

1 Original Research Article

2 Antifungal potential of acetone and ethyl acetate extracts of *Thevetia peruviana* on
3 development of *Phytophthora colocasiae*, causal agent of late blight of taro (*Colocasia*
4 *esculenta* (L.) Schott) from three Agro-ecological zons of Cameroon.
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ABSTRACT

Aims: This study was conducted 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: Mention the design of the study here.

Place and Duration of Study: 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.

Methodology: acetone and ethyl acetate extracts of *T. peruviana* were prepared and used at concentrations of 12.5, 25 and 50 µg/ml. *P. colocasiae* was isolated from infected taro leaf cultivars "Macumba or Ibo coco" located in three different regions: west, Littoral and Centre. The different explants 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, active *against P. colocasiae* could be used as alternative to fungicides for the control of taro leaf blight.

Keywords: *Extracts of Thevetia peruviana, antifungal potential, Phytophthora colocasiae, taro*

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1. INTRODUCTION

24 Cocoyams (*Colocasia esculenta*) are well adapted food crops across many agro-ecological zones of
25 sub-Saharan Africa. They rank third in importance, after cassava and yam, among the root and tuber
26 crops cultivated and consumed in many West and Central Africa countries. Cocoyams are
27 nutritionally superior to both cassava and yam in the possession of higher protein, mineral and

28 vitamin contents as well as easily digestible starch (Liu and *al.*, 1997 cit Imar Dibrime, 2011). Africa
29 in the last three decades has consistently accounted for an increasing percentage of global cocoyam
30 production, which currently stands at about 10 million tonnes per annum (FAO, 2012). Cocoyam is
31 therefore undoubtedly an important food crop in sub-Saharan Africa (SSA), particularly in Nigeria,
32 Ghana and Cameroon. Global production is estimated at about 10.64 million tonnes on a cultivated
33 area of 1.67 million hectares (Anonymous 1, 2020). In addition, 77% of global taro production comes
34 from sub-Saharan Africa (Anonymous 1, 2020). However, the increasing production in the region has
35 depended largely on farming more land rather than increasing crop yields. This is contrary to the
36 projections of FAO that the 70% growth in global agricultural production needed to feed an additional
37 2.3 billion people by 2050 must be achieved by increasing yields and cropping intensity on existing
38 farmlands, rather than by increasing the amount of land brought under agricultural production (FAO,
39 2009). This could be due to the enemies of this crop such as diseases that hinder its production. One
40 of the most important is late blight caused by *Phytophthora colocasiae* (Guarion 2010; Asseng et al.,
41 2017). It was first described in Java by Marian Raciborski in 1900 (Adinde et al., 2016). The disease
42 mainly affects the leaves of the taro (Fig. 1B), and can completely destroy susceptible cultivars in
43 less than 10 days and cause yield losses in the range of 50 to 100% (Fullerton & Tyson, 2004;
44 Brooks, 2005; Guarion, 2010; Manju et al, 2017a). This loss of yield has a remarkable impact on
45 farmers' incomes as well as on the food security of human populations. pH 7 and temperature of
46 27°C are the optimal conditions for the pathogen to grow in the field (Tsompbeng et al., 2014a; Cabi,
47 2016).



48 **Fig.1. (A): *colocasia esculenta* plants. (B): attacked plants showing symptoms of taro late**
49 **blight on the upper leaf surface.**

51
52 Control strategies for this pathogen are most often focused on the use of metalaxyl-based chemical
53 fungicides (Ashok & Saikia, 1996; Carmichael et al., 2008); but due to the problems of residues in
54 groundwater (Ndongo, 1999), the development of resistance in the target organism and the danger
55 to man and the environment, alternative control methods are increasingly being considered.
56 Currently, considerable efforts are directed towards the exploration of plant extracts with pesticide
57 potential as alternative or complementary sources to synthetic pesticides. Plant extracts have the

58 advantage of being not only available to farmers, but also non-toxic and easily biodegradable and
59 therefore healthy for the environment (Okigbo and Nmeke, 2005; Okigbo and Omdamiro, 2006).
60 Several studies have shown the antifungal effects of plant extracts on *Phytophthora infestans*, the
61 causative agent of late blight in potatoes, tomatoes and black nightshades (Fontem et al., 2005;
62 Goufo et al., 2010; Djeugap et al., 2011), but no information is available on the effect of seed extracts
63 of plants such as *Thevetia peruviana* on *P. colocasiae* in Cameroon. The seeds, leaves, fruits and
64 roots of the Yellow Oleander (*Thevetia peruviana*) are considered potential sources of biological
65 compounds active as insecticides (Ambang et al., 2005; Gatsi et al., 2020), fungicides (Ambang et
66 al., 2010, 2011; Ngoh Dooh et al., 2014a and b; Mboussi et al., 2016), virucides (Tewtrakul et al.,
67 2002) and bactericides (Singh et al., 2012). Thus, the present work aims to evaluate the antifungal
68 potential of acetone and ethyl acetate extracts of *Thevetia peruviana* seeds on the in vitro
69 development of *P. colocasiae* from three agro-ecological zones of Cameroon.

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71 **2. MATERIAL AND METHODS**

72 **2.1. Plant and chemical material**

73 The plant material consists of the seed pits of *Thevetia peruviana* collected in the city of Yaoundé
74 where the tree serves as a house fence; and leaves of *Colocasia esculenta* collected in peasant
75 plantations located in the locality of: Bafang in the Department of Haut-Nkam in West (OU123), Penja
76 in the department of Mounjo in Littoral (LT122) and Yaoundé in the department of Mfoundi in Central
77 Cameroon (CE111); and taken to the lab. The chemical material is a product with the trade name
78 Callomil Plus 72 WP consisting of 12% metalaxyl and 60% copper oxide.

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80 **2.2. Methods**

81 **2.2.1. Preparation of extracts of *Thevetia peruviana* seeds**

82

83 The plant of *Thevetia peruviana* has been identified according to the botanical systematics key of the
84 species with reference to the recent version of the International Code of Botanical Nomenclature
85 (Greuter et al., 2003; Spichiger et al., 2002). The mature *T. peruviana* fruits were picked, the kernels
86 extracted from the fruits were crushed and the resulting almonds were dried at room temperature for
87 3 to 4 weeks in the laboratory and then crushed using a manual mill to obtain a paste. The organic
88 extract was prepared by maceration of 1 kg of paste in 5 L of solvent for 48 hours and then filtered.
89 The resulting filtrate was concentrated at 60 °C using a rotary evaporator and the solvent extract
90 obtained was stored in the refrigerator at 4 °C until use. Doses of extracts of 12.5; 25 and µl/ml were
91 obtained following a progression geometry of reason 2 (Derradji-Heffaf, 2013) from a stock solution of
92 500 µl/ml.

93 The extraction yield of each extract was calculated using the formula cited by Ngho
 94 Dooh (2014a and b):

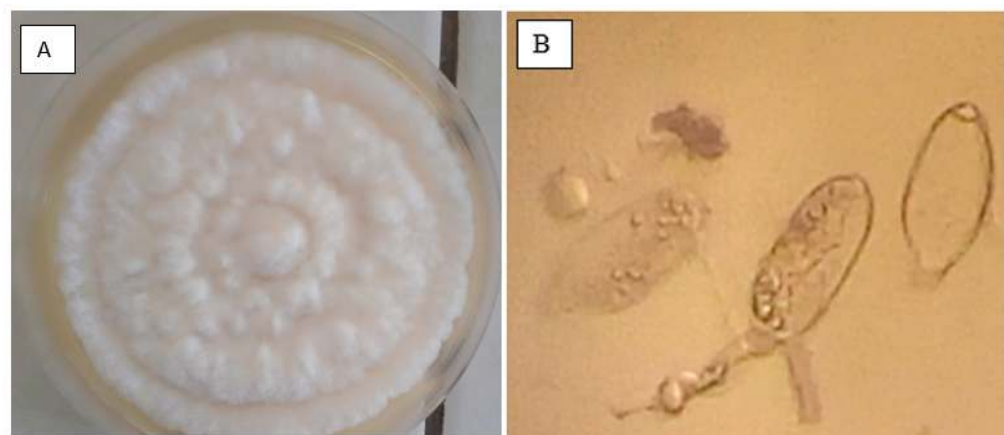
$$95 \quad Rd\% = \frac{\text{Mass of extract}}{\text{Mass of powder}} \times 100$$

96

97 **2.2.2. Isolation and purification of *Phytophthora colocassiae***

98 The infected leaves of the harvested taro variety "Macumba" were cut into fragments of about 2 cm²
 99 at the growth front of the pathogen and disinfected superficially in a solution of 5% sodium
 100 hypochlorite for 2 minutes. After three rinses with sterilized distilled water (EDS), the fragments were
 101 dried on hydrophilic paper and then deposited at the rate of four fragments in a petri dish poured in
 102 the gelled V8 culture medium supplemented with a solution of antibiotics composed of penicillin (250
 103 mg / l), ampicillin (250 mg / l) and nystatin (20 mg / l) (Djeugap et al., 2009, Tsopmbeng et al., 2012).
 104 After three days of incubation in the laboratory at a temperature of 23±1°C, colonies of the pathogen,
 105 visible around the fragments, were taken and transplanted into new petri dishes containing the PDA
 106 culture medium. This process was repeated several times until pure morphological cultures of the
 107 mycelium (not septate) and fruiting (sporangia) as described by Brooks, (2005) and Scot *et al.* (2011)
 108 were obtained (Fig. 2)

109



110

111

112 **Fig.2. *Phytophthora colocassiae*: pure culture of mycelium (A) and sporangia (B).** (Gr: X20)

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114 The isolates obtained are characterized according to morphological criteria such as pathogenicity
 115 and growth rate (Ondo, 2006).

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117 **2.2.3. In vitro evaluation of the antifungal activity of the crude extracts**

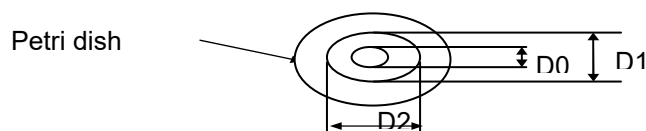
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119 The in vitro evaluation of the antifungal activity of the extracts was done at concentrations of 12.5; 25
 120 and 50 µl/ml for the two extracts from the stock solutions of 500 µl/ml for each. A synthetic fungicide

121 (Callomil Plus 72 WP) was used as a positive control by taking from a 50g sachet, 1 g of powder per
 122 5 ml of distilled water. Mycelial explants of *P. colocasiae* about 8 mm in diameter were taken with a
 123 cookie cutter from a pure fruiting culture seven days old and placed in the center of the petri dish
 124 containing the media enriched with the different extracts or fungicide. A negative control not
 125 supplemented with extract was developed. Each treatment was repeated 3 times. Incubation was
 126 carried out at $23\pm 1^{\circ}\text{C}$ under a photoperiod of 12/12 for one week. A daily measurement of the radial
 127 growth diameter of each cultured explant was taken and continued until the mycelium filled the
 128 control dishes. The radial (D) growth of the pathogen was assessed by measuring two perpendicular
 129 diameters traced to the back of the petri dish. The average of the two perpendicular measurements
 130 removed from the diameter of the explant represents the measure of the radial growth of the fungus.
 131 It is obtained by the formula described by Dohou et al. (2004):

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

132
 133 Where: D0 is the diameter of the explant; D1 and D2 are the culture diameters measured in
 134 both perpendicular directions (Fig. 3).



135

136

137 **Fig.3. Measurement diagram of mycelial growth in petri dish on medium V8.**

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139 The percentage of inhibition (I%) due to each extract is evaluated in relation to the mycelial growth in
 140 the control boxes according to the formula developed by Dohou et al. (2004):

141

$$I\% = \frac{D_{to} \text{ mm} - D_{xi} \text{ mm}}{D_{to} \text{ mm}} \times 100$$

142 With I (%): percentage of inhibition; D_{to} is the average diameter of the control batch and D_{xi} is the
 143 average diameter of the batches in the presence of the extract.

144

145 **2.2.4. Correlation between concentration and inhibition percentages**

146

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

153 In this case if $r < 0$ then the relationship is inversely proportional and the correlation is negative. If $r >$
 154 0 then the relationship is positive; if r between 0.7 and 1 then the correlation is perfect and positive; if
 155 r between -0.7 and -1 then the correlation is perfect and negative; if $r < 0.7$ then the correlation is
 156 positive but imperfect; if $r > -0.7$ then the correlation is negative and imperfect (Ngho Dooh, 2015).

157

158 **2.2.5. Fungicide or fungistatic activity of extracts and fungicide**

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160 At the end of each test, the mycelium explants from the boxes where the growth was completely
 161 inhibited, were taken and deposited aseptically on the culture medium containing no extract. After 7
 162 days of waiting, depending on whether or not the fungus has resumed growth, the starting extract
 163 was identified as fungistatic or fungicide respectively (Essome et al., 2021).

164

165 **2.2.6. Statistical analysis**

166

167 The percentages of inhibition of the radial growth of the pathogen were transformed into
 168 probits and the values obtained were regressed on the logarithm of the concentration of
 169 plant extracts. The efficacy of the extracts was evaluated on the basis of the inhibiting
 170 concentration value of 50% (CMI_{50}) and 90% (CMI_{90}) determined after 8 days of growth
 171 according to the formula developed by Finney (1971). Inhibition percentage data were
 172 subjected to analysis of variance using R analysis software version 5.1.0 and means
 173 separated by Duncan's multiple test at the 5% probability threshold.

174

174 **3. RESULTS AND DISCUSSION**

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175 **3.1. Results**

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176 **3.1.1 Extraction yield**

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178 The yield, volume, colour and appearance of the different extracts obtained varied depending on the
 179 extraction solvent used. Extraction with ethyl acetate yielded slightly higher (28.5%) than acetone
 180 (23.3%). The ethyl acetate extract has an oily appearance and pale yellow color while the acetone
 181 extract has a viscous appearance and brown color (Table 1).

182

183 **Table 1: Extraction yield (%) and characteristics of extracts per 1kg of seeds.**

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

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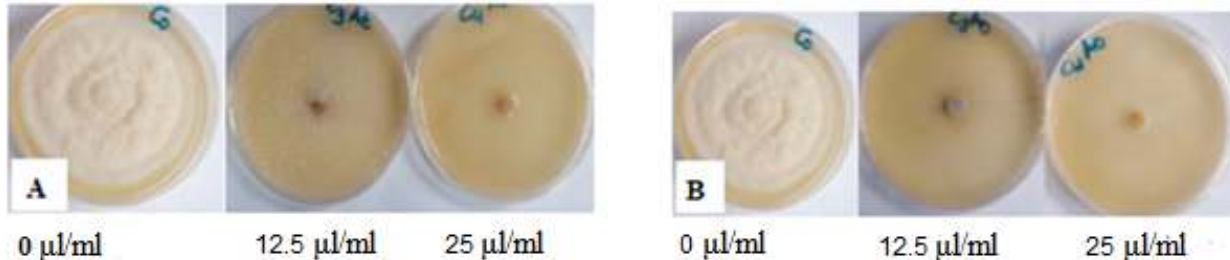
186 **3.1.2 Effect of *Thevetia peruviana* extracts on the *in vitro* growth of *P. colocasiae***

187

188 The seed extracts tested significantly inhibited the radial growth of *P. colocasiae*. The
 189 diameter of the fungal colony that received the high concentrations of the extracts was very small

190 and zero at the highest concentrations. Total inhibition was achieved at a concentration of 25 $\mu\text{l/ml}$
 191 for acetone and ethyl acetate extract. On the other hand, in the control boxes, the growth of *P.*
 192 *colocasiae* was significantly higher compared to the different concentrations of the extracts tested
 193 (Fig. 4).

194



195
 196 **Fig.4** In vitro inhibitory activity of *Thevetia peruviana* extracts on the radial growth of the *P. colocasiae*
 197 strain (OU123) after 8 days of incubation on PDA medium; A: ethyl acetate extract, B: acetone extract.
 198

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

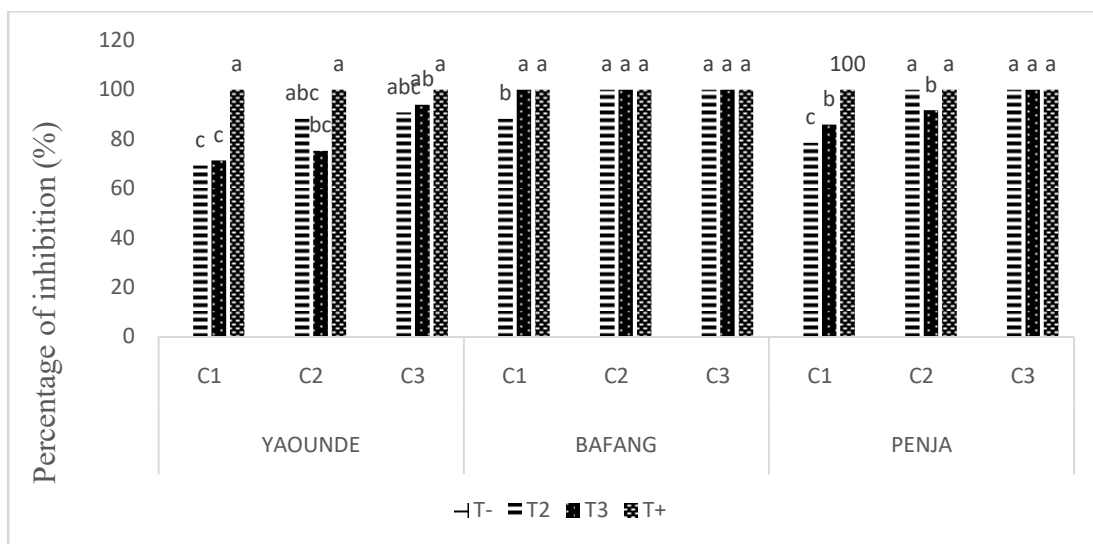
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206

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

207 The EAE showed inhibition on the growth of *P. colocasiae* strains. With the CE111 strain we
 208 had the lowest percentage of inhibition, 90.78% at the highest dose C3, (Fig. 5) against 100% for
 209 OU123 and LT122 (Fig. 5). Inhibition was also proportional to concentration. EAE was found to be
 210 effective in the same way as Callomil at dose C3 with 100% growth inhibition on two strains
 211 compared to the control ($P<0.05$). The OU123 strain was more sensitive to the extract at the smallest
 212 dose of 12.5 $\mu\text{l/ml}$ with more than 88% reduction in mycelial growth.

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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 mg/ml); C1 = 12.5 mg/ml; C2 = 25 mg/ml; C3 = 50 mg/ml; T+ (12.5 mg/ml)

3.1.5 Fungicidal or Fungistatic Activity of the Extracts

The fungi tested showed different behaviors vis-à-vis 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).

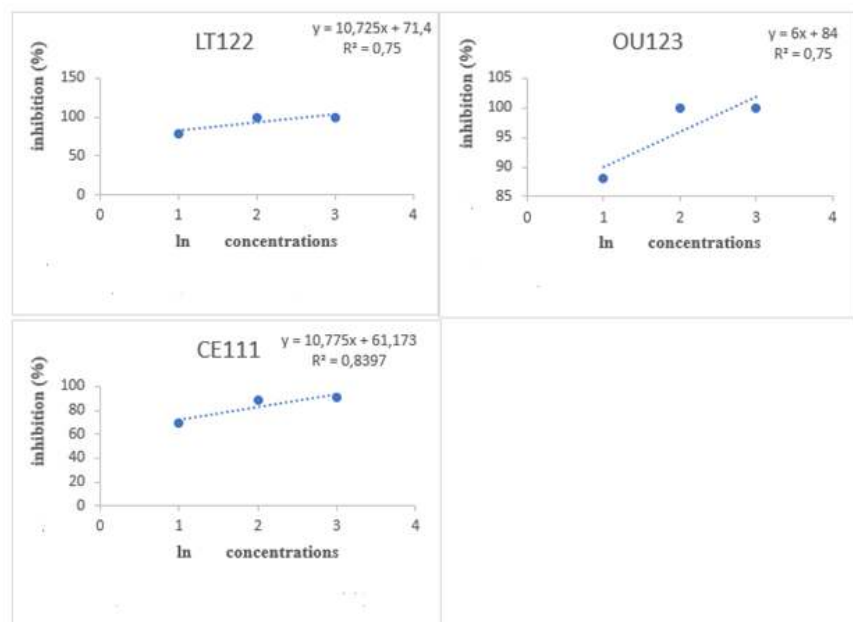
Table 2. Fungicide or fungistatic activity of extracts and synthetic fungicide

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

232

233 **3.1.6 Correlation test between concentrations of ethyl acetate extract and percentages of**
 234 **inhibition of *Phytophthora colocasiae* growth**

235 The equations obtained with the EAE showed increasing linear relationships. Indeed, all regression
 236 lines obtained with the strains showed positive slopes. The correlation coefficients were all between
 237 0.7 and 1. The strains OU123, CE111 and LT122 showed respectively the following coefficients: $r =$
 238 0.75 , $r = 0.84$ and $r = 0.75$ which is a perfect and positive correlation (Fig. 6).

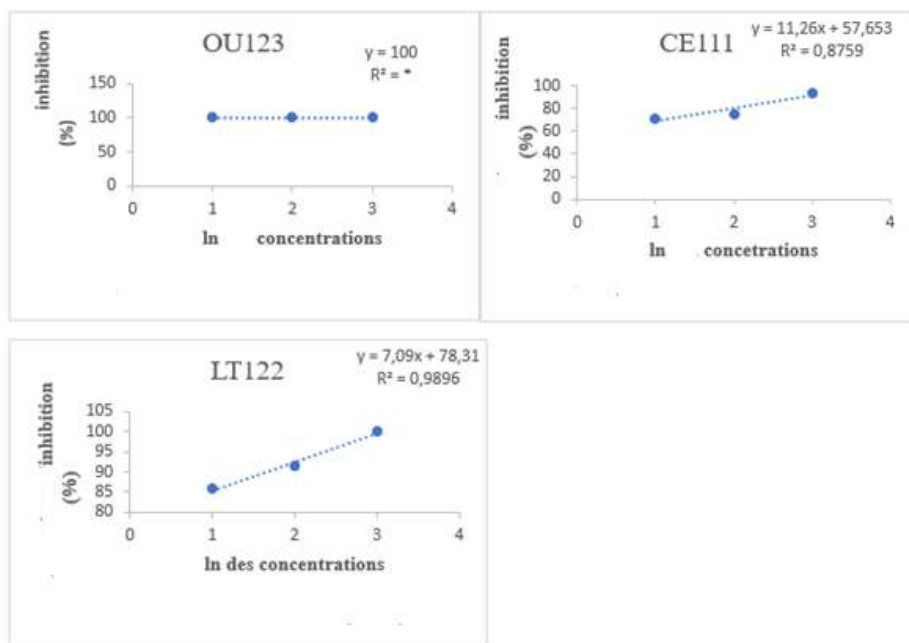


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 240
 241 **Fig.6. Regression lines of growth of *Phytophthora colocasiae* strains after treatment with**
 242 **ethyl acetate extract.**

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 244 **3.1.6 Correlation test between acetone extract concentrations and *phytophthora colocasiae***
 245 **growth inhibition percentages**

246 *P. colocasiae* strains acted differently with acetone extract. A strong positive correlation was
 247 obtained with some strains, the correlation coefficient r was greater than 0.7; this is the case with
 248 CE111: $r = 0.88$, LT122 : $r = 0.99$ is a positive and perfect correlation (Fig. 7). With the OU123 strain
 249 ($y=100$) the equation obtained showed a constant linear relationship which highlights an absence of
 250 correlation ($r=0$). The lines obtained with isolates CE111 and LT122 showed positive slopes,
 251 respectively $y=11.26x + 57.7$ and $y=7.09x + 78.31$.

252



253
254

255 **Fig.7. Regression lines of growth of *Phytophthora colocassiae* strains after treatment with**
256 **acetone extract.**

257 **3.1.7 Minimal Inhibitory Concentrations of the Different Extracts**

258 The MICs of the growth of *P. colocassiae* strains varied by extract. MICs₉₀ are higher with EAE and
259 range from 15 to 90 µl/ml. The MICs obtained with EAc range from 11.25 to 47.83 µl/ml. The smallest
260 CMI₉₀ (11.25) was obtained with EAc on strain OU123. However, no CMI₅₀ was obtained with the two
261 extracts (Table 3).

262
263 **Table 3: CMI₅₀ and CMI₉₀ (in µl/ml) of the mycelial growth of *P. colocassiae* in the presence of**
264 ***T. peruviana* extracts.**

265

		Isolats		
		OU123	LT122	CE111
EAE	CMI ₉₀	15	22,5	90
	CMI ₅₀	*	*	*
EAc	CMI ₉₀	11,25 ^a	20	47,83
	CMI ₅₀	*	*	*

266 * Represents values that are not set to be at zero statistically
267 ^a smaller concentration inhibiting 90% mycelial growth.

268
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3.2 Discussion

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

272 The extraction of 1000 g of the seeds of *T. peruviana* produced different yields. These yields ranged
273 from 28.5% with ethyl acetate to 23.3% with acetone. This variation can be attributed to the nature of
274 the solvent. These results are different from those obtained by Ngoh Dooh (2014) who, after
275 extraction, using the same amounts of *T. peruviana* seed paste with the same solvent volumes,
276 obtained a yield of 33.16% with ethyl acetate and 9.43% with acetone. Indeed, Svoboda and
277 Hampson (1999) and Smallfield (2001) report that environmental conditions, harvest period and age
278 of plant material can influence extraction yields.

279

280 EAE and EAc significantly inhibited the growth of *P. colocassiae* strains compared to the control.
281 These extracts would contain substances that inhibit or delay the growth of the fungus. Indeed,
282 Pamo et al. (2003) reported that plant extracts from a number of plants contain compounds such as
283 tannins, flavonoids and alkaloids that have fungicidal properties. The different concentrations of
284 extracts significantly influenced the radial growth of the fungus; high concentrations being more
285 inhibitory. This reduction in growth was more pronounced with acetone extract than with ethyl
286 acetate extract. The effectiveness of these extracts on the growth of *P. colocassiae* could be
287 explained by the presence in these extracts of bioactive molecules revealed by phytochemical
288 screening, such as essential oils, saponifiable oils, coumarins, sterols, alkaloids, triterpenes, tannins,
289 sugars, phenols, saponins and anthocyanins. All these molecules have antifungal properties, as
290 demonstrated by the work of Ngoh Dooh (2014).

291

292 The results obtained show that some strains have been shown to be more resistant to certain
293 extracts used compared to others, which would be due to the nature of the specificity they would
294 present at the membrane level. In general, antifungals can be contact: acting at the level of the
295 fungus membrane or systemic: acting inside the cell (Zacchino et al., 1998). In both cases, specific
296 membrane or intracellular receptors may be essential for the expression of the biological activity of
297 the antifungal. Some chemical constituents have the ability to recognize sites of action in the
298 pathogen, others do not. They would thus act through a concentration effect and once fixed on their
299 receptors, would elicit responses such as inhibition of general metabolism (fungistatic effect) or
300 alteration of the plasma membrane of the fungus and inhibition of respiration (fungicidal effect)
301 (Mboussi et al., 2016). Callomil Plus 72 WP was very effective against *P. colocassiae* at all doses
302 with inhibition percentages of around 100% on strain growth. Its effectiveness would be due to the
303 presence of copper oxide major active ingredient (60%), which is known for its action on cellular
304 respiration. This result is similar to those of Tsompbeng et al., (2014b) who showed in vitro the
305 efficacy of Callomil on strains of *P. colocassiae*. The low MIC values obtained with the acetone and
306 ethyl acetate extract highlight the effectiveness and fungicidal properties of these different extracts
307 on the growth of the fungus tested. These results are consistent with those of Tsompbeng et al.,

308 (2014b) and Ngoh Dooh et al., (2014a) who showed that low MIC values of *Callistemon viminalis* and
309 *T. peruviana* extracts respectively inhibit the development of *P. colocasiae*.

310

311 **4. CONCLUSION**

312

313 The study showed that *T. peruviana* extracts inhibited the radial growth of *P. colocasiae* in vitro.

314 These extracts have been shown to be active on *P. colocasiae* and may therefore be an alternative

315 for the fight against taro late blight. Although their activity is comparable to that of the reference

316 fungicide (Callomil Plus 72 WP), the fact remains that these crude extracts contain a large number of

317 different compounds which, once purified, would have a higher effectiveness than the chemical

318 fungicide.

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