the relationship between the concentrations used and the inhibition percentages obtained for each extract. In other words, it was a question of establishing a linear relationship model to predict the percentage of inhibition from the concentrations of each extract, for each type of fungus, and for each stage of life. In each case, the correlation coefficient was determined in order to provide information on the degree of linear dependence between the two variables.

In this case if a < 0 then the relationship is inversely proportional and the correlation is negative. If a > 0 then the relationship is positive; if r between 0.7 and 1 then the correlation is perfect and positive; if r between -0.7 and -1 then the correlation is perfect and negative; if r < 0.7 then the correlation is positive but imperfect; if r > -0.7 then the correlation is negative and imperfect" [38].

2.2.5 Fungicidal or fungistatic activity of extracts and chemical fungicide

"At the end of each test, the mycelium explants from the boxes where the growth was completely inhibited, were taken and deposited aseptically on the culture medium containing no extract. After 7 days of waiting, depending on whether or not the fungus has resumed growth, the starting extract was identified as fungistatic or fungicidal respectively" [39].

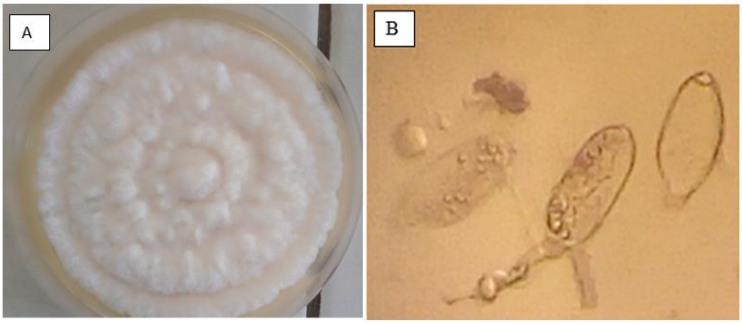

Fig. 2. *Phytophtora colocassiae*: Pure culture of mycelium (A) and sporangia (B). (Gr: X20)

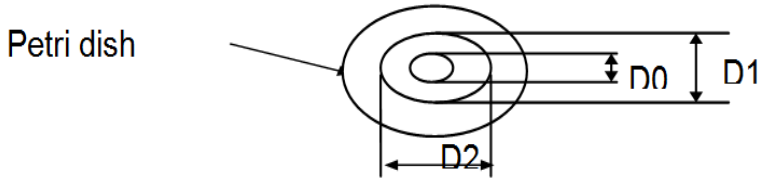

Fig. 3. Diagram of measurement of mycelial growth in petri dish on V8 medium

2.3 Statistical Analysis

The inhibition percentages of the radial growth of the pathogen were transformed into probits and the values obtained were regressed on the logarithm of the concentration of plant extracts. The efficacy of the extracts was evaluated on the basis of the inhibiting concentration value of 50% (CMI_{50}) and 90% (CMI_{90}) determined after 8 days of growth according to the formula developed by [40]. Inhibition percentage data were subjected to analysis of variance using R analysis software version 5.1.0 and means separated by Duncan's multiple test at the 5% probability threshold.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Extraction yield

The yield, volume, colour and appearance of the different extracts obtained varied depending on the extraction solvent used. Extraction with ethyl acetate yielded slightly higher (28.5%) than acetone (23.3%). The ethyl acetate extract has an oily appearance and pale yellow color while the acetone extract has a viscous appearance and brown color (Table 1).

3.1.2 Effect of *Thevetia peruviana* extracts on the *in vitro* growth of *P. colocasiae*

The seed extracts tested significantly inhibited the radial growth of *P. colocasiae*. The diameter of the fungal colony that received the high concentrations of the extracts was very small and zero at the highest concentrations. Total inhibition was achieved at a concentration of 25 µl/ml for acetone and ethyl acetate extract. On the other hand, in the control boxes, the growth of *P. colocasiae* was significantly higher compared to the different concentrations of the extracts tested (Fig. 4).

3.1.3 Effect of EAc on the growth of *P. colocasiae* strains

The EAc showed inhibition on the growth of *P. colocasiae* strains. With the CE111 strain we had the lowest percentage of inhibition, 93.88% at the highest (C3) dose, (Fig. 5) compared to 100% for OU123 and LT122 (Fig. 5). Inhibition was proportional to concentration. EAc was found to be effective in the same way as Callomil at dose C3 with 100% growth inhibition on both strains compared to control (P>0.05). The OU123 strain was more sensitive to the extract at the 12.5 µ/ml dose with 100% reduction in mycelial growth.

3.1.4 Effect of EAE on the growth of *P. colocasiae* strains

The EAE showed inhibition on the growth of *P. colocasiae* strains. With the CE111 strain we had the lowest percentage of inhibition, 90.78% at the highest dose (C3), (Fig. 5) compared to 100% for OU123 and LT122 (Fig. 5). Inhibition was also proportional to concentration. EAE was found to

be effective in the same way as Callomil at dose C3 with 100% growth inhibition on two strains compared to the control (P<0.05). The OU123 strain was more sensitive to the extract at the smallest dose of 12.5 µl/ml with more than 88% reduction in mycelial growth.

3.1.5 Fungicidal or Fungistatic Activity of the Extracts

The fungi tested showed different behaviors towards the extracts and depending on the doses. For strain CE111, EAE was found to be fungistatic at C2 and fungicidal at C3 while EAc was found to be fungistatic at both doses. However, with the OU123 strain, EAE and EAc were found to be fungicidal at both doses. For LT122, EAE and EAc were found to be fungistatic at C2 and fungicidal at C3 (Table 2).

3.1.6 Correlation test between ethyl acetate extract concentrations and inhibition percentages of *Phytophthora colocasiae* growth

The equations obtained with the EAE showed increasing linear relationships. Indeed, all regression lines obtained with the strains showed positive slopes. The correlation coefficients were all between 0.7 and 1. The strains OU123, CE111 and LT122 showed respectively the following coefficients: r = 0.75, r = 0.84 and r = 0.75 which is a perfect and positive correlation (Fig. 6).

3.1.7 Correlation test between acetone extract concentrations and *phytophthora colocasiae* growth inhibition percentages

P. colocasiae strains behaved differently with acetone extract. A strong positive correlation was obtained with some strains, the correlation coefficient r was greater than 0.7; this is the case with CE111: r = 0.88, LT122 : r = 0.9, a positive and perfect correlation (Fig. 7). With the OU123 strain (y=100) the equation obtained showed a constant linear relationship which highlights an absence of correlation (r=0). The lines obtained with isolates CE111 and LT122 showed positive slopes, respectively y=11.26x + 57.7 and y=7.09x + 78.31.

3.1.8 Minimal Inhibitory Concentrations of the Different Extracts

The MICs of the growth of *P. colocassiae* strains varied by extract. $MICs_{90}$ are higher with EAE and range from 15 to 90 µl/ml. The MICs obtained with EAc range from 11.25 to 47.83 µl/ml. The smallest CMI_{90} (11.25) was obtained with EAc on strain OU123. However, no CMI_{50} was obtained with the two extracts (Table 3).

3.2 Discussion

This work was based on the evaluation of the antifungal potential of the ethyl acetate and acetone extract of *T. peruviana* seeds on *phytophthora colocasiae* strains responsible of taro late blight.

The extraction of 1000 g of the seeds of *T. peruviana* produced different yields. These yields ranged from 28.5% with ethyl acetate to 23.3% with acetone. This variation can be attributed to the nature of the solvent. These results are different from those obtained by [41].who, after extraction, using the same amounts of *T. peruviana* seed paste with the same solvent volumes, obtained a yield of 33.16% with ethyl acetate and 9.43% with acetone. Indeed, [42] and Smallfield [43] report that "environmental conditions, harvest period and age of plant material can influence extraction yields".

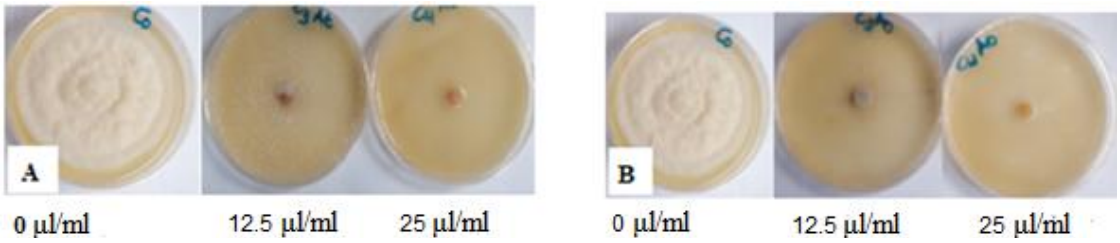

Fig. 4. In vitro inhibitory activity of *Thevetia peruviana* extracts on the radial growth of the *P. colocasiae* strain (OU123) after 8 days of incubation on PDA medium; A: Ethyl acetate extract, B: Acetone extract

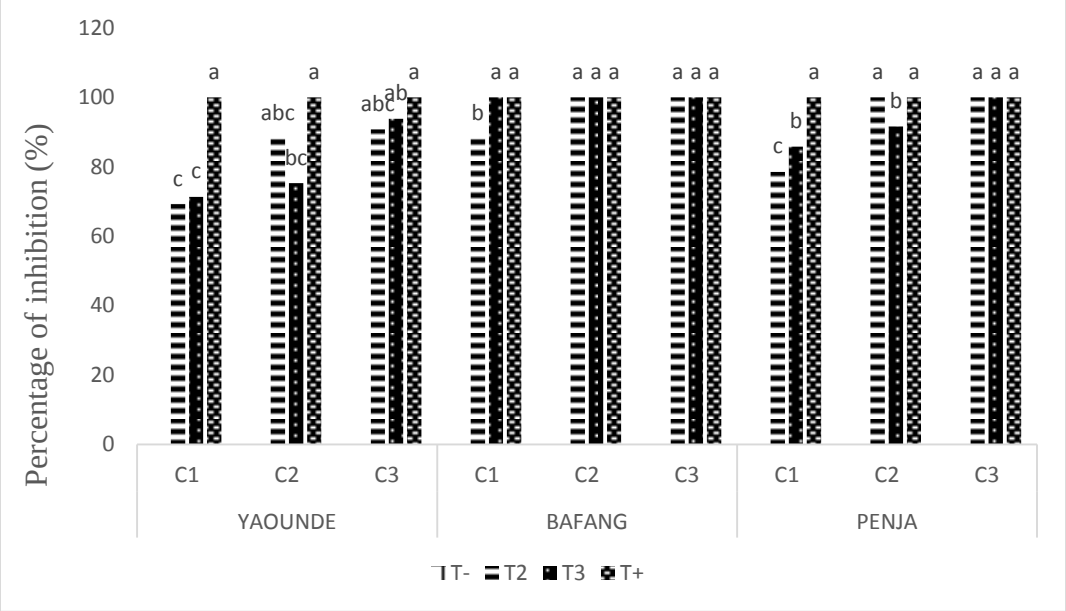

Fig. 5. Effect of extracts on the growth of *P. colocasiae* strains

For each strain, the assigned values of the same letter do not differ significantly according to the Newman-Keuls test.

T-= Negative control; T2= Ethyl acetate; T3= Acetone; T+=Fungicide

T- (0 µl/ml); C1=12.5 µl/ml; C2=25 µl/ml; C3=50 µl/ml; T+ (12.5 µl/ml l)

Table 1. Extraction yield (%) and characteristics of extracts for 1kg of seeds

Extract with	Yield	Caracteristics
Ethyl acetate (EAE)	28,5	Oily and pale yellow in colour
Acetone (EAc)	23,3	Brown and viscous

Table 2. Fungicidal or fungistatic activity of extracts and synthetic fungicide

Especies	Isolates	Extracts	Concentrations	Effect
P. colocasiae	CE111	EAE	C2 (25 µl/ml)	Fungistatic
			C3 (50 µl/ml)	Fungicidal
		EAc	C2 (25 µl/ml)	Fungistatic
			C3 (50 µl/ml)	Fungistatic
		Callomil	C1 (12,5 µl/ml)	Fungicidal
	OU123	EAE	C2 (25 µl/ml)	Fungicidal
			C3 (50 µl/ml)	Fungicidal
		EAc	C2 (25 µl/ml)	Fungicidal
			C3 (50 µl/ml)	Fungicidal
		Callomil	C3(5025 µl/ml)	Fungicidal
	LT122	EAE	C2 (25 µl/ml)	Fungistatic
			C3(5025 µl/ml)	Fungicidal
		EAc	C2 (25 µl/ml)	Fungistatic
			C3 (50 µl/ml)	Fungicidal
		Callomil	C1 (12,5 µl/ml)	Fungicidal

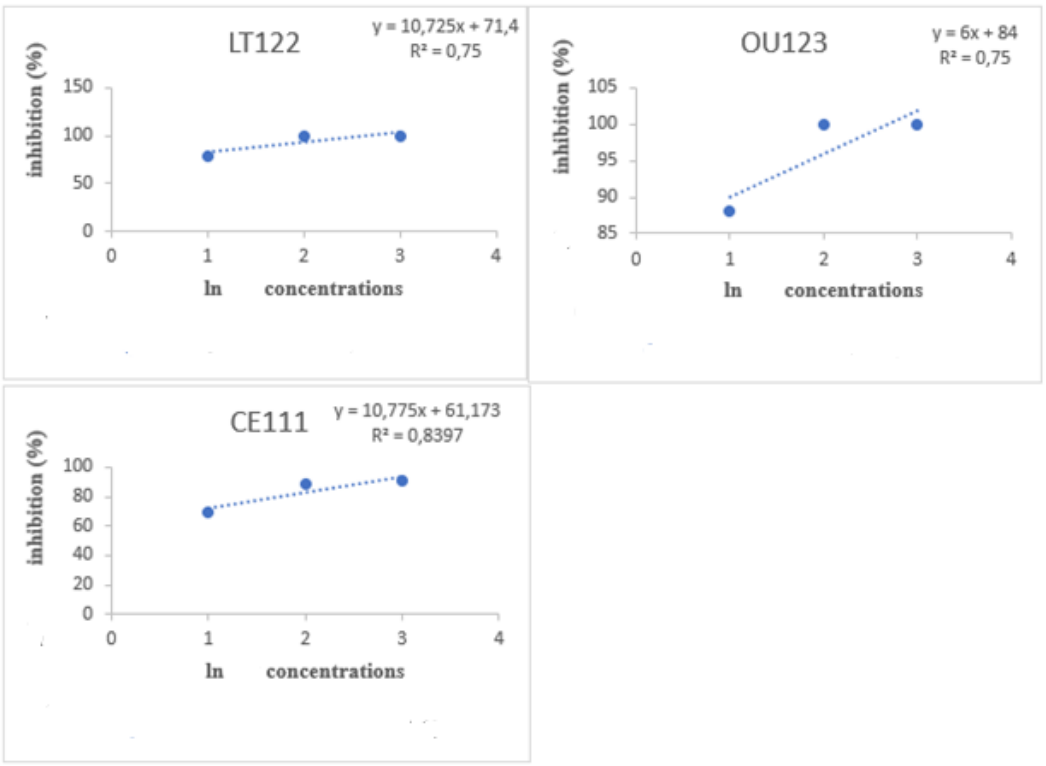

Fig. 6. Regression lines of growth of *Phytophthora colocasiae* strains after treatment with ethyl acetate extract

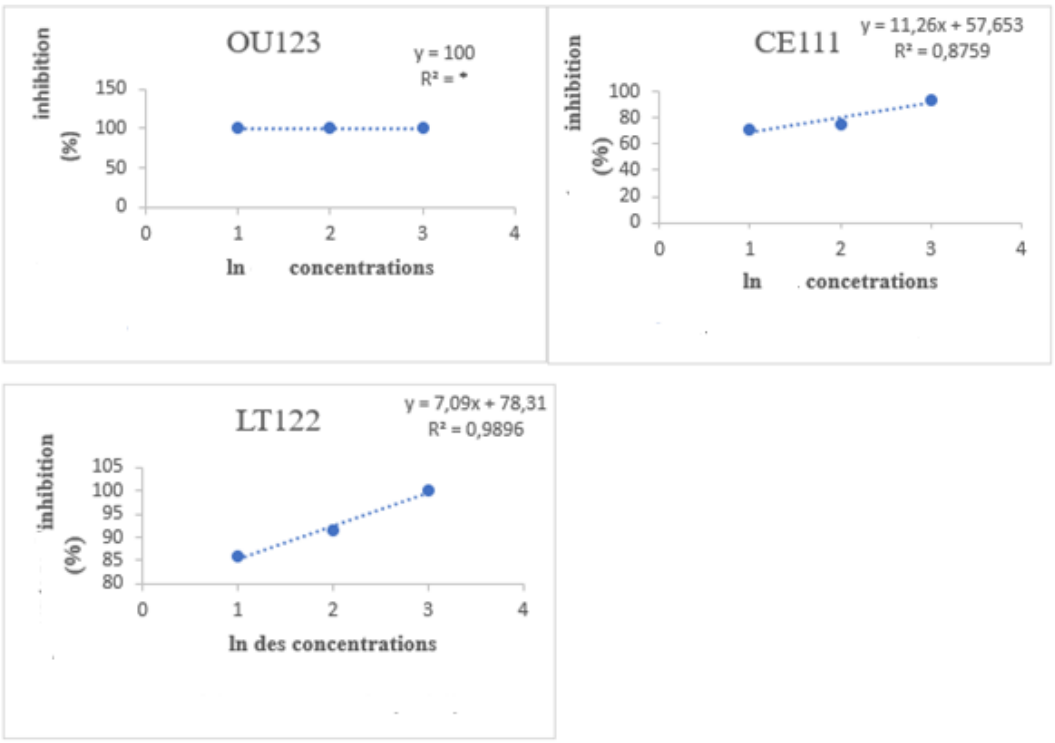

Fig. 7. Regression lines of growth of *Phytophthora colocasiae* strains after treatment with acetone extract

Table 3. CMI_{50} and CMI_{90} (µl/ml) of the mycelial growth of *P. colocassiae* in the presence of *T. peruviana* extracts

		Isolats		
		OU123	**LT122**	**CE111**
EAE	CMI_{90}	15	22.5	90
	CMI_{50}	*	*	*
EAc	CMI_{90}	11.25[a]	20	47.83
	CMI_{50}	*	*	*

□ *Represents values that are not set to be at zero statistically*
[a] *smaller concentration inhibiting 90% mycelial growth*

EAE and EAc significantly inhibited the growth of *P. colocassiae* strains compared to the control. These extracts would contain substances that inhibit or reduce the growth of the fungus. Indeed, [44,45] reported that "plant extracts from a number of plants contain compounds such as tannins, flavonoids and alkaloids that have fungicidal properties. The different concentrations of extracts significantly influenced the radial growth of the fungus; high concentrations being more inhibitory. This reduction in growth was more pronounced with acetone extract than with ethyl acetate extract". The effectiveness of these extracts on the growth of *P. colocassiae* could be explained by the presence in these extracts of bioactive molecules revealed by phytochemical screening, such as essential oils, saponifiable oils, coumarins, sterols, alkaloids, triterpenes, tannins, sugars, phenols, saponins and anthocyanins. All these molecules have antifungal properties, as demonstrated by [46].

The results obtained show that some strains have been shown to be more resistant to certain extracts used compared to others, which would be due to the nature of the specificity they would present at the membrane level. In general, antifungals can be contact: acting at the level of the fungus membrane or systemic: acting inside the cell [47,48]. In both cases, specific membrane or intracellular receptors may be essential for the expression of the biological activity of the antifungal. Some chemical constituents have the ability to recognize sites of action in the pathogen, others do not. They would thus act through a concentration effect and once fixed on their receptors, would elicit responses such as inhibition of general metabolism (fungistatic effect) or alteration of the plasma membrane of the fungus and inhibition of respiration (fungicidal effect) [49,50,51]. Callomil Plus 72 WP was very effective against *P. colocassiae* at all doses with inhibition percentages of around 100% on strain growth. Its effectiveness would be due to the presence of copper oxide major active ingredient (60%), which is known for its action on cellular respiration. This result is similar to those of [52,53] who showed in vitro the efficacy of Callomil on strains of *P. colocassiae*. The low MIC values obtained with the acetone and ethyl acetate extract highlight the effectiveness and fungicidal properties of these different extracts on the growth of the fungus tested. These results are similar with those of [54,55] who showed that low MIC values of *Callistemon viminalis* and *T. peruviana* extracts respectively inhibit the development of *P. colocasiae*.

4. CONCLUSION

The study showed that *T. peruviana* extracts inhibited *in vitro* the radial growth of *P. colocasiae***.** These extracts have been shown to be active on *P. colocasiae* and may therefore be an alternative for the fight against taro late blight. Despite the fact that these crude extracts exhibit activity that is comparable to the reference fungicide (Callomil Plus 72 WP), they still include a variety of distinct chemicals that, after being purified, would be more effective than the chemical fungicide.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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