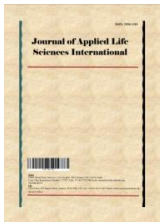

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Efficacy of Methanolic and Aqueous Extracts of *Thevetia peruviana* (pers.) K. Schum on Growth of *Phytophthora colocasiae* Racib, Causal Agent of Taro Late Blight in Cameroon

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Authors' contributions

This work was carried out in collaboration among all authors. Author CES designed the study, wrote the protocol and wrote the first draft of the manuscript. Author ZA designed the study, wrote the protocol, wrote the first draft of the manuscript and reviewed all drafts of the manuscript. Author JPND, Authors WKT and DMN managed the experimental process and identified the fungal strains. Authors PZN and WKT performed the statistical analysis and reviewed all the drafts of the manuscript. Authors GC And AH managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study aims to evaluate the antifungal activities of methanolic and aqueous extracts of *Thevetia peruviana* seeds on the *in vitro* growth of *Phytophthora colocasiae*. A randomized sample block design containing four treatments (T-: absolute control, AE, ME and Callomil Plus at the dose of

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12.5 µL/ml) 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 study took place 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.
Aqueous and methanolic extracts of *T. peruviana* were prepared and used at the concentrations of 12.5, 25 and 50 µL/ml. *P. colocasiae* was isolated from infected taro leaf cultivars "Macumba/Ibo coco" located in three different regions in Cameroon: West, Littoral and Centre. The different leaf explants of taro 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. Methanolic and aqueous extracts have completely inhibited the growth of West and Littoral strains at 25 µL/mL while total inhibition of the pathogen was not obtained with strain of Centre region. The lowest inhibition was obtained with the strain of Centre region (85.1%) for aqueous extract and (70.95%) for methanolic extract compare to 100% for West and Littoral region at highest concentration. The aqueous extract at the concentration of 25 µL/ml totally inhibited the *in vitro* radial growth of some strains of *P. colocasiae*. This extract, active *against P. colocasiae* could be used as alternative to fungicides for the control of taro leaf blight. In other hand, the strain of Littoral region was most sensible to extracts than the others. This strain could be used to provide a genetic resource for future trials in natural conditions in greenhouse and in the field.

Keywords: Thevetia peruviana; antifungal activities; Phytophthora colocasiae; taro.

1. INTRODUCTION

Taro leaf blight caused by *Phytophthora colocasiae* is the most devastating disease in taro production in Cameroon since one decade, responsible for production losses ranging from 50 to 100% in plantations [1,2]. It was first described in Java by Marian Raciborski in 1900 [3]; H 7 and a temperature of 27°C are optimal conditions for pathogen growth in the field [4,5].

Taro (*Colocasia esculenta* (L.) Schott) is an important staple food for millions of people in African, Asian and Central American countries [6,4]. In Cameroon, taro is cultivated for its tubers rich in starch (73-80%) and highly digestible with an amylose content of 30.62% [7,8]. It is thus richer in amylose than cassava (16.89%) or maize (22.4%) [9,10]. The highly digestible starch of the tubers makes taro an excellent food for diabetics [10]. The world production of taro is estimated in 2018 at about 10.64 million tons on a cultivated area of 1.67 million hectares [11]. In addition, 77% of global taro production comes from sub-Saharan Africa [11]. The taro leaf is the fourteenth most consumed vegetable in the world [12]. Cameroon is the third largest producer of taro in the world, and the second largest producer in Africa after Nigeria, with a production of 1.9 million tons over 7.87 million tons for the all Africa so 24.14% of African production [11].

Despite the economic, nutritional and socio-cultural importance of taro, its cultivation is affected by late blight, which mainly affects the leaves and can completely destroy sensible cultivars in less than 10 days and cause yield losses of 50-100% [12,13,1,14]. This loss of yield has a remarkable impact on farmers' incomes and the food security of the human population. Several strategies have already been used to reduce attacks of this pathogen; including chemical control, which often focuses on the use of synthetic metalaxyl-based fungicides [15,16]. Due to the problems of residues in groundwater [17], the development of resistance in the target organism and the danger to humans and the environment due to synthetic products, alternative control methods are increasingly being considered. Currently, considerable efforts are directed towards exploring plant extracts with pesticidal 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 environmentally acceptable [18,19]. Several studies have shown the antifungal effects of plant extracts on *Phytophthora infestans*, the causal agent of potato, tomato and black nightshade blight [20,21,22], but very little informations are available on the effect of plant seed extracts such as *Thevetia peruviana* on *P. colocasiae* in Cameroon. Seeds, leaves, fruits and roots of

Yellow oleander (*Thevetia peruviana*) are considered potential sources of biologically active compounds as insecticides [23,24], fungicides [25,26,27,28,29], virucides [30] and bactericides [31]. Thus, the present work proposes to evaluate the efficacy of aqueous and methanolic extracts of *Thevetia peruviana* on the *in vitro* growth of *P. colocasiae* from three agro-ecological zones of Cameroon.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

The plant material consists of kernels of *Thevetia peruviana* collected in the city of Yaoundé where the trees are used as a house fence; and leaves of *Colocasia esculenta* collected in peasant plantations located in Bafang, Haut-Nkam Division (West Region); Penja, Moungo Division (Littoral Region) and Yaoundé, Mfoundi Division (Central Region of Cameroon). The leaves have been carried to the laboratory for immediate use.

2.1.2 Chemical material

The chemical material is a Callomil Plus 72 WP trade name product consisting of 12% metalaxyl and 60% copper oxide, obtained in market.

2.2 Methods

2.2.1 Preparation of the extracts of *Thevetia peruviana* seeds

The plant of *Thevetia peruviana* has been identified according to the key of botanical systematics of the species by referring to the recent version of the International Code of Botanical Nomenclature [32,33]. Mature *T. peruviana* fruits were collected, the stones extracted from the fruits were crushed and the resulting kernels were dried at room temperature for 3-4 weeks in the laboratory and then grind using a hand grinder to obtain a paste. The organic extract was prepared by macerating 1 kg of paste in 5 L of solvent (methanol) for 48 h and then filtered. The resulting filtrate was concentrated at 60°C using a rotary evaporator and the resulting solvent extract was kept refrigerated at 4°C until use. For the aqueous extraction, the resulting paste was wrapped in muslin cloth and soaked directly in sterilized distilled water for 12 h (on a basis of 36 g paste in 72 mL water) and then dewatered to extract as much product as possible [29]. The aqueous extract thus prepared is used directly. Extract doses of 12.5; 25 and 50 µL/ml were obtained following a geometric progression of reason 2 from a stock solution of concentration 500 µL/ml.

The extraction yield of each extract was calculated according to the formula used by Ngoh Dooh [27,28]:

$$\text{Extraction yield } (\%) = \frac{\text{Mass of extract}}{\text{Mass of powder}} \text{ X } 100$$

2.2.2 Isolation and purification of *Phythopthora colocasiae*

The infected leaves of the harvested taro variety "Macumba" were cut into fragments of approximately 2 cm^2 at the growth front of the pathogen and superficially disinfected in a 5% sodium hypochlorite solution for 2 minutes. After three rinsings with sterilized distilled water (SDW), the fragments were dried on hydrophilic paper and then four fragments were placed in a Petri dish poured with 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) [34,35]. After 3 days of incubation in the laboratory at 23±1°C, the mycelia of the pathogen, visible around the fragments, were collected and transferred to new Petri dishes containing Potato Dextrose Agar (PDA) culture medium. This process was repeated several times until pure morphological cultures of the mycelium (not septate) and fruiting bodies (sporangia) were obtained as described by [13,36]. The resulting isolates were characterized according to morphological criteria such as pathogenicity and growth rate [37].

2.2.3 *In vitro* evaluation of the antifungal activity of 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 methanolic and aqueous extract from the stock solutions of concentration 500 µL/mL for each. A synthetic fungicide (Callomil Plus 72 WP) was used as a positive control by taking 1 g of powder per 5 mL of distilled water from a 50 g sachet. *P. colocasiae* mycelial explants of approximately 8 mm

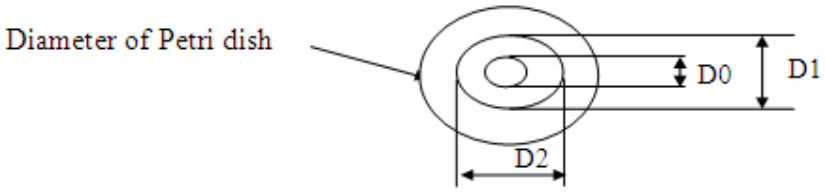

Fig. 1. Diagram of measurement of mycelial growth in a Petri dish on V8 medium

diameter were picked from a seven-day old pure fruiting culture and placed in the center of the petri dish containing the media enriched with the various extracts or fungicide. A negative control without extract enrichment 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. Radial growth (D) of the pathogen was assessed by measuring two perpendicular diameters plotted on the back of the petri dish (Fig. 1). The average of the two perpendicular measurements minus the explant diameter represents the measure of radial growth of the fungus. It is obtained by using the formula of Dohou:

$$D = \frac{D1+D2}{2} - D0 \text{ [38]}$$

Where: D0 is the diameter of the explant; D1 and D2 are the culture diameters measured in the two perpendicular directions.

The percentage of inhibition (I%) due to each extract is evaluated compared to the mycelial growth in the control dishes according to the formula:

$$I(\%) = \frac{Dto\ (mm) - Dxi\ (mm)}{Dto\ (mm)} X100 \text{ [38]}$$

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 Fungicidal or fungistatic activity of extracts

At the end of each test, the mycelium explants from the Petri dishes where growth was totally inhibited were removed and deposited aseptically on the culture medium containing no extract. After 7 days of waiting, depending on whether or not growth of the fungus had resumed, the starting extract was identified as fungistatic or fungicidal, respectively [39,29].

2.2.5 Statistical analysis

The percentages of radial growth inhibition of the pathogen were transformed into probits and the values obtained were regressed on the logarithm of the concentration of the plant extracts. The efficacy of the extracts was evaluated on the basis of the 50% (MIC_{50}) and 90% (MIC_{90}) inhibitory concentration value determined after 8 days of growth [40]. The data for percentage of inhibition, MIC_{50}, MIC_{90} were subjected to an analysis of variance using Analysis Software R version 3.5.1. and the means separated by Tukey test at the 5% threshold.

3. RESULTS AND DISCUSSIONS

3.1 Results

3.1.1 Extraction yield

The yield, volume, colour and appearance of the various extracts obtained depend on the extraction solvent used. Extraction with methanol gave the highest yield: 35% and extraction with water the lowest yield: 20.33% (Table 1). The methanol extract has a viscous appearance and brown colour while the aqueous extract has a liquid appearance and whitish colour.

3.1.2 Effect of *T. peruviana* extracts on the *In vitro* growth of *Phytophthora colocasiae*

The seed extracts tested significantly inhibited the radial growth of *P. colocasiae*. The diameter of the fungal mycelia that received the high concentrations of extracts was very small and was zero at the highest concentrations. Total inhibition was achieved at the 25 µL/mL concentration with both aqueous and methanolic extracts. However, in the control dishes, the growth of *P. colocasiae* was significantly higher compared to the different concentrations of the tested extracts (Fig. 3).

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

Extract	Yield (%)	Characteristics
Methanol (ME)	35	Light brown and highly viscous
Water (AE)	20.33	Liquid and whitish

Fig. 2. Pure strain of *Phytophthora colocassiae*

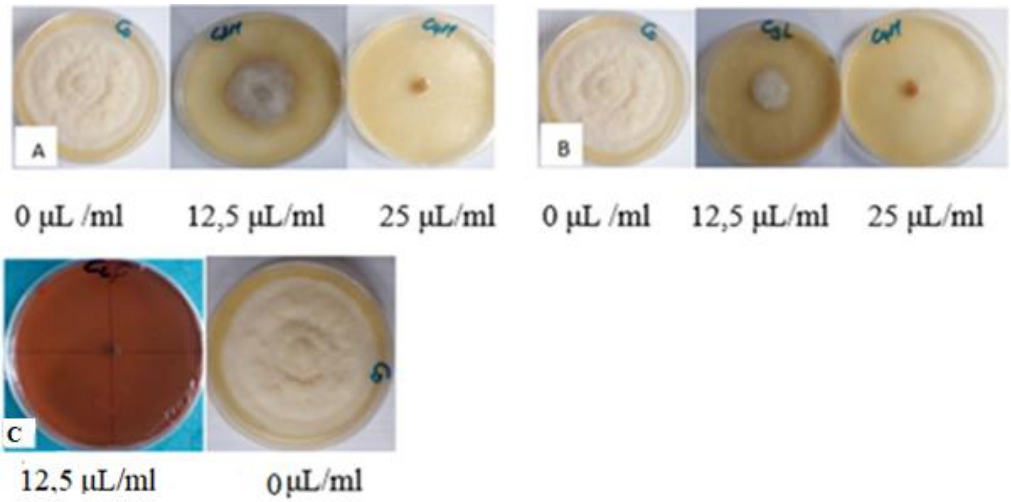

Fig. 3. *In vitro* inhibitory activity of *T. peruviana* extracts on the radial growth of *P. colocasiae* after 8 days incubation on PDA medium

With A: Methanol extract, B: Aqueous extract, C: Callomil.

3.1.3 Effect of aqueous extract (AE) on the growth of *Phytophthora colocasiae* strains

Aqueous extract has inhibited the growth of *P. colocasiae* strains. The Central Region strain (CE111) had the lowest percentage of inhibition: 85.01% at the highest concentration (C3), (Fig. 4) compared to 100% for the West (OU123) and Littoral (LT122) strains (Fig. 4). Inhibition was proportional to concentration. Aqueous extract was as effective as Callomil at concentration C3 (50 µL/mL) with 100% growth inhibition on both strains compared to the control ($P < 0.05$). LT122 was more sensible to extract at 12.5 µL/mL with over 89% reduction in mycelial growth.

3.1.4 Effect of methanol extract (ME) on growth of *Phytophthora colocasiae* strains

Methanol extract showed inhibition on the growth of *P. colocasiae* strains. Strain CE111 had the lowest percentage inhibition: 70.95% at the highest concentration C3, (Fig. 4) compared to 100% for OU123 and LT122 (Fig. 4). Inhibition was proportional to concentration. Methanol extract was as effective as Callomil at concentration C3 with 100% growth inhibition on both strains compared to the control ($P<0.05$). Strain LT122 was more sensitive to extract at 12.5 µL/mL with more than 69% reduction in mycelial growth.

3.1.5 Fungicidal or fungistatic activity of extracts and fungicide

The fungi tested showed different behaviors towards the extracts and according to the concentrations. For strain CE111, aqueous extract was fungistatic at concentration C2 and fungicidal at concentration C3, whereas methanol extract was fungistatic at concentrations C2 and C3. However, with strain OU123, methanol extract was fungistatic at both concentrations; aqueous extract was fungistatic at concentration C2 and fungicidal at concentration C3. For strain LT122, aqueous extract was fungicidal at the C2 and C3 concentration; methanol extract was fungistatic at the C2 concentration, however, the mycelial pastilles were unable to resume growth in the Petri dishes at C3 concentration and even after transfer to neutral culture medium (Table 2).

3.1.6 Determination of minimum inhibitory MIC_{50} and MIC_{90} concentrations for the growth of *Phytophthora colocasiae* strains

The MICs for the growth of *P. colocasiae* strains varied between extracts. MIC_{90}s are higher with aqueous extract and range from 22.5 to 105 µL/mL. The MIC_{90}s obtained with methanol extract range from 18.37 to 90 µL/mL. Only one MIC_{50} was determined; the one of the methanol extract with strain CE111 (50.5 µL/mL) (Table 3).

3.2 Discussion

The present work was based on the evaluation of the antifungal power of the aqueous and methanol extract of *T. peruviana* seeds on the strains of *Phytophthora colocasiae*, the causal agent of taro late blight.

The extraction of 1 kg of *T. peruviana* seeds produced different yields. These yields varied according to the solvents: 35% with methanol and 20.33% with water. This variation can be attributed to the nature of the solvent. The difference in yield observed between aqueous and methanolic extract could be explained by the fact that organic solvents would bind more compounds compared to water and therefore increase the extraction yield [41]. In addition, the high polarity of solvents such as methanol allows

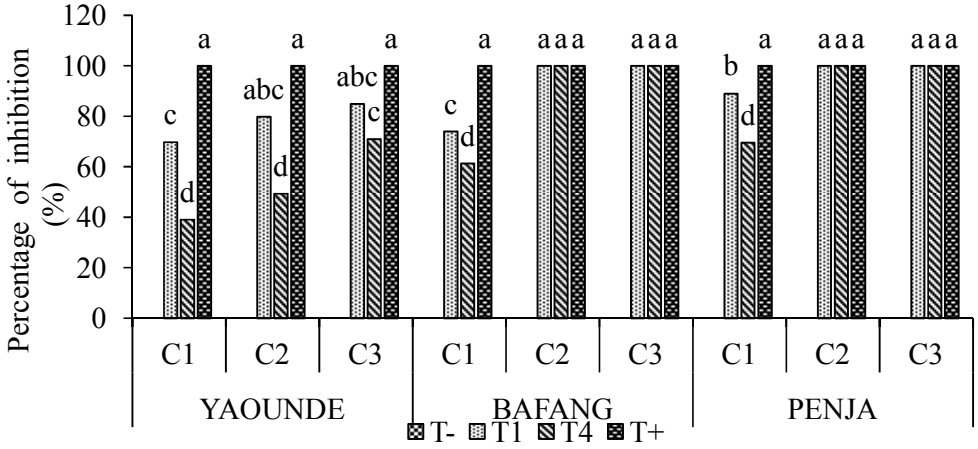

Fig. 4. Effect of extracts on the growth of *Phytophthora colocasiae* strains

For each strain, the values assigned the same letter does not differ significantly in the Tukey test.
T- = Negative control ; T1 = Aqueous extract; T4 = Methanol extract; T+ = Fungicide
T- (0 µL/mL) ; C_1=12.5 µL/ml; C_2=25 µL/mL; C_3=50 µL/mL; T+ (12.5 µL/mL)

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

Species	Isolates	Extracts	Concentrations	Effect
P.colocasiae	CE111	ME	C2 (25 µL/mL)	Fungistatic
			C3 (50 µL/mL)	Fungistatic
		AE	C2 (25 µL/mL)	Fungistatic
			C3 (50 µL/mL)	Fungicidal
		Callomil	C1 (12.5 µL/mL)	Fungicidal
	OU123	ME	C2 (25 µL/mL)	Fungistatic
			C3 (50 µL/mL)	Fungistatic
		AE	C2 (25 µL/mL)	Fungistatic
			C3 (50 µL/mL)	Fungicidal
		Callomil	C3 (12.5 µL/mL)	Fungicidal
	LT122	ME	C2 (25 µL/mL)	Fungistatic
			C3 (50 µL/mL)	Fungicidal
		AE	C2 (25 µL/mL)	Fungicidal
			C3 (50 µL/mL)	Fungicidal
		Callomil	C1 (12.5 µL/mL)	Fungicidal

CE111: Isolates from Central Region; OU123: Isolates from West Region; LT122: Isolates from Littoral Region

Table 3. MIC_{50} and MIC_{90} (µL/mL) for mycelial growth of *Phytophthora colocasiae* in the presence of *Thevetia peruviana* extracts

Treatments	Minimal inhibitory concentrations	Isolates		
		OU123	LT122	CE111
AE	MIC_{90}	22.25	12.62	105
	MIC_{50}	*	*	*
ME	MIC_{90}	18.37	16.12	90
	MIC_{50}	*	*	50.5

**Represents values that are not set to be statistically zero; [a]Represents the lowest concentration that inhibits mycelial growth by 90%*

it to be more efficient in the extraction of many compounds [42]. These yields are different from those obtained by [27,28] who, after extraction using the same quantities of *T. peruviana* paste with the same volumes of solvent, had obtained a yield of 7.44% with methanol and 23% with water. Indeed, Svoboda and Hampson [43] and Smallfield [44] report that environmental conditions, harvest period and age of plant material can influence extraction yields.

Aqueous and methanolic extract significantly inhibited the growth of *P. colocassiae* strains compared to the control. This reduction was more pronounced with the aqueous extract than with the methanolic extract. These extracts would contain substances that would inhibit or retard the growth of the fungus. Indeed, Pamo [45] and Ngoh Dooh [27] reported that plant extracts from a number of plants contain compounds such as tannins, flavonoids and alkaloids that have fungicidal properties. Different concentrations of extracts significantly influenced the radial growth of the fungus; higher concentrations were more inhibitory. Different results were reported by Tsompbeng [41] who, using aqueous and methanolic extracts of *Laggera pterodonta* and *Cupressus lusitanica*, on *Phytophthora colocasiae* had obtained very high percentages of inhibition with methanolic extracts as opposed to aqueous extracts. This could be due to the fact that the chemical composition of the extracts could vary according to the nature of the plants and also according to the organs used. Similarly, Reddy [46] obtained a reduction in the growth of several fungi (*Aspergilus, penicillium*) with alcoholic extracts from the leaves of *Thevetia peruviana*. On the other hand, dichloromethane and methanol extracts from the leaves of *T. peruviana* inhibited the growth of *Cladosporium cucumerinum* [47].

The results obtained on the fungicidal or fungistatic activity of the extracts show that some strains proved to be more resistant than 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 fungal membrane or systemic: acting inside the cell [48]. In either

case, specific membrane receptors or intracellular receptors may be essential for the expression of the biological activity of the antifungal agent. 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, elicit responses such as an inhibition of general metabolism (fungistatic effect) or alteration of the plasma membrane of the fungus and inhibition of respiration (fungicidal effect) [29].

Callomil Plus 72 WP was very effective against *P. colocassiae* at all concentrations with inhibition percentages of the order of 100% on strain growth. Its efficacy is thought to be due to the presence of the major active ingredient copper oxide (60%), which is known to affect cell respiration. Indeed, Tsompbeng [41] showed *In vitro* the efficacy of Callomil on *P. colocassiae*.

The low MIC values obtained with the aqueous and methanolic extracts highlight the efficacy and fungicidal properties of these different extracts on the growth of the fungus tested. These results are in agreement with those of Tsompbeng [41] and Ngoh Dooh [28] who showed that the low MIC values of the extracts of *Callistemon viminalis* and *Thevetia peruviana* respectively inhibited the development of *P. colocasiae* and *P. megakarya.*

4. CONCLUSION

The study showed that extracts of *T. peruviana* inhibited the radial growth of *P. colocasiae in vitro*. These extracts were found to be active on *P. colocasiae* and may therefore provide an alternative for the control of taro blight. Although their activity was comparable to that of the reference fungicide (Callomil Plus 72 WP), the fact remains that these crude extracts would contain a large number of different compounds which, when purified, would have a higher activity than fungicides. This preliminary study provides a basis for future trials under natural greenhouse and field conditions.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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