Agricultural Science Research Journal Vol. 7(9): 267 – 276, September 2017
Available online at http://resjournals.com/journals/agricultural-science-research-journal.html
ISSN: 2026 –6073 ©2017 International Research Journals

Full Length Research Paper

Phenolics from *Lippia multiflora* Moldenke as potential bioactive agents against peanut pathogens

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Abstract

The phenolic composition of aqueous extract from dried leaves of *Lippia multiflora* Moldenke was analyzed by liquid chromatography- mass spectrometry (LC-MS) before inverse solid phase fractionation. The crude extract and the different fractions (from FL1 to FL5) obtained after fractionation were then tested *in vitro* for their effect on two fungi responsible of peanut foliar diseases, *Phaeoisariopsispersonata* and *Pucciniaarachidis*. The chemical analysis of the aqueous *L. multiflora* crude extract indicated the presence of seven phenolic compounds (nuomiosideA, isonuomiside A, samioside, verbascoside, isoverbascoside, alyssonoside and leucoseptoside A) and one monoterpene (geniposide). The antifungal activity, estimated by the efficient rate of the fungal appressorium formation, varied from 33.33% to 58.33% and 5.56% to 38.89% for *P. personata*and *P. arachidis* spores, respectively. By spraying the different fractions on artificially contaminated leaves, the best antifungal activity was noticed using FL3 and FL4 fractions, the only one in which the presence of samioside was described. The results of this study suggest that the protecting effect of *Lippia*extracts against peanut pathogens may be related to the presence of four phenylethanoid glucosides: verbascoside, nuomoside A, leucoseptoside A and samioside.

Keywords: plant extracts, phenolics, monoterpenes, antifungal activity, *Lippia multiflora,* peanut.

Introduction

Peanut has an extreme socio economic importance in Africa. Indeed, as a food crop providing a substantial source of income, peanut helps widely to human and animal nutrition. Owing to the robustness and plasticity of the plant associated to the multiplicity of its uses, this oilseed and protein crop is particularly appreciated (Schilling *et al*. 1996). However, peanut remains a plant which is strongly attacked, notably by fungi involved in foliar diseases. Three of them, the rust and the early and late spots, are detrimental to the plant's growth. Studies have shown that the combination of these diseases may generate losses which can reach 70% of the production (Sunkade *et al*. 2005). Controlling their development appeared therefore essential but, in order to answer the global objectives of sustainable development, it cannot be done using the synthetic antifungal currently already marketed. One of the promissing alternatives consist in selecting plants extracts limiting plant pathogen activity.

Different laboratories, either in Africa (Burkina Faso and west african sub region) or elsewhere throughout the world, have successfully tested the antimicrobial (fungal, bactericidal, virocid) activities of some local plants. More particularly, the *Lippia* genus that belongs to the Verbenaceae family and composed of almost 200 species (Burkill 2000) has been the subject of several studies. It has been noted that *Lippia multiflora* essential oil had a bactericidal activity on *Staphylococccus aureus, Escherichia coli* (Bassolet*et al*. 2001) and *Bacillus cereus* (Owolabi *et al*. 2009) whereas that of *L. rugosa*led to a total inhibition of *Aspergillus flavus*growth (Tatsadjieu *et al*. 2009). Studies on *L. rehmannii* (Linde*et al*. 2010) indicated a real efficiency of essential oil against *Rhizoctonia solani,Fusarium oxysporum* and *Penicillium digitatum* fungi, which are respectively

potato, maize and orange tree pathogens. More or less, these studies generally focus on the biological activity of the plant extracts, neglecting to give information on the nature of the bioactive compounds herein. Conversely, recent works related to the chemical composition of *L. multiflora* essential oil (Tchobo *et al.* 2013; Bayala *et al.* 2014) helped identify the most important chemical families present in extracts of this plant, without giving details about the compound activity. Yet, a complete and in-depth scientific study of the biological activities and molecular composition of these plant extracts would offer possibilities to optimize their use and increase in value (Stevenson *et al*. 2009). Another characteristic of this research is that it concerns exclusively oil extracts from plants. The biochemical techniques developed to obtain these extracts lead to isolate mainly hydrophobic and volatile compounds, leaving aside most of the phenolic compounds. However, this family of compounds offers a great diversity of molecules presenting important biological and technological interests (Macheix *et al*. 2005) with numerous pharmaceutical applications considering, among other, their potent antioxidant activity (Kone 2009). Finally, the use of essential oils presents some disadvantages related notably to the rapid evaporation of the species particles and the instability of isolated compounds. Compared to essential oils, aqueous preparations of plants extracts require less extraction technical nature; they also offer more facility to be used to implement phytosanitary treatments. In such a context, this study proposes to carry out the phytochemical analysis of *L. multiflora* on samples obtained by aqueous extraction and to test their *in vitro* efficiency on *Phaeoisariopsispersonata* and *Pucciniaarachidis*, two fungi causing late leaf spot and rust on peanut.

Materials and methods

Plant and fungal material

Lippia multiflora leaves have been collected in Gampêla, a village located in the center of Burkina Faso. Peanut leaves uninjured from any infection about 30 days after seedling (DAS) have been used to carry out artificial contaminations during the biological test. They come from TS32-1 susceptible variety to late leaf spot and rust (Fig 1). This variety is grown in the greenhouse of the Unit for Training and Research on Life and Land Science of UniversitéOuaga I. Fungal spores of *P. arachidis*and *P. personata*have been gathered in Gampêla on leaves naturally infected in fields which haven't received antifungal treatments. Foliar stains presented themselves under the form of reddish brown pustules for rust and black lesions for late spot.

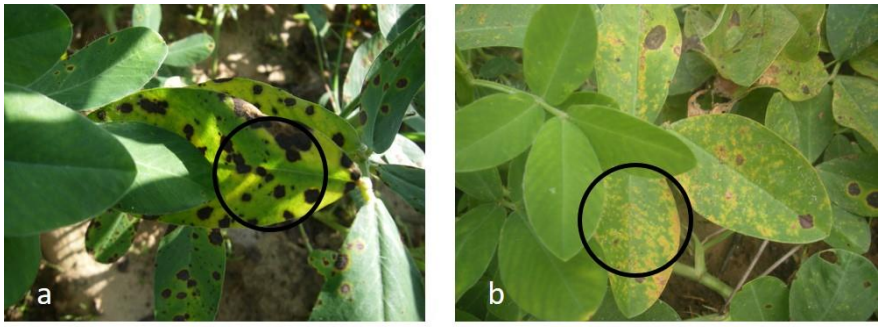


Figure 1: illustrations of symptoms of late leaf spot (a) and rust (b) of peanut

Obtaining Lippia multiflora aqueous extract

The leaves collected from *Lippia multiflora* species have been washed and dried in the shade, finely crushed and kept in a fresh and dry place, sheltered from light.

The extraction has been carried out adding 4l of purified water (MilliQ) to 500g of powder. After shaking during 2 hours at room temperature (15-17° C) the mixture was placed at +4°C for 10 hours for decantation. The solution was then distributed in 100 mL pots to be centrifuged (Centrifuge trademark Sorvall RC 26 Plus) at 15000 rpm for 15 min. Supernatants were collected and then frozen with liquid nitrogen before being lyophilized at -50°C in a freeze dryerAlpha 1-2 (Christ) equipped with a RZ 2.5 vacuum pump (Vacuubrand) to obtain a dried extract.

From the lyophilized product, 0.5g has been taken off and 50mL of purified water have been added. After 30 min. in an ultrasound bath (Branson 5510 trademark), the whole is centrifuged 15000 rpm for 5 min. and the supernatant used for the LC-MS analyses.

Fractional of Lippia multiflora dry extract

The fractional distillation of crude extract was carried out on extraction columns in inverse solid phase (Bond Elut LR C C_{18} VARIAN, 100 x 4.6 mm). The floating (15mL) was passed through each column followed by 5 mL of purified water thus constituting the fraction 1 (FL1, 100% water fraction or aqueous fraction). Four other fractions were obtained through elution using decreasing polarity solvents composed of water/acetonitrile mixture (95/5; 90/10; 85/15; 10/90). The 5 fractions obtained (FL1, FL2, FL3, FL4 and FL5) were vacuum-evaporated (60° C, 30min) using a rotary evaporator (Rotavapor R3, BUCHI). After the acetonitrile evaporation, the residue was frozen using liquid nitrogen before being lyophilized. The dry extracts were diluted (20µg/mL) before their analysis using LC-MS.

Chemical analysis of Lippia multiflora extracts

The analyses have been carried out using liquid chromatography-mass spectrometry (LC-MS) (ESI-MS, Waters 1525 m and Waters Micromass ZQ ESCi multimode ionization mass spectrometry (Micromass

Ltd, UK)). These experiments were execute at University of Montpellier II in Laboratory of Biochemistry and Plant Physiology. The absorbance data were recorded using a diode module detector, covering between 200 and 800 nm on 4 nm step. All the eluents used, acetronitrile (Carlo-Erba) and methanol (VWR Hi Per Solv Chromanorm), were of HPLC quality. The columns were of C18 type (XTerra MS (Waters), 2.1 mm x100 mm, 3.5 µm particle size) and protected by pre-columns of the same nature. The mobile phase was given at a flow rate of 0.21mL /min and composed of permuted water (solvent A) and acetonitrile (solvent B), both phases acidified by 0.1% (v/v) formic acid in order to prevent the ionization of phenolic acids. The gradient elution program was as follows: 0-8 min, 99-90% A; 8-17 min, 90-83% A; 17-26 min, 90-73% A; 26-55 min, 73-0% A and 55-60min, 0-95% A. Molecules have been identified taking into consideration the retention time of their peak, and their absorbance and mass spectra. When the standards were available, the compound identity was confirmed by a retention time (*tr*) and spectra identical to that of standard. In other respects, a comparison of the results is made with previous investigations carried out by other authors.

Tests for antifungal activity

The spore suspensions were prepared (density of 100 spores/mL assessed using Malassez counting chamber) from harvests made on necrotic stains of peanut leaves naturally contaminated. Crude extract solutions of *Lippia multiflora* and lyophilized extracts fractions (FL1, FL2, FL3, FL4 and FL5) were prepared (10 mg of dried extract in 2 mL distilled water) to be used in the tests. An aqueous solution of verbascoside at 1 mg/mL (FL6) and an aqueous solution of verbascoside and isoverbacoside mixture (FL7) at 0.5mg/mL of each compound (Sigma, France) have been used as references for efficiency tests. The use of both compounds as positive controls is explained by the fact that numerous studies have confirmed their antifungal efficiency (Hennebelle (2006); Shikanga *et al*. (2010); Oyourou *et al*. (2013)). A negative control consisted of distilled water (FL8).

Spore germination tests

The spore germination tests have been carried out by placing 2 mL of each plant extract fraction solution in a test tube into which 2 mL of the adjusted spores' suspension at 1.10^2 spores/mL has been added. The negative control has been obtained by mixing 2 mL distilled water with 2 mL spores suspension. Reference solutions (FL6 and FL7) were brought at the rate of 2 mL to the same spores' volume. The incubation has been made in complete darkness at 22° C during 8 hours for rust spores and 25° C during 24 hours for those of late spot. The fungal appressoria has been then measured on 50 spores with a microscope of Zeiss Primostar trademark. Observations have been made at 40 x magnification. The efficiency rate (E) of each extract fraction have been calculated using the formula proposed by Greche and Hajjaji (2000):

$$\mathrm{E}(\%) = 100 \times [\mathrm{MLC} - \mathrm{MLE}] \div \mathrm{MLC}$$

Where MLC is the mean length of the germinal tube of the spore with the negative control and MLE is the mean length of the germinal tube of the spore with the tested plant extract.

Foliar stain inhibition test

Leaves from the TS32-1 susceptible variety grown in greenhouse have been harvested by cutting the petiole at its bottom, washed and planted in boxes full of sterile sand at the rate of ten leaves per box. The infection was carried out by pulverizing the abaxial part of the leaf with either one or the other of both inocula. After twelve hours of darkness to favor fungal appresorium formation (Subrahmanyam*et al*. (1980); Sankara (1997)), the boxes have been incubated at 22° C under 12 hours photoperiod by night and 12 hours photoperiod by day. The sand contained in the boxes was daily watered with distilled water and recovered by a transparent plastic for maintaining a strong humidity (between 80 and 90%).

The treatment with the inoculum was repeated each five days and observations made during 30 days. The evolution of rust and leaf spot symptoms on the leaflets in presence of extracts fractions has been appreciated using the 9 classes' severity scale of ICRISAT (Subrahmanyam *et al*. 1982).

Statistical analysis

The average of the results has been calculated and the data as a whole have been subjected to a variance analysis and to the smallest significant difference test at 5% threshold using XLSTAT software, 2010 version.

Results

Phytochemical analysis of *Lippia multiflora* aqueous extract

The analysis of aqueous extracts of *L. multiflora* by liquid chromatography-mass spectrometry (LC-MS) furnished a complex chromatographic profile presented in Figure 2. Peaks presenting an absorbance between 200 and 800 nm were only detectable during the first 25 minutes of analysis. More than 15 peaks were observable, but only eight of them were identified by mass spectrometry analysis.

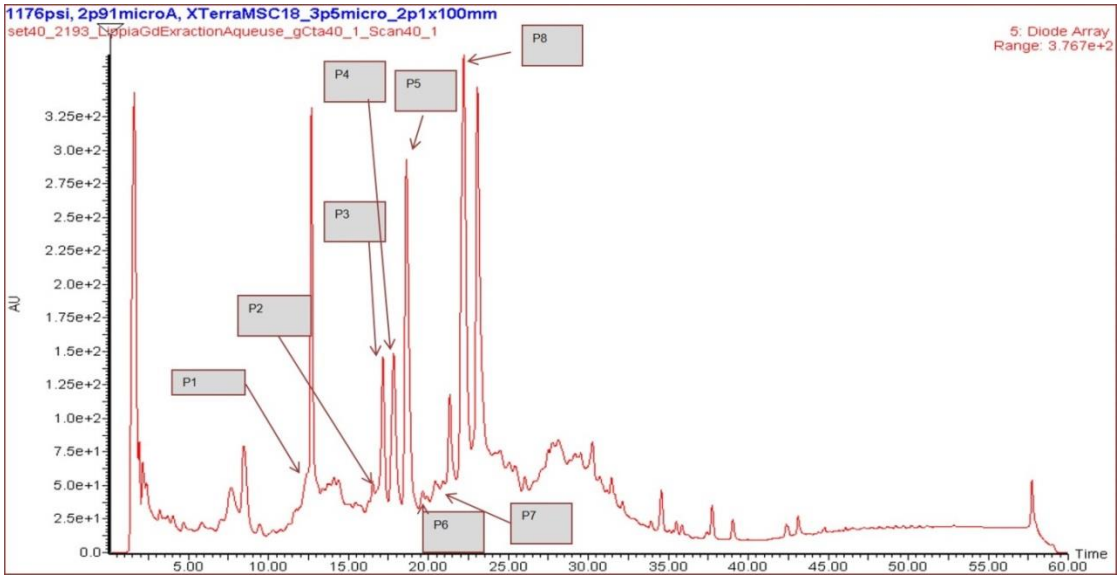


Figure 2: Complete chromatographic profile (absorbance between 200 and 800 nm) of *L. multiflora* aqueous extract.
Legend.Peaks of identified compounds.P1: Geniposide, P2: Nuomioside A, P3: Samioside, P4: Verbascoside, P5: Alyssonoside, P6: Isoverbascoside, P7: Isonuomioside, P8: Leucosceptoside A.

Concerning peak 1 (P1), analysis in negative electrospray (ES^-) and low fragmentation voltage (30V) mode showed the presence of a parent ion *m/z* 387.3 with a UV absorption spectrum showing a maximum at 284 nm and 326 nm. This peak corresponds to geniposide. This compound is a monoterpene from the iridoid family. It is composed of a cyclopentane merged to a heterocycle of 6 atoms among which one is oxygenated.

For the second peak (P2), analysis in negative electrospray and high fragmentation voltage (60V) mode allowed observing the presence of a parent ion *m/z* 609 and a fragment ion *m/z* 161. This compound was detected by UV absorption at 285 nm and 326 nm. This peak corresponds to nuomioside A (or calceolarioside E). The isomer of this compound, the isonuomoside, is also found in the extract and is represented by the peak 7 (P7) at the retention time of 20.42 min. Both compounds are phenolic compounds from the group of the phenylethanoid glucosides.

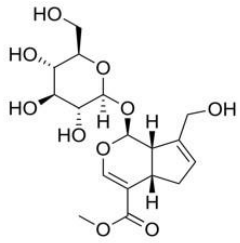
The compound corresponding to peak 3 (P3), at a retention time of 17.26 min, included a parent ion *m/z* 755 and fragment ions *m/z* 593.2 and 461 in mass spectrometry using negative electrospray mode (ES^-), and at high voltage (60 V). The UV absorbance spectrum showed a maximum at 290 nm and 330 nm. It corresponds to the samioside, a phenylethanoid glucoside.

The peak 4 (P4), at the retention time of 17.68 min., showed a parent ion *m/z* 623 and three major fragments ions *m/z* 461, 315 and 161, for a mass spectrometry analysis using negative electrospray mode (ES^-), at high voltage (60 V). The UV spectrum exhibited maxima at 288 nm and 330 nm. These characteristics indicate that this peak is the verbascoside or acteoside, another phenylethanoid glucoside.

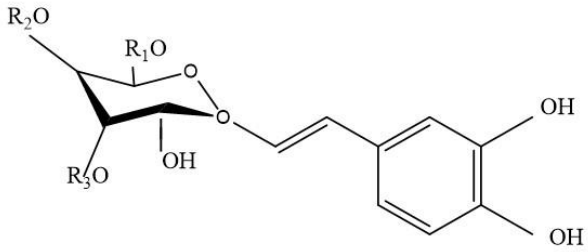
Peak 5 (P5), recorded at retaining time of 18.89 min, presented relative parent ion *m/z* 769 and fragment ions *m/z* 593 and 491 using negative electrospray mode (ES^-) at high voltage (60 V). The absorbance spectrum of this compound records peaks at 250 nm, 275 nm and 340 nm. This compound is identified as being the alyssonoside, another phenylethanoid glucoside.

Peak 6 (P6) obtained at retention time of 19.7 min., showed characteristics which are similar to the verbascoside: parent ion *m/z* 623 and two fragment ions *m/z* 167 and 345, and a UV absorbance showing two peaks at 228 nm and 327 nm. This compound is the isoverbascoside, an isomer of the verbascoside.

Peak 8 (P8), at a retention time of 22.1min., presented a parent ion *m/z* 637 and two fragment ions *m/z* 475 and 381in mass spectrometry analysis using negative electrospray mode (ES-) at low fragmentation voltage (30V). Its UV absorbance presented three maxima at 250 nm, 270 nm and 340 nm. This compound is the leucosceptoside A, a phenylethanoid glucoside. The chemical structures of identified compounds are presented in Figure 3.



Compound 1 : Geniposide



Compound 2: Nuomioside A (calceolarioside E, cusianoside A): R1 = H, R2 = (E)-caffeoyl, R3 = β-D-apioside

Compound 3: Samioside: R1 = H, R2 = (E)-caffeoyl, R3 = β -D-apiofuranoside

Compound 4: Verbascoside (acteoside): R1 = H, R2 = (E)-caffeoyl, R3 = α-L-rhamnoside

Compound 5: Alyssonoside: R1 = β -D- apiopyranoside, R2 = α- L-rhamnopyranoside, R3 = β-D-glucopyranoside

Compound 6: Isoverbascoside (isoacteoside): R1 = (E)-caffeoyl, R2= H, R3 = α-L-rhamnoside

Compound 7: Isonuomioside A: R1 = (E)-caffeoyl, R2 = H, R3 = β-D-apioside

Compound 8: Leucoseptoside A: R1 = H, R2 = (E)-caffeoyl, R3 = β-D-glucopyranoside

Figure 3: Chemical structures of the major compounds isolated from *L. multiflora* leaves

Phytochemical analysis of fractions obtained from Lippia multiflora aqueous extract

A separation has been carried out on solid phase to isolate the different compounds present in *Lippia multiflora* extract. Table 1 shows the extraction output and the chemical analysis results of the crude extract and different fractions. Most of the compounds were eluted by water, as the dry mass of the first fraction (FL1) represents 47% of the total dry mass. Verbascoside and leucoseptoside were present in each fraction. The fractionation helped obtain three fractions with few or no geniposide (FL2, FL3 and FL4). However, the technique was useful for three of the eight compounds: nuomoside A was concentrated in FL3, samioside in FL4 (a presence was also noticed in FL3) and alyssonoside in FL5.

Table 1: Phytochemical content of different *Lippia multiflora* leaf extracts obtained without (crude extract) or after fractionation (FL1, FL2, FL3, FL4 and FL5) on inverse solid phase

Fraction	Mass (g)	Percentage of total mass (%)	Identified compounds							
			geniposide.	nuomioside A	samioside	verbascoside.	alyssonoside	isoverbascoside	isonuomioside	leucosceptoside A
Crudeextract	0.5	100%	+++	++	+++	+++	+++	++	++	+++
FL1	0.2350	47%	++	nd	nd	+-	nd	nd	+-	++
FL2	0.1050	21%	++	nd	nd	++	nd	nd	++	+-
FL3	0.0200	4%	+-	++	+-	++	nd	nd	nd	+-
FL4	0.0140	2.8%	+-	nd	++	++	nd	nd	+ +	++
FL5	0.0105	2.1%	nd	nd	nd	++	+-	++	nd	++

+++: highly present; ++ : present, +- : trace ; nd :not detected
FL1: 100% water, FL2: 95/5 Acétonitrile/water, FL3: 90/10 Acétonitrile/water, FL4: 85/15 Acétonitrile/water; FL5 : 10/90 Acétonitrile/water

Except in fraction 5 (FL5), both iridoids and phenylethanoid glucosides were present. On the other hand, the aqueous fraction (100% H_20) FL1 recorded the highest percentage of iridoids in the fractioned total mass, or 47%. Only fraction FL3 pointed out the presence of nuomioside A among its eluted compounds. On the contrary, the verbascoside was present in all the fractions. Fraction FL5 revealed the presence of alyssonoside (as traces). Fractions FL4 and FL5 recorded the lowest gathered masses (about 2% of the total mass). The compounds not collected in the fractions, corresponding to the substances retained in the column, represent about 23% of the total mass.

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Effect of Lippia extracts on spore germination

In vitro efficiency of *L. multiflora* extracts on the inhibition of *P. personata* and *P. arachidis*spore germination are presented in Table 2. Whatever the spores, the aqueous fraction (FL1) showed the lowest inhibition effect on spore germination, even though the spores of *P. personata* and *P. arachidis*didnot demonstrate the samesensitivity to the treatments. In this way, the spores of *P. arachidis*seemed to be less sensitive to the applied treatments with an average inhibition rate varying from 22% to 39%, whereas those of *P. personata* varied from 33% to 58%. For the positive control (verbascoside FL6 and a mix verbascoside/ isoverbascoside (1:1), FL7), the same observation was done, the percentage of inhibition ranging from 62.5 to 66.7% and 38.9 and 61.1 % for *P. personata* and *P. arachidis*, respectively. For both fungi, FL3 fraction (also FL4 for *P. arachidis*) presented the highest efficiency rate.

Table 2: Efficiency rate of *L. multiflora* extract fractions (FL1, FL2, FL3, FL4 and FL5), standards (FL6 and FL7) and a negative control (FL8) on inhibition of *P. arachidis* and *P. personata* spores germination

Treatment	Efficiency rate of treatment on spore germination (%)	
	Phaeoisariopsis personata	*Puccinia arachidis*
FL1	33.33^{c}	5.56 ^{c}
FL2	50.00^{b}	33.33^{b}
FL3	58.33^{ab}	38.89^{b}
FL4	54.17^{ab}	33.33^{b}
FL5	45.83^{b}	22.22^{b}
FL6	66.67^{a}	61.11^{a}
FL7	62.50^{ab}	38.89^{b}
FL8	0.00^{d}	0.00^{d}
Probability	< 0.0001**	< 0.0001**

FL1: 100% water, FL2: 95/5 Acétonitrile/water, FL3: 90/10 Acétonitrile/water, FL4: 85/15 Acétonitrile/water; FL5: 10/90 Acétonitrile/water; FL6: verbascoside; FL7: verbascoside and isoverbacoside mixture; FZ8: negative control.Values are expressed in percentage and correspond to the mean of 5 measurements. **: highly significant different. Means followed by the same letter were not significantly different between treatment at $p \leq 0.05$ (Student Newman-Keul test).

Effect of Lippia extract fractions on disease evolution

This effect was measured evaluating the extension of late spot or leaf rust stains on leaves previously inoculated by *P. personata* and *P. arachidis*, respectively. As observed in Figure 4,

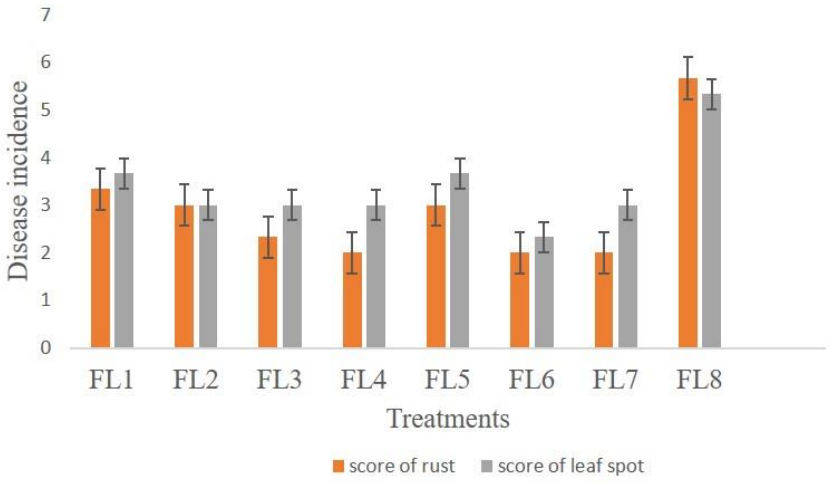

Figure 4: Extension of foliar diseases related to rust and late spot stains on peanut leaves artificially contaminated, in presence of *L. multiflora* extracts (FL1, FL2, FL3, FL4 and FL5), positive control (FL6 and FL7) and negative control (FL8). Error bars represent ± standard deviation of mean (n = 3). FL1 : 100% water, FL2 : 95/5 Acétonitrile/water, FL3 : 90/10 Acétonitrile/water, FL4 : 85/15 Acétonitrile/water; FL5 : 10/90 Acétonitrile/water; FL6 : verbascoside; FL7 : verbascoside + isoverbascoside; FL8: negative control. Extension is expressed using the 9 classes' severity scale of ICRISAT

FL8 (control) presented the highest evolution with a mark which is above 5.00 for both diseases. For leaf rust, the fractions FL3 and FL4 showed the lowest foliar necrosis development, with an average mark of 2.5 and 2, respectively. The effect of FL4 was similar to that obtained with the positive control FL6 and FL7. For late spot, the fractions FL2, FL3 and FL4 recorded the lowest evolutions of foliar stains with an average note of 3.00, equivalent to the positive control FL7 but inferior to the FL6 control.

Discussion

The phytochemical analysis carried out on *Lippia multiflora* leaves has revealed a high content and diversity in phenol compounds. However, most of the identified compounds have been previously described in *Lippia* or plants from the same group. Indeed, the nuomioside A identified in this sample has been found in species such as *Paraboeaglutinosa* (Wang *et al.* 2011) and *Lantana radula* (Oyourou *et al*. 2013), plants which belong to the same order as *Lippia multiflora*. In the

same way, the verbascoside and the isoverbascoside, compounds generally found in the plants from Lamiales order (Kirmizibekmez *et al,* (2005); Boudjelal *et al.* (2012)), have been isolated from *Lippia multiflora* (Hanson *et al.* 2011). The presence of these compounds has also been pointed out in another species of*Lippia*, *L. javanica* (Olivier *et al,* 2010). In other respects, samioside, alyssonoside and leucosceptoside A, other phenolics noted in this study, have been previously identified in plants from *Phlomis* genus, which belongs to the same order as *Lippia* genus (Kirmizibekmez*et al.* 2005). Moreover, as a great diversity was observed in the phenylethanoid glycoside content of the leaves of five *Phlomis*species (*Phlomisnissolii*, *P. leucophracta*, *P. bruguieri*, *P. russeliana* and *P. kurdika*), the authors proposed to use these compounds for the chemotaxonomy of the genus. The presence of leucosceptoside was previously reported in *Lippiadulcis* and *L. canescens* (Abe *etal*. 2002). Finally, this study allows confirming the synthesis of an iridoid, the geniposide, in species from *Lippia*genus, this compound being identified previously in *Lippiaalba* extracts (Hennebelle 2006). Isolated for the first time from *Gardenia jasminoides* (Inouye *et al*. 1969), this iridoid belongs to the monoterpene family, often described as a group of intermediates in alkaloid biosynthesis. The presence of iridoids has been observed in several medicinal plants from Rubiaceae (*Gardenia* and *Coffea*genus), Lamiaceae (*Melissa* and *Thymus* genus) or Verbenaceae (*Lippia* and *Verbena* genus) families. Several biological properties, notably antimicrobial (antifungal and antibacterial) (Nitiema *et al*. (2012); Yala *et al.* (2016)), anti-inflammatory and antiseptic properties (Coulibaly, 2012), are attributed to phenolic compounds.

The antifungal activity of the aqueous extract of *Lippia multiflora* against two peanut pathogens have been tested by two techniques and using the crude extract or the different factions obtained by its fractionation. The different responses obtained between plant extracts, controls, and standard molecules testify that *L. multiflora* leaf extracts possess antifungal activity attributable to the presence of some of the phenolic compounds accumulated. In order to identify what compounds were the most effective against fungi, the crude extract was fractionated on an inverse solid phase. Certainly because of their close molecular relationship which can confer nearby physicochemical properties to the molecules, this technique appeared to be poorly useful for the individualization of the compounds. Results indicated that the aqueous fraction (FL1) contains most of the identified molecules, except nuomioside A, samioside, alyssonoside and isoverbascoside, and represents 47% of the total mass of the crude extract. However, the chemical composition of the intermediate fractions FL2, FL3 and FL4 appeared richer, even though relatively close, than that of FL1 and FL5 fractions. FL3 and FL4 fractions present the most original biochemical profile. FL3 appeared as the only one extract that contain nuomioside A and FL4 seemed to be particularly rich in samioside, leucoseptoside A and isoniomoside. Interestingly, it is in these two fractions that the stronger efficiency against fungi was recorded. Associated with verbascoside (its antifungal activity is shown by the FL6 activity), these four compounds could, taken alone or in combination, play a major role in the antifungal activity of *Lippia multiflora* extract. Antifungal properties of some compounds identified in *Lippia multiflora* aqueous extract have been tested by studies before ours. In this way, the verbascoside has been subject of in-depth investigations and its antifungal properties have been confirmed. It would give a higher inhibition when applied at pure state. Recent studies on this molecule have permitted to extract it from *Lippia javanica* and *Lantana camara* leaves which would be an excellent source with possibilities of collection up to 83 mg per gramme of dry leaves (Oyourou *et al*. 2013). Besides, these authors report an antifungal efficiency of this molecule against *Penicillium digitatum*, a fungus causing citrus fruits rotting. A concentration of 1g/l would involve an inhibition of the disease between 90 and 100%. These results agreed with ours in which the verbascoside, represented by FL6, holds the record in the germination inhibition of *Phaeoisariopsis personata* (66.67%) and *Puccinia arachidis* (61.11 %) and stops the foliar stain evolution. The fractionation allowed obtaining the presence of this compound in an evident way in FL2, FL3, FL4 and FL5 as a whole and only as trace in FL1. Fractions FL2, FL3, FL4 and FL5 present nearly the same kinetics of activity. The presence of the verbascoside in these fractions could explain partially the correlation with the antifungal activity observed. This hypothesis has also been formulated by Shikanga *et al*. (2009) whose fractions of *Lippia javanica* and *Lippia rhmanii* enriched with verbascoside have given the highest inhibitions of *Penicillium digitatum* growth. However, the verbascoside stability has been studied by Vertuani*et al.* (2011) and Oyourou *et al*. (2013). According to these authors, the compound would be instable under solar light, except when maintained in acidic environment (pH5). This result indicates that verbascoside cannot be used as a natural antifungal agent.

Other compounds identified in *Lippia multiflora* aqueous extract have been described as antifungal by several authors. The geniposide, one of the iridoids identified in *Lippia multiflora* is part of them. In *Gmelinaarborea* (Verbenaceae), it has been reported by Kawamura and Ohara (2005) that an iridoid has an antifungal activity against *Trametes versicolor*, vector of the wood white rot. In the same way, Da Silva *et al.* (2007) have shown the inhibition of *Cladosporium sphaerospermum* and *C. cladosporioides* byiridoids derived from *Alibertia sessilis.* In our study, it can be noted that the most active fractions (FL3 and FL4) have a low iridoid content, indicating a weak effect of this compound against peanut pathogens.

Conclusion

The antifungal activity of aqueous extracts from *Lippia multiflora* Moldenke leaves on *Phaeoisariopsis personata* and *Puccinia arachidis* are reported for the first time. The inhibitory effects of aqueous extract can be attributed to active chemical composites as phenolic compounds or monoterpene within this plant.

In order to specify the role of each of the different compounds accumulated in *Lippia multiflora* leaves, *in vitro* and *in situ* studies are planned, using aqueous solutions, at different concentrations, of samioside, leucoseptoside or isoniomoside, mixed or not with verbascoside. Overall, results obtained in this study

underlined the possible used of aqueous extract of *L. multiflora* by smallholder farmers to several fungi diseases.

Acknowledgements

This work was supported by the project RESEAU (Renforcement de l'Enseignement Supérieur et de l'Action Universitaire) of the Franco-Burkinabe University Cooperation.

Authors acknowledge the laboratory of Biochemistry and Plant Physiology (University of Montpellier II) for allowing to carry out the phytochemistry.

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