

# A <sup>1</sup>H NMR-BASED METABOLOMIC APPROACH TO STUDY THE PRODUCTION OF ANTIMALARIAL COMPOUNDS FROM *PSIADIA ARGUTA* LEAVES (PERS.) VOIGT

Keshika Mahadeo<sup>a,h</sup>, Isabelle Grondin<sup>a</sup>, Gaëtan Herbette<sup>b</sup>, Tony L. Palama<sup>c</sup>, Nadia Bouchemal<sup>c</sup>, Joyce Soulange<sup>d</sup>, Sabina Jhaumeer Laulloo<sup>e</sup>, Joelle Sadeyen<sup>f</sup>, Laurence Humeau<sup>f</sup>, Michel Frederich<sup>g</sup>, Anne Gauvin-Bialecki<sup>a</sup>, Hippolyte Kodja<sup>h</sup>

<sup>a</sup> *Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments, Faculté des Sciences et Technologies, Université de La Réunion, 15 Avenue René Cassin, CS 92 003, 97 744, St Denis Cedex 9, La Réunion, France*

<sup>b</sup> *Aix-Marseille Univ, CNRS, Centrale Marseille, FSCM, Spectropole, Campus de St Jérôme-Service 511, 13397, Marseille, France*

<sup>c</sup> *Université Sorbonne Paris Nord, Laboratoire de Chimie, Structures, Propriétés de Biomatériaux et d'Agents Thérapeutiques, CSPBAT, CNRS, UMR 7244, F, 93430, Villetaneuse, France*

<sup>d</sup> *Department of Agriculture and Food Science, Faculty of Agriculture, The University of Mauritius, Mauritius*

<sup>e</sup> *Department of Chemistry, Faculty of Science, The University of Mauritius, Mauritius*

<sup>f</sup> *UMR Peuplement Végétaux et Bio-agresseurs en Milieu Tropical, Université de La Réunion, 15 Avenue René Cassin, CS 92 003, 97 744 St Denis Cedex 9, La Réunion, France*

<sup>g</sup> *Université de Liège, Département de Pharmacie, Centre Interfacultaire de Recherche sur le Médicament (CIRM), Laboratoire de Pharmacognosie, Campus du Sart-Tilman, Quartier Hôpital, Avenue Hippocrate, 15 B36 4000 Liège, Belgium*

<sup>h</sup> *UMR Qualisud, Université de La Réunion, 15 Avenue René Cassin, CS 92 003, 97 744, St Denis Cedex 9, La Réunion, France*

## KEYWORDS:

*Psiadia arguta*

Asteraceae

Antimalarial diterpenes

Metabolomic

NMR

Multivariate data analysis

## ABSTRACT

*Psiadia arguta* (Asteraceae) is endemic to the island of Mauritius in the Indian Ocean. The species is traditionally used to treat various ailments, such as its use as an expectorant or for the treatment of bronchitis and asthma. Preliminary biological screenings have displayed the antimalarial (*Plasmodium falciparum*) and anticancer (HeLa human cell line) potential of *P. arguta* leaves. The phytochemical investigation of this plant has led to the isolation and characterization of sixteen compounds including five antiplasmodial molecules. The accumulation of the antiplasmodial

compounds during the growth of the plant was studied by a  $^1\text{H}$  NMR-based metabolomic approach. In order to identify factors influencing the production of bioactive compounds, young plants of *P. arguta* were multiplied using *in vitro* culture techniques, and micro-propagated plants at different stages of development were acclimatized and followed for the experiments. The multivariate data analysis showed an accumulation of four bioactive compounds in the leaves of *P. arguta* when these plants were challenged with a biotic stress: labdan-13(*E*)-en-8 $\alpha$ -ol-15-yl acetate, labdan-8 $\alpha$ -ol-15-yl acetate, labdan-13(*E*)-ene-8 $\alpha$ -ol-15-diol, and (8*R*,13*S*)-labdan-8,15-diol.

## 1. Introduction

*Psiadia arguta* Pers. (Voigt) (Asteraceae) commonly known as “Baume de l’île Plate” belongs to the Asteraceae family. This shrub is endemic to Mauritius and is used in folk medicine as an expectorant and for treatment of bronchitis and asthma (Sussman, 1980; Wang et al., 1992). Its presence has been reduced due to urbanisation, propagation of invasive plants, and difficulties of seed germination (Kodja et al., 1998). Some wild specimens can still be found on Ilot Gabriel, a small islet off the north coast of Mauritius. However, this species is completely extinct in the natural habitat on Mauritius. For this reason, *P. arguta* has been registered in 1997 in the IUCN Red List of Threatened Species. For its conservation, Kodja et al. (1998) provided a micro-propagation method through cotyledonary axillary bud culture and conservation efforts have led to the exclusion of *P. arguta* from the IUCN Red List. Recent research on wild specimens of *P. arguta* have demonstrated the antimicrobial, anti-inflammatory, antiplasmodial, and anticancer potential of this species (Jonville et al., 2011, 2008; Kauroo et al., 2016; Recio et al., 1995). Jonville et al. (2008, 2011) reported that *P. arguta* crude extracts have displayed a high antiplasmodial activity in comparison to the other biological activity tested (anti-inflammatory and anticancer). Indeed, the dichloromethane leaf extract displayed a strong activity against *Plasmodium falciparum* 3D7 and W2 strains with  $\text{IC}_{50}$  of 10.1 and 8.4  $\mu\text{g}/\text{mL}$ , respectively. Besides, this extract exhibited a moderate antiplasmodial activity *in vivo* in mice infested by *P. berghei* (Jonville et al., 2008).

Despite the medicinal and ecological interest in *P. arguta*, there are only few studies on *Psiadia arguta*, particularly describing the chemical composition. This might be due to the low abundance of this species in its natural habitat. Recently, phytochemical investigations on a wild specimen of *Psiadia arguta* led to the isolation and characterization of 16 terpenoids including the known compounds  $\beta$ -amyryn,  $\alpha$ -amyryn, labda-13(*E*)-en-8 $\alpha$ -ol-15-yl acetate, labdan-8 $\alpha$ -ol-15-yl acetate, anticopalic acid, physanicandiol, 14-*epi*-physanicandiol, 13-*epi*-sclareol, labda-13(*E*)-en-8 $\alpha$ ,15-diol, 8*R*,13*S*-labdane-8,15-diol, labdanolic acid, labd-8(20)-en-15-ol and the undescribed diterpenes, labdan-8 $\alpha$ -ol-15-yl-(formate), labdan-8 $\alpha$ -ol-15-yl-(2-methylbutanoate), labdan-8 $\alpha$ -ol-15-yl-(3-methylpentanoate), and labdan-8 $\alpha$ -ol-15-yl-(labdanolate). Of all isolated compounds, labdan-13(*E*)-en-8 $\alpha$ -ol-15-yl acetate, labdan-8 $\alpha$ -ol-15-yl acetate, 13-*epi*-sclareol, labdan-13(*E*)-ene-8 $\alpha$ -ol-15-diol, and (8*R*, 13*S*)-labdane-8,15-diol showed promising antiplasmodial activity (Mahadeo et al., 2019). Further studies on *P. arguta* are difficult due to its low abundance and the degree of protection in natural parks (Kodja et al., 1998; Walter and Gillett, 1998). Vegetative

micropropagation represents an efficient tool for the sustainable production of this species for scientific experiments. Thus, in our continuing research on *P. arguta*, we conducted a greenhouse experiment in order to understand the conditions for accumulation of the antiplasmodial compounds. To achieve these objectives, *in vitro* plantlets were micropropagated and then acclimatized. Three parameters have been taken into account in order to identify the conditions of accumulation of the antiplasmodial compounds: the age of plants, the conditions of culture and the effect of the environment. Besides, metabolomics has been demonstrated to be a powerful tool to analyse the metabolic profile of biological samples (Ayouni et al., 2016). Therefore, in the present study, a  $^1\text{H}$  NMR based metabolomic analysis was performed to determine the differences between plants according to each studied factor and rapidly recognize the metabolites involved in the antiplasmodial activity of *P. arguta* leaf crude extract.

## 2. Results and discussion

*P. arguta* is endangered due to its low abundance and difficulties for seed germination both in its natural habitat and in artificial conditions (Kodja et al., 1998). In the present study, given that *P. arguta* seed germination is difficult, it was impossible to obtain sufficient quantities of plant material for all experiments. Thus, young plants were micropropagated using tissue culture techniques as previously reported (Kodja et al., 1998). In order to investigate the circumstances for accumulation of the antiplasmodial compounds, plants of 6, 7, 8, and 9 months old were studied. Besides, two conditions of culture were studied: *in vitro* and acclimatized plants. Three independent repetitions were performed for the acclimatization of plants as these could be subjected to various stresses during this process. Indeed, some acclimatized plants were attacked by mealybugs and aphids. In natural habitat, insects were observed on the leaf surfaces of *P. arguta* young plants on Ilot Gabriel (islet off the north coast of Mauritius) and in the botanical garden of the University of La Reunion Island (Kodja, comm pers). By removing the phloem sieve elements in the leaves, aphids introduce toxins, viruses, and other pathogens into the plant. Moreover, the honeydew secreted by the aphids is an ideal culture medium for various fungi on the leaf. Hence, in order to study the possible influence of the environment on the accumulation of antiplasmodial compounds, an elicitation step was performed on the acclimatized plants using *Botrytis cinerea* culture filtrate. The protocol for obtaining all plants from different conditions of culture is summarized in Fig. 1. The antiplasmodial activity of all crude extracts was evaluated, the chemical profile was analysed by  $^1\text{H}$  NMR, and a multivariate data analysis was performed to identify active compounds.

### 2.1. IN VITRO ANTIPLASMODIAL ACTIVITY OF P. ARGUTA LEAF CRUDE EXTRACTS CULTIVATED IN DIFFERENT CONDITIONS

The antiplasmodial potential of each leaf crude extract was determined against *Plasmodium falciparum* (3D7 strain) and the results are shown in Table 1.

### 2.1.1. THE EFFECT OF THE AGE OF PLANTS ON THE ANTIPLASMODIAL ACTIVITY

The influence of the antiplasmodial activity according to the age of the plants was first analysed for axenic plants as these were produced in controlled and axenic conditions (Table 1). The results showed a weak antiplasmodial activity ( $IC_{50}$  of 31.3-41.7  $\mu\text{g/mL}$ ). The comparison of the antiplasmodial activity using parametric analysis of variance showed no significant values relating to the age of plants (P-value = 0.548). Therefore, this factor does not have a significant influence on the antiplasmodial activity of *P. arguta* plants that are up to 8 months old.

### 2.1.2. THE EFFECT OF THE CONDITIONS OF CULTURE ON THE ANTIPLASMODIAL ACTIVITY

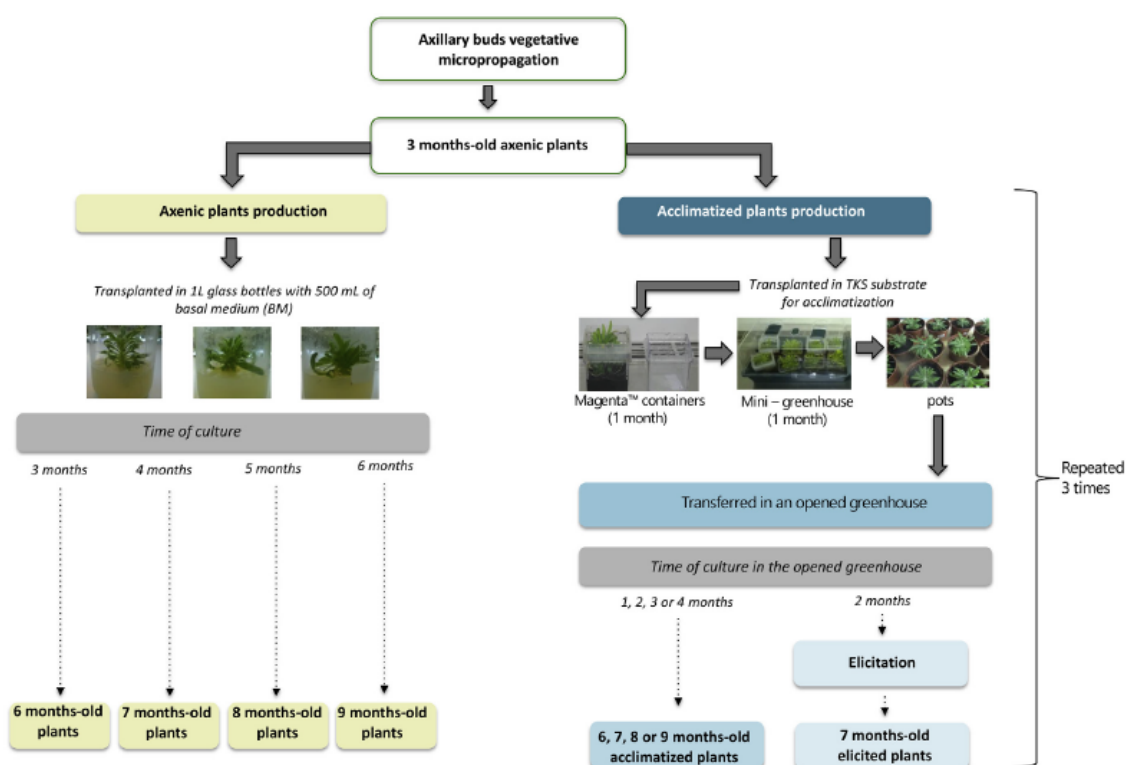
As only plants obtained from the first repetition and up to 8 months old were not attacked, the effect of the conditions of culture was analysed by comparison of the results obtained from leaf crude extracts of axenic and healthy acclimatized plants. At this stage, the plants transferred in the opened greenhouse were no longer in sterile conditions. The air and the TKS substrate contained microorganisms that might have influenced the plants' metabolism. For both conditions of culture, the crude extracts displayed low antiplasmodial activity, with  $IC_{50}$  of 31.3-41.7  $\mu\text{g/mL}$  for axenic plants and 28.9-39.7  $\mu\text{g/mL}$  for healthy acclimatized plants (Table 1). The results showed no significant difference in the antiplasmodial activity for both conditions (Student t-test, P-value = 0.374). Therefore, the conditions of culture used in this study appeared to have no significant effect on the antiplasmodial activity of plants up to 8 months of age.

### 2.1.3. THE EFFECT OF A BIOTIC STRESS ON THE ANTIPLASMODIAL ACTIVITY

The effect of the environment on the antiplasmodial activity of *Psiadia arguta* plants was studied by the comparison of healthy and attacked acclimatized plants. Insects were observed on some plants transferred in the opened greenhouse (Fig. 2). Plant damage from these insects on *P. arguta* consisted of yellowing leaves, dark spots of necrosis, and leaf wilt (Fig. 2B). The identification of these insects was attempted, and the results showed two different specimens: mealybugs from the Pseudococcidae family (voucher number: AFRA00185\_01) and aphids from the *Aphis* genera (voucher number: AFRA00184\_01) (Fig. 2C and D). These voucher specimens are registered in the reference collection of the UMR PVBMT (Unité Mixte de Recherche Peuplement Végétaux et Bio-agresseurs en Milieu Tropical, CIRAD of La Réunion). The analysis of the antiplasmodial activity of the crude extracts obtained from the infected plants revealed that these plants exhibited a higher antiplasmodial activity, with  $IC_{50}$  of 16.1-32.1  $\mu\text{g/mL}$  (Student t-test, P-value =  $7.52 \times 10^{-7}$ ), in comparison to the crude extracts of healthy acclimatized plants (Table 1). Those results could be explained by the observation of aphids and mealybugs on the plants. The population growth of these insects increases rapidly during warm conditions (Irwin and Thresh, 1988), which was the case when the greenhouse experiments were performed. Aphids and mealybugs are plant damaging pests that consume large amounts of phloem sieve elements (Nalam et al., 2018). Besides, the variation of the antiplasmodial activity for infected plants could be explained by the intensity of the attack. As reported on sorghum species, plant damage from aphids results from the loss of nutrients and sugars during feeding, which can cause plant water stress and a metabolic imbalance (Bowling et al., 2016). Besides, symptoms of aphid damage

include the purpling and twisting of the young leaves, and as feeding injury intensifies, leaf health declines and leaf colour changes to yellow or brown. These kinds of symptoms were observed on the leaves of *P. arguta* contaminated plants (Fig. 2A and B). In order to overcome biotic stresses, plants have evolved complex defence systems including the synthesis of diverse specialised metabolites, named phytoalexins (Zaynab et al., 2018). Those includes alkaloids, terpenoids, and phenolic compounds that affect the performance of herbivores (Zaynab et al., 2018). Thus, our results suggested that the physical and biotic stresses induced by the attack of *P. arguta* plants by insects activated a defence reaction, which resulted in the accumulation of bioactive compounds in the leaves.

**Fig. 1.** Process for obtaining all experimental plants in the present study.



#### 2.1.4. THE EFFECT OF ELICITATION ON THE ANTIPLASMODIAL ACTIVITY

In order to confirm the results obtained and evaluate the effect of a biotic stress on the metabolome of *P. arguta* plants, we decided to focus on elicited plants of *P. arguta* in controlled conditions. A preparation of *Botrytis cinerea* extract was chosen because this fungus is the prevalent one that damages plants. In response, plants have developed the ability to recognize the elicitors and to react to them by activation of defence responses. Besides, this elicitor has been successfully used in other species such as *Vitis* spp to induce protection against major pathogens (Poinssot et al., 2003; Saigne-Soulard et al., 2015).

**Table 1** - Antiplasmodial activity of *P. arguta* leaf crude extracts from different conditions of culture.

Antiplasmodial activity (IC <sub>50</sub> , µg/mL)		
Age of plants after axillary buds' subculturing	Crude extract of axenic plants <sup>a</sup>	Crude extract of acclimatized plants <sup>b</sup>
6 months	41.7 ± 5.7	39.5 ± 3.0
		33.1 ± 2.5
		20.8 ± 5.2 <sup>c</sup>
7 months	31.3 ± 9.5	39.7 ± 0.5
		24.3 ± 1.0 <sup>c</sup>
		31.5 ± 4.5 <sup>c</sup>
8 months	38.9 ± 14.2	28.9 ± 2.4
		17.5 ± 1.0 <sup>c</sup>
		21.5 ± 2.7 <sup>c</sup>
9 months	40.1 ± 3.0	32.1 ± 3.9 <sup>c</sup>
		14.2 ± 5.1 <sup>c</sup>
		16.1 ± 4.5 <sup>c</sup>
Artemisinin (positive control)	0.004 ± 0.001	—

Values are the mean ± standard deviation calculated from three independent assays in duplicate for each extract; Artemisinin was used as positive control (n = 3).

<sup>a</sup> After 3 months of culture in the Erlenmeyer containing the MS30 solid medium, all microshoots were subcultured in flasks containing 500 mL of MS30 solid medium until the target age was reached; the extract was realised with leaves collected from one biological replicate.

<sup>b</sup> The extract was performed with leaves collected from 4 to 5 biological replicates pooled together.

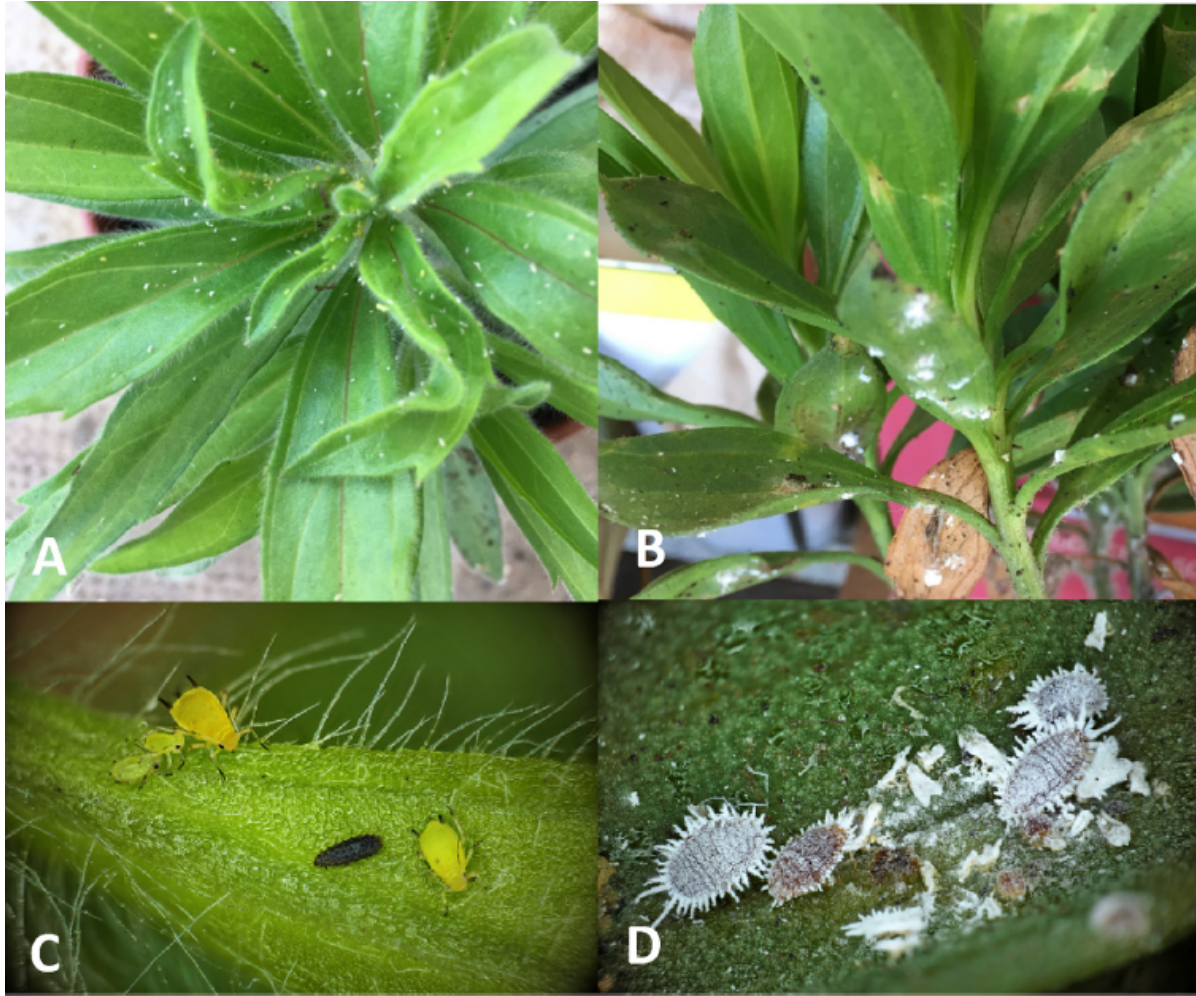
<sup>c</sup> Plants infected by mealybugs and aphids.

Therefore, in the present study, plants of *P. arguta* were acclimatized in a greenhouse and were then elicited with a solution of *Botrytis cinerea* culture filtrate. The latter contains β-glucan oligosaccharides (including 50% of DP6, 32% of DP5, and 18% of DP2-3) (Saigne-Soulard et al., 2015). Oligosaccharide fragments have previously been reported to induce resistance reactions in plants (Aziz et al., 2007). Thus, two concentrations of *B. cinerea* culture filtrate (8 and 5 g/L) and two times of incubation (48 h and 72 h) were tested. After pulverization of the solution of *B. cinerea*, leaves of treated plants were observed for any morphological differences. Any damage on the leaves was perceived. The antiplasmodial activity of all leaf crude extracts was evaluated (Table 2).

The results showed that all elicited plants were active in comparison to the control, which was inactive (IC<sub>50</sub> > 50 µg/mL). Besides, the time of incubation affected the antiplasmodial activity (analysis of variance, P-value = 0.0062); whereas, no significant difference was perceived for both tested concentrations (analysis of variance, P-value = 0.3645). There was no interaction between the two factors (analysis of variance, P-value = 0.5246). The antiplasmodial activity evaluated for

the elicited plants is slightly higher than those measured for plants attacked by aphids and mealybugs, suggesting that *P. arguta* plants reacts rapidly to a biotic stress.

**Fig. 2.** (A) Aphids on leaves of *P. arguta* acclimatized plants, (B) Mealybugs on leaves of *P. arguta* acclimatized plants, (C) Aphids and (D) mealybugs.



**Table 2** - Antiplasmodial activity of *P. arguta* leaf crude extracts of 7 months-old elicited plants.

Conditions of culture	Antiplasmodial activity (IC <sub>50</sub> , µg/mL)
Elicited (8 g/L) - 48 h	16.9 ± 2.7
Elicited (8 g/L) - 72 h	14.4 ± 2.1
Elicited (5 g/L) - 48 h	18.6 ± 4.2
Elicited (5 g/L) - 72 h	14.7 ± 2.6
Non-elicited (control)	> 50
Artemisinin (positive control)	0.004 ± 0.001

Values are the mean ± standard deviation calculated from three independent assays in duplicate for each extract. Artemisinin was used as positive control (n = 3).

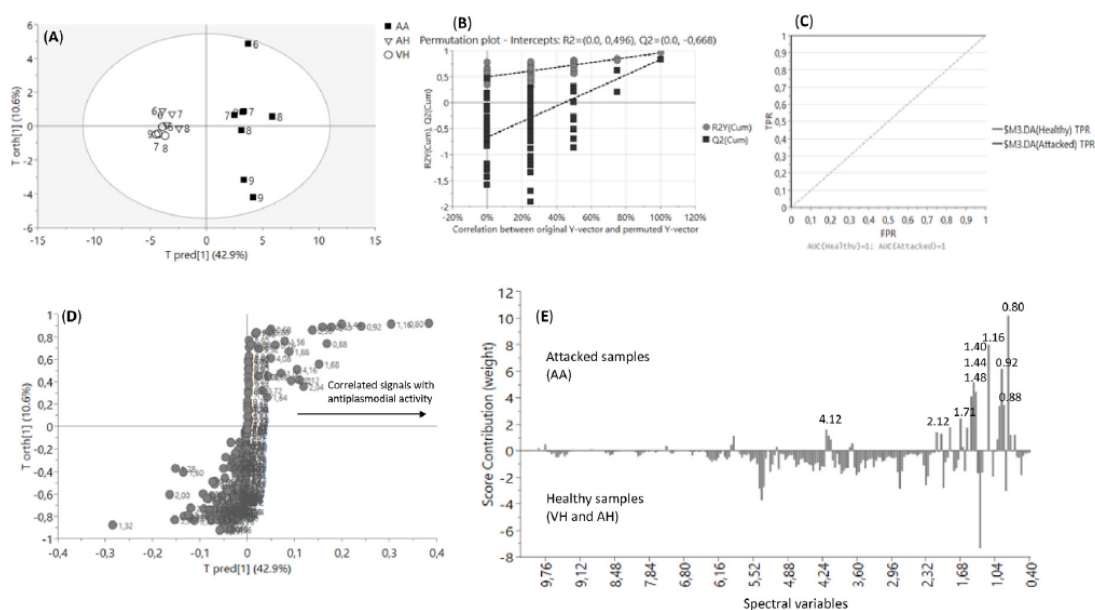
## 2.2. <sup>1</sup>H NMR PROFILES AND MULTIVARIATE DATA ANALYSIS

The chemical profile of all crude extracts was analysed by <sup>1</sup>H NMR and a multivariate data analysis was performed in order to identify the bioactive metabolites. An unsupervised Principal Component Analysis (PCA) was applied on <sup>1</sup>H NMR spectral data of all extracts to visualize variations between the samples (Fig. S1, supporting information). A three-component model explained 82.7% of the variance, with the first two components explaining 70%. From a general point of view, we noticed a trend of samples as grouping in two clusters along the first component: healthy samples (axenic and acclimatized ones) and attacked samples. In order to confirm these two groups discrimination, the orthogonal projection to latent structures discriminant analysis (OPLS-DA) was applied on the <sup>1</sup>H NMR spectral data of healthy and attacked samples. The OPLS-DA model set of 1 predictive and 2 orthogonal components (RX<sup>2</sup> = 66.3%, RY<sup>2</sup> = 95.5% and Q<sup>2</sup> = 83.2) (Fig. 3A). The permutation test confirmed the validity of the model (Fig. 3B). The receiver operating characteristic (ROC) curves showed areas under curve (AUC) of 1 (Fig. 3C). The comparison of spectral variables from both groups and the S-plot (Fig. 3D) has led to the identification of the variables contributing to this discrimination. Indeed, the contribution plot (Fig. 3E) highlighted the variables 0.80, 0.88, 0.92, 1.16, 1.40, 1.44, 1.48, 1.71, 2.12 and 4.12 that are found in the <sup>1</sup>H NMR spectra of infected samples (AA). These specific signals in the region from 0.5 to 2.5 ppm were present at higher concentration in attacked samples (AA) but also at a lower intensity or completely absent in axenic plants (VH) and healthy acclimatized plants (AH) as observed in the <sup>1</sup>H NMR spectra (Fig. 4). The scattering of attacked samples on the OPLS-DA score plot may be associated with the intensity of the attack and hence the variation of levels of specific metabolites in their crude extracts. These signals might be related to the higher antiplasmodial activity observed for attacked plants. As they were perceived in the aliphatic region of the NMR spectrum, they might be attributed to terpenes. Indeed, the existence of terpenoids can be determined by the presence of several singlets between 0.5 and 2.0 ppm in the <sup>1</sup>H NMR spectrum, as observed on *P. arguta* attacked plants spectra (Liu, 2011). Besides, terpenes are a class of phytoalexins known to



display a wide range of biological activity including an antiparasitic effect (Ludwiczuk et al., 2017; Mbaveng et al., 2014; Nevzorova et al., 2017; Šarac et al., 2014).

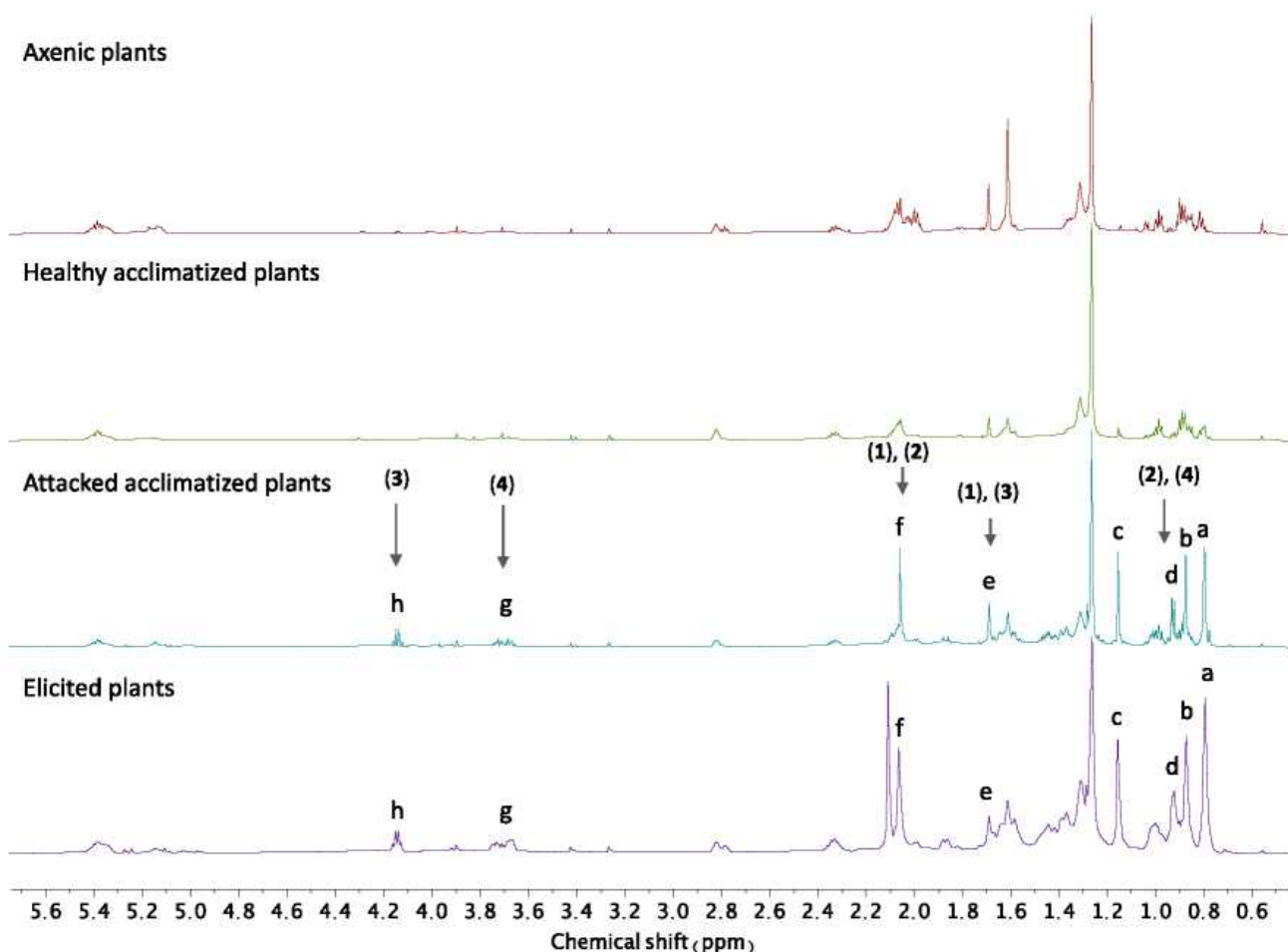
**Fig. 3.** (A) OPLS-DA score plot, (B) permutation plot, (C) ROC plot and (D) S-plot generated from the  $^1\text{H}$  NMR spectra (600 MHz) of healthy and attacked *P. arguta* plantlets crude extracts. (E) Contribution plot generated from the comparison of spectral variables of attacked samples vs healthy samples. The labels 6, 7, 8 and 9 on the PCA score plot correspond to the age of the plant; AA: attacked acclimatized plants; AH: healthy acclimatized plants and VH: healthy axenic plants.



## 2.3. IDENTIFICATION OF BIOACTIVE COMPOUNDS

The analysis of the  $^1\text{H}$  NMR spectra of *P. arguta* extracts from all conditions of culture highlighted two distinct chemical profiles: the first one is related to healthy samples and the second one to attacked samples. The differences observed were mainly qualitative. The comparison of spectral data realised with the contribution plot revealed that these two groups were distinguished mainly by chemical variables at  $\delta_{\text{H}}$  0.76, 0.84, 0.92, 1.12, 1.16, 1.44, 1.64, 2.08, 3.73, and 4.13 ppm (Figs. 3 and 4).

**Fig. 4.**  $^1\text{H}$  NMR spectra ( $\text{CDCl}_3$ , 600 MHz) of the ethyl acetate extracts from *in vitro*, healthy, attacked, and elicited acclimatized plants of *P. arguta*. Assignments: signals a ( $\delta_{\text{H}}$  0.80,  $\text{H}_3$ -19 and  $\text{H}_3$ -20), b ( $\delta_{\text{H}}$  0.88,  $\text{H}_3$ -18), and c ( $\delta_{\text{H}}$  1.16,  $\text{H}_3$ -17) are characteristic of the labdane bicyclic ring; d ( $\delta_{\text{H}}$  0.92,  $\text{H}_3$ -16), e ( $\delta_{\text{H}}$  1.71,  $\text{H}_3$ -16), f ( $\delta_{\text{H}}$  2.12,  $\text{H}_3$ -20), g ( $\delta_{\text{H}}$  3.69,  $\text{H}_3$ -15), and h ( $\delta_{\text{H}}$  4.13,  $\text{H}_3$ -15) are assigned to labdan-13(*E*)-en-8 $\alpha$ -ol-15-yl acetate (1); labdan-8 $\alpha$ -ol-15-yl acetate (2); labdan-13(*E*)-ene-8 $\alpha$ -ol-15-diol (3); (8*R*,13*S*)-labdan-8,15-diol (4).



Since all observed signals in attacked samples might be related to the terpenoids family, the  $^1\text{H}$  NMR signals were assigned by comparison to those of terpenes previously isolated (labdane diterpenes and triterpenes) from the wild specimen extract (Mahadeo et al., 2019). The results provided evidence of labdan-13(*E*)-en-8 $\alpha$ -ol-15-yl acetate (1), labdan-8 $\alpha$ -ol-15-yl acetate (2), labdan-13(*E*)-ene-8 $\alpha$ -ol-15-diol (3) and (8*R*,13*S*)-labdan-8,15-diol (4) in the extracts of contaminated plants (Fig. 4). Major signals such as H-19/H-20 at  $\delta_{\text{H}}$  0.76 (s), H-18 at  $\delta_{\text{H}}$  0.84 (s), and H-17 at  $\delta_{\text{H}}$  1.16 (s), characteristic of the labdane bicyclic ring, were clearly distinguished in the  $^1\text{H}$  NMR spectra. In addition to these, signals of the lateral chain of labdan-13(*E*)-en-8 $\alpha$ -ol-15-yl acetate were detected at  $\delta_{\text{H}}$  2.12 (s) and  $\delta_{\text{H}}$  1.71 (s). Resonances at  $\delta_{\text{H}}$  2.12 (s) and  $\delta_{\text{H}}$  0.92 (d,  $J = 6.6$  Hz) were assigned to the labdan-8 $\alpha$ -ol-15-yl acetate. Moreover, characteristic signals of the labdan-13(*E*)-ene-8 $\alpha$ -ol-15-diol at  $\delta_{\text{H}}$  4.13 (q,  $J = 6.7$  Hz) and  $\delta_{\text{H}}$  1.71 (s), and the (8*R*,13*S*)-labdan-8,15-diol at

$\delta_{\text{H}}$  0.92 (d,  $J = 6.6$  Hz) and  $\delta_{\text{H}}$  3.69 (m) were also identified. In order to confirm those hypotheses, the 1D and 2D NMR spectra of one attacked plant crude extract were obtained (supporting information). The NMR data showed signals characteristic of the labdan bicyclic ring (Table S1, supporting information). Characteristic signals of the lateral chain provided evidence of compounds 1-4 in the crude extract of attacked plants. Indeed, the analysis of the  $^{13}\text{C}$  and  $^1\text{H}$  NMR spectra revealed resonances consistent with those of an acetate moiety ( $\delta_{\text{C}}$  171.4 for C-1',  $\delta_{\text{C}}$  20.3 for C-2' and  $\delta_{\text{H}}$  2.0 for H-2'). In addition, the signals  $\delta_{\text{C}}$  137.4 and  $\delta_{\text{C}}$  30.6 associated with C-13 confirmed the presence of compounds 1 and 2, respectively. Furthermore, the HSQC cross-peaks between C-15 ( $\delta_{\text{C}}$  58.3) and H-15 ( $\delta_{\text{H}}$  3.6) and C-15 ( $\delta_{\text{C}}$  63.0) and H-15 ( $\delta_{\text{H}}$  4.1) are assigned to compounds 3 and 4.

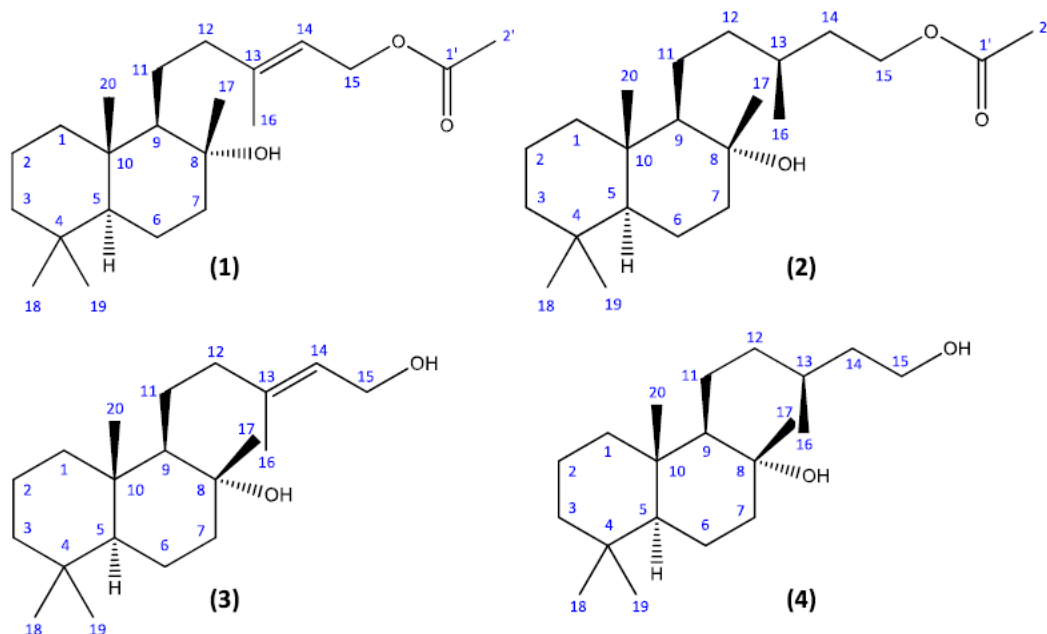
As demonstrated in our previous study on the isolation of antiplasmodial compounds from a wild specimen of *P. arguta*, the compounds 1-4 (Fig. 5) were the most active ones with  $\text{IC}_{50}$  of 10.2, 11.7, 11.3, and 6.9  $\mu\text{g}/\text{mL}$ , respectively (Mahadeo et al., 2019). Therefore, their accumulation in the contaminated plant extracts could explain their highest antiplasmodial activity compared to those of uninfected ones.

## 2.4. ELICITATION

In order to confirm the accumulation of these compounds in stressed plants, the  $^1\text{H}$  NMR spectra of all elicited plants were analysed. The inspection of all  $^1\text{H}$  NMR spectra revealed the presence of the same signals as those found in attacked plants' spectra (Fig. 4). The results confirmed the production of these four compounds following a biotic or a physical stress.

The identification of these four diterpenes in elicited plants as well as in acclimatized plants confirmed that they were synthesized in response to a biotic stress and without any specificity. Thus, those results demonstrated that labdan-13(*E*)-en-8 $\alpha$ -ol-15-yl acetate, labdan-8 $\alpha$ -ol-15-yl acetate, labdan-13(*E*)-ene-8 $\alpha$ -ol-15-diol and (8*R*,13*S*)-labdan-8,15-diol are accumulated in the leaves of *P. arguta* plants after the first interaction with the environment, particularly after phytopathogenic attacks. Indeed, terpenes are phytoalexins that are biosynthesized in response to plant aggression (Schmelz et al., 2014). Many studies have demonstrated the induction of monoterpenes, sesquiterpenes or di-terpenes after fungal or herbivore attacks including aphid attacks (Dicke et al., 2003; Du et al., 1998; Loughrin et al., 1994; Niu et al., 2012; Pontin et al., 2015; Raffa and Smalley, 1995; Ren and West, 1992; Salomon et al., 2016). The induction of plant volatile biosynthesis is regulated by the jasmonic acid (JA) and salicylic acid (SA) pathways, which are plant defence signalling pathways (Arimura et al., 2005; Ozawa et al., 2000). It is also established that the JA-signalling pathway regulates the biosynthesis of volatile terpenoids (Dicke et al., 2003). Moreover, phloem-feeding insects such as aphids or mealybugs mainly trigger the SA- and JA-signalling pathways, which could explain our results on the accumulation of terpenes in the leaves of attacked plants (Stam et al., 2014). To the best of our knowledge, this is the first report of these four compounds produced by plants in response to attack by herbivorous insects.

**Fig. 5.** Compounds identified in *P. arguta* attacked plants.



### 3. Conclusions

In this work we have been able to establish the acclimatization of *P. arguta in vitro* plantlets providing a biotechnological tool for the production of this plant and its metabolites. Besides, the NMR-based metabolomic approach used in the present study was a powerful tool to determine the metabolomic variation of plants in response to environmental perturbations. With this methodology, labdan-13(*E*)-en-8 $\alpha$ -ol-15-yl acetate, labdan-8 $\alpha$ -ol-15-yl acetate, labdan-13(*E*)-ene-8 $\alpha$ -ol-15-diol, and (8*R*,13*S*)-labdan-8,15-diol were identified as the major compounds contributing to the discrimination between attacked and healthy plants. These four compounds were considered to be biotic stress-related chemicals that were also responsible for the antiplasmodial activity of attacked plants.

## 4. Experimental section

### 4.1. PLANT MATERIAL AND CULTIVATION

The axenic plants of *P. arguta* Pers. (Voigt) (Asteraceae) were obtained from seeds that were collected in 2008 from a specimen in culture in the botanical garden of the University of Reunion Island. The micropropagation of the axenic plants was performed using the method of Kodja et al. (1998).

These axenic plantlets were cultured in 250 mL Erlenmeyer flasks on basal medium (BM). The BM consisted of macro and microsalts of (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, Maurel's vitamins (Morel and Wetmore, 1951) and 0.75% agar. The medium's pH was adjusted to 5.8, and then it was autoclaved at 121 °C. Every 3 months, well-developed plantlets containing multiple axillary buds were used for vegetative micropropagation on basal medium. Thus, the multiplication of axenic plantlets was performed by excision of axillary buds with few leaves (each consisting of a microshoot). All microshoots were placed on the surface of the basal medium.

In the present work, three months old well-developed axenic plantlets were used to obtain plant materials for all experiments. Thus, three protocol experiments were followed to obtain 6-9 months-old plants depending on the conditions of culture: (1) *in vitro* axenic plants, (2) plants acclimatized in a greenhouse, and (3) acclimatized and elicited plants in a greenhouse. The protocols are explained in Fig. 1.

- (1) For the axenic plants, three months old well-developed plants were transplanted in 1 L glass bottles containing 500 mL of basal medium. These were allowed to grow for 3, 4, 5 or 6 months on this growth medium until they reached 6–9 months old. All axenic plants were kept in a growth room at  $25 \pm 1$  °C and under a 14 h photoperiod provided by 400 Wm<sup>-2</sup> cool fluorescent tubes. There was 1 biological replicate for each age of plant. When the plants were 6–9 months old, the leaves were collected and freeze-dried.
- (2) For acclimatized plants, excess culture medium was removed from the roots of three months old well-developed plants, and the plantlets were then transplanted into Magenta™ containers with sterile TKS®1 substrate (growing medium, Floragard). After one month, all Magenta™ containers were opened in sterile conditions and transferred in a mini greenhouse. All plants were kept in this greenhouse and the level of humidity was reduced gradually within for 4 weeks (acclimatization step). All the plants were at that point five-months-old. Acclimatized plants were afterwards transplanted in pots (10×10 cm) containing non-sterile TKS®1 substrate. All the plants were transferred in an opened greenhouse; located in the botanical garden of the University of Reunion Island. The plants were allowed to grow 1–4 months on the non-sterile TKS substrate until they reached 6–9 months old. There were 4–5 biological replicates for each age of plants. For each age, the leaves of all specimens were collected and pooled together before freeze-drying.
- (3) For elicited plants, after the acclimatization step, the plants were transferred in the greenhouse and were allowed to grow for 2 months until they reached seven-months-old. Then, the plants were elicited with *Botrytis cinerea* culture filtrate, which was provided by the Institut des Sciences de la Vigne et du Vin (Villenave d'Ornon, France). The preparation of the culture filtrate is described in Saigne-Soulard et al. (2015). Two solutions of 8 g/L and 5 g/L of *B. cinerea* culture filtrate were prepared in sterile water. Plants leaves were sprayed with these solutions, and 48 h or 72 h after treatment, the leaves were collected and freeze-dried.

## 4.2. EXTRACTION

Five grams of freeze-dried leaves of each sample were ground and exhaustively extracted by maceration with ethyl acetate (analytical grade, Carlo Erba) at room temperature with magnetic agitation. The extraction was repeated three times. The extracts were pooled and evaporated under reduced pressure and kept at 4 °C until analysis. The amount of crude extract obtained varied from 0.3 to 0.5 g.

## 4.3. SAMPLE PREPARATION FOR NMR ANALYSIS

For each sample,  $15 \pm 0.2$  mg of ethyl acetate crude extract were transferred to a 2 mL Eppendorf tube. A volume of 1.5 mL of deuterated chloroform ( $\text{CDCl}_3$ , D 99.9 atom %, Eurisotop) containing 0.03% TMS (tetramethylsilane) was added to each sample. The mixture was vortexed at room temperature for 1 min. An aliquot of 0.6 mL was transferred into 5 mm-NMR tubes for NMR analysis.

## 4.4. NMR ANALYSIS

$^1\text{H}$  NMR spectra were recorded at 298 K on a Bruker 600 MHz Avance III spectrometer (600.13 MHz proton NMR frequency) equipped with a TCI cryoprobe. Each NMR spectrum was recorded in  $\text{CDCl}_3$  and consisted of 64 scans.  $\text{CDCl}_3$  was used as the internal lock. Each  $^1\text{H}$ -NMR spectrum was recorded with the following parameters: standard one-pulse sequence with  $30^\circ$  flip angle and relaxation delay (RD) = 1.0 s. Free induction decays (FIDs) were Fourier-transformed with line broadening (LB) = 0.3 Hz. The resulting spectra were manually phased, baseline-corrected, and calibrated to TMS at 0.00 ppm using MestReNova 10.0 (Mestrelab Research S.L.). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of  $\delta$  0.32-10.02. The region of  $\delta$  7.05-7.49 was removed prior to further analysis because of the residual signal of solvent. The matrix size consisted then of 231 variables.

1D and 2D NMR spectra ( $^1\text{H}$ ,  $^{13}\text{C}$ , COSY and HSQC) of the crude extract of the infected plant were recorded at 298 K in  $\text{CDCl}_3$  on a Bruker 500 MHz Avance III spectrometer.

## 4.5. MULTIVARIATE DATA ANALYSIS

Chemometric analysis was performed with SIMCA version 12.0 (Umetrics, Umea, Sweden) in the form of an unsupervised principal component analysis (PCA) and a supervised orthogonal partial least squares discriminant analysis (OPLS-DA). The scaling was based on Pareto method. With PCA, class differences were determined from a multivariate dataset.  $R_x^2$  and  $Q^2$  values described the quality of the model.  $R_x^2$  indicated goodness of fit and is defined as the proportion of the variance in the data observed in the model.  $Q^2$  is defined as the proportion of variance in the data that was predictable by the model.

## 4.6. ANTIPLASMODIAL ACTIVITY

The effect of each crude extract on parasite growth of the *P. falciparum* strain 3D7 was measured in a 48 h growth assay as previously reported (Mahadeo et al., 2019). Briefly, ring stage

parasite cultures were grown for 48 h in the presence of increasing concentrations of crude extract at 37 °C. After 48 h in culture, parasite viability was evaluated by the lactate dehydrogenase inhibition colorimetric test. The half inhibitory concentration (IC<sub>50</sub>) values are the average of three in-dependent assays with each determination in duplicate and are expressed ± standard deviation. Artemisinin (Sigma-Aldrich, Machelen, Belgium) at an initial concentration of 100 ng/mL was used as positive control in all experiments.

#### **4.7. STATISTICAL ANALYSIS**

Preliminary tests of distribution normality and homogeneity of variances were performed using the Shapiro-Wilk test and Bartlett test, respectively. The effect of plant age on the antiplasmodial activity was analysed using parametric one-way analysis of variance. The effect of conditions of culture and environment on the antiplasmodial activity was examined using Student t-tests. Differences in antiplasmodial activity between two concentrations of oligosaccharide solution and two times of incubation were investigated using parametric two-way analysis of variance. All these analyses were performed with R 3.4.2 (R Core Team, 2017).

#### **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### **Acknowledgements**

This study was supported by the Regional Council of La Réunion. The authors thank Pr. Stéphanie Cluzet (Institut des Sciences de la Vigne et du Vin, Villenave d'Ornon, France) for providing the *B. cinerea* culture filtrate. We acknowledge the NMR-PF facility (University Paris 13 France).

#### **Appendix A. Supplementary data**

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.phytochem.2020.112401>.

## **References**

- Arimura, G., Kost, C., Boland, W., 2005. *Herbivore-induced, indirect plant defences*. *Biochim. Biophys. Acta* 1734, 91-111.
- Ayouni, K., Berboucha-Rahmani, M., Kim, H.K., Atmani, D., Verpoorte, R., Choi, Y.H., 2016. *Metabolomic tool to identify antioxidant compounds of Fraxinus angustifolia leaf and stem bark extracts*. *Ind. Crops Prod., The Second Mediterranean Symposium on Medicinal and Aromatic Plants (MESMAP-2)* 88, 65-77.
- Aziz, A., Gauthier, A., Bézier, A., Poinssot, B., Joubert, J.-M., Pugin, A., Heyraud, A., Baillieul, F., 2007. *Elicitor and resistance-inducing activities of beta-1,4 cellodextrins in grapevine, comparison with beta-1,3 glucans and alpha-1,4 oligogalacturonides*. *J. Exp. Bot.* 58, 1463-1472.

Bowling, R.D., Brewer, M.J., Kerns, D.L., Gordy, J., Seiter, N., Elliott, N.E., Buntin, G.D., Way, M.O., Royer, T.A., Biles, S., Maxson, E., 2016. Sugarcane aphid (Hemiptera: aphididae): a new pest on sorghum in north America. *J. Integr. Pest Manag.* 7.

Dicke, M., van Poecke, R.M.P., de Boer, J.G., 2003. Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic Appl. Ecol.* 4, 27-42.

Du, Y., Poppy, G.M., Powell, W., Pickett, J.A., Wadhams, L.J., Woodcock, C.M., 1998. Identification of semiochemicals released during aphid feeding that attract parasitoid *Aphidius ervi*. *J. Chem. Ecol.* 24, 1355-1368.

Irwin, M.E., Thresh, J.M., 1988. Long-range aerial dispersal of cereal aphids as virus vectors in North America. *Phil. Trans. Roy. Soc. Lond. B* 321, 421-446.

Jonville, M.C., Kodja, H., Humeau, L., Fournel, J., De Mol, P., Cao, M., Angenot, L., Frédérick, M., 2008. Screening of medicinal plants from Reunion Island for anti-malarial and cytotoxic activity. *J. Ethnopharmacol.* 120, 382-386.

Jonville, M.C., Kodja, H., Strasberg, D., Pichette, A., Ollivier, E., Frédérick, M., Angenot, L., Legault, J., 2011. Antiplasmodial, anti-inflammatory and cytotoxic activities of various plant extracts from the Mascarene Archipelago. *J. Ethnopharmacol., Immunomodulators* 136, 525-531.

Kauroo, S., Govinden-Soulange, J., Marie, D.E.P., 2016. Endemic Asteraceae from Mauritius islands as potential phytomedicines. *Int. J. Chem. Environ. Biol. Sci.* 4, 23-27.

Kodja, H., Robene-Soustrade, I., Humeau, L., Figier, J., Govinden-Soulange, J., Gurib-Fakim, A., 1998. Micropropagation of *Psiadia arguta* through cotyledonary axillary bud culture. *Plant Growth Regul.* 25, 75-80.

Liu, W.J.H., 2011. *Traditional Herbal Medicine Research Methods: Identification, Analysis, Bioassay, and Pharmaceutical and Clinical Studies.* John Wiley & Sons.

Loughrin, J.H., Manukian, A., Heath, R.R., Turlings, T.C., Tumlinson, J.H., 1994. Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plant. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11836-11840.

Ludwiczuk, A., Skalicka-Wozniak, K., Georgiev, M.I., 2017. Chapter 11 - terpenoids. In: Badal, S., Delgoda, R. (Eds.), *Pharmacognosy.* Academic Press, Boston, pp. 233-266.

Mahadeo, K., Herbette, G., Grondin, I., Jansen, O., Kodja, H., Soulange, J., Jhaumeer-Laulloo, S., Clerc, P., Gauvin-Bialecki, A., Frederich, M., 2019. Antiplasmodial diterpenoids from *Psiadia arguta*. *J. Nat. Prod.* 82, 1361-1366.

Mbaveng, A.T., Hamm, R., Kuete, V., 2014. 19 - harmful and protective effects of terpenoids from african medicinal plants. In: *Toxicological Survey of African Medicinal Plants.* Elsevier, pp. 557-576.

Morel, G., Wetmore, R.H., 1951. Fern callus tissue culture. *Am. J. Bot.* 38, 141-143.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and Bio assays with tobacco tissue cultures. *Physiol. Plantarum* 15, 473-497.

Nalam, V., Louis, J., Shah, J., 2018. Plant defense against aphids, the pest extraordinaire. *Plant Sci.*

Nevzorova, Y.A., Grossmann, J., Trautwein, C., 2017. Anti-tumorigenic and anti-angiogenic effects of natural conifer *Abies sibirica* terpenoids in vivo and in vitro. *Biomed. Pharmacother.* 89, 386-395.



Niu, H., Zhao, L., Lu, M., Zhang, S., Sun, J., 2012. The ratio and concentration of two monoterpenes mediate fecundity of the pinewood nematode and growth of its associated fungi. *PLoS One* 7.

Ozawa, R., Arimura, G., Takabayashi, J., Shimoda, T., Nishioka, T., 2000. Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. *Plant Cell Physiol.* 41, 391-398.

Poinssot, B., Vandelle, E., Bentéjac, M., Adrian, M., Levis, C., Brygoo, Y., Garin, J., Sicilia, F., Coutos-Thévenot, P., Pugin, A., 2003. The endopolygalacturonase 1 from *Botrytis cinerea* activates grapevine defense reactions unrelated to its enzymatic activity. *Mol. Plant-Microbe Interact. MPMI* 16, 553-564.

Pontin, M., Bottini, R., Burba, J.L., Piccoli, P., 2015. *Alium sativum* produces terpenes with fungistatic properties in response to infection with *Sclerotium cepivorum*. *Phytochemistry* 115, 152-160.

Raffa, K.F., Smalley, E.B., 1995. Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. *Oecologia* 102, 285-295.

Recio, M.C., Giner, R.M., Manez, S., Rios, J.L., Marston, A., Hostettmann, K., 1995. Screening of tropical medicinal plants for antiinflammatory activity. *Phytother Res.* 9, 571-574.

Ren, Y.Y., West, C.A., 1992. Elicitation of diterpene biosynthesis in rice (*Oryza saliva* L.) by chitin. *Plant Physiol.* 99, 1169-1178.

Saigne-Soulard, C., Abdelli-Belhadj, A., Téléf-Micouleau, M., Bouscalt, J., Cluzet, S., Corio-Costet, M.-F., Mérillon, J.-M., 2015. Oligosaccharides from *Botrytis cinerea* and elicitation of grapevine defense. *Polysaccharides* 939-958.

Salomon, M.V., Purpora, R., Bottini, R., Piccoli, P., 2016. Rhizosphere associated bacteria trigger accumulation of terpenes in leaves of *Vitis vinifera* L. cv. Malbec that protect cells against reactive oxygen species. *Plant Physiol. Biochem.* 106, 295-304.

Šarac, Z., Matejic, J.S., Stojanovic-Radic, Z.Z., Veselinovic, J.B., Dzamic, A.M., Bojovic, S., Marin, P.D., 2014. Biological activity of *Pinus nigra* terpenes—evaluation of FtsZ inhibition by selected compounds as contribution to their antimicrobial activity. *Comput. Biol. Med.* 54, 72-78.

Schmelz, E.A., Huffaker, A., Sims, J.W., Christensen, S.A., Lu, X., Okada, K., Peters, R.J., 2014. Biosynthesis, elicitation and roles of monocot terpenoid phytoalexins. *Plant J.* 79, 659-678.

Stam, J.M., Kroes, A., Li, Y., Gols, R., van Loon, J.J.A., Poelman, E.H., Dicke, M., 2014. Plant interactions with multiple insect herbivores: from community to genes. *Annu. Rev. Plant Biol.* 65, 689-713.

Sussman, L.K., 1980. Herbal medicine on Mauritius. *J. Ethnopharmacol.* 2, 259-278.

Walter, K.S., Gillett, H.J., 1998. 1997 IUCN Red List of Threatened Plants. IUCN.

Wang, Y., Hamburger, M., Gueho, J., Hostettmann, K., 1992. Cyclohexanecarboxylic-acid derivatives from *Psiadia trinervia*. *Helv. Chim. Acta* 75, 269-275.

Zaynab, M., Fatima, M., Abbas, S., Sharif, Y., Umair, M., Zafar, M.H., Bahadar, K., 2018. Role of secondary metabolites in plant defense against pathogens. *Microb. Pathog.* 124, 198-202.