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ORIGINAL ARTICLE

Phytochemical potential of *Ficus* species for the control of the phytonematode *Meloidogyne javanica*

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Abstract

Root-knot nematodes, genus *Meloidogyne,* are among the most plant damaging pathogens worldwide. The action of natural products against plant pathogens has been investigated to assess their effectiveness in the control of diseases. Thus, the present study aimed to evaluate the phytochemistry potential of the *Ficus* species for the control of *Meloidogyne javanica*. *In vitro* inhibitory activity assays were performed with crude ethanolic extracts of leaves and branches from 10 *Ficus* species. Among these, *Ficus carica* extracts exhibited strong paralysis activity against second stage juveniles (J2) (EC_{50} = 134.90 μg · ml^{-1}), after 72 hours. In addition, high efficacy was observed in egg-hatching inhibition at different embryonic stages. Microscopy analysis revealed severe morphological alterations in the nematode tissues at the J2 stage, as well as immotility of juveniles released from eggs in the presence of *F. carica* extracts. The efficacy of the treatments for the other species was very low. These differences were supported by the variation in the compound classes, mainly for alkaloids and metabolite profiles by Gas Chromatography/Mass Spectrometry (GC/MS) when *F. carica* was compared with the other species. The results indicated that *F. carica* is a promising source for the isolation and identification of molecules capable of acting in the control of *M. javanica.*

Keywords: gas chromatography/mass spectrometry (GC/MS), metabolite profiling, pest control, phytochemistry

Introduction

Alternative sources of pesticides for pest control are among the main challenges for the establishment of sustainable agriculture. The application of synthetic and highly toxic compounds is the main form of pest management for several agricultural crops. However, inappropriate and prolonged use of these substances can jeopardize the ecosystem, as well as human health (Nicolopoulou-Stamati *et al.* 2016). In Brazil, which is the largest consumer of pesticides in the world, the use of agrochemicals in agriculture is a public health problem, given the contamination of the environment, foods and human intoxications (Pignati *et al.* 2017). It is estimated that more than 50% of the world's synthetic nematicides is used to control nematodes of the genus *Meloidogyne*, also known as root-knot nematodes. *Meloidogyne javanica* is one of the most important pathogens affecting agricultural crops (Jones *et al.* 2013). Thus, alternatives for the management of this phytonematode must be developed.

In this context, the bioprospection of natural products could generate alternative sources to replace conventional pesticides. Therefore, it is an important tool for new environmentally friendly bioactive molecules (Cantrell *et al.* 2012). The use of natural products for biological pest control, such as plant metabolites, fungal and bacterial products, has already been reported (Ntalli and Caboni 2012; Lee *et al.* 2013; Caboni *et al.* 2015; Gouveia *et al.* 2017; Sufiate *et al.* 2017).

In the search for alternatives to replace synthetic agrochemicals, strategies based on the use of biodiversity should be considered as sources of new bioactive compounds, such as from the Brazilian Atlantic Forest. This is the case for many species of the genus *Ficus* spp., which belong to the Moraceae family, popularly known as "worm control agents" for their anthelmintic properties (Amorin *et al.* 1999). The diversity of this genus in Brazil has been described (Berg and Carauta 2002) including native and exotic species of *Ficus*. However, few studies have explored the phytochemical properties of these species.

The anthelmintic activity of the *Ficus* species has been reported since antiquity. Thus, many studies have focused on its medicinal properties. However, its use for pest control in agriculture has been limited (Mawa *et al.* 2013). Extracts from *Ficus* spp. and other members of the family Moraceae have been applied for the control of phytonematodes (Ahmad and Siddiqui 2009; Liu *et al.* 2011). However, their use for the control of *M. javanica* has not been evaluated. Thus, the application of metabolomic approaches could also be used for the identification of compounds present in these species. With the use of gas chromatography/mass spectrometry (GC/MS) based platforms, numerous metabolites can be identified in a single chromatographic run. The use of metabolomic technologies has been crucial in the discovery of bioactive natural products (Cox *et al.* 2014). Therefore, techniques such as mass spectrometry emerge as powerful tools in the identification of new molecules of agronomic interest, which also contribute to the expansion of the knowledge about the Brazilian genetic patrimony.

In order to understand nematotoxic properties and the development of alternative methods for the control of phytonematodes, the toxicities of extracts of the *Ficus* species were evaluated, as well as their potential as a source of bioactive compounds. In addition, metabolite profiles of the 10 species were evaluated by GC/MS. *Ficus carica* branch extracts significantly reduced the motility of J2s and the hatching of nematode eggs and presented distinct metabolite profiles. Therefore, bioactive molecules present in *F. carica* constitute an alternative to be explored for the control of *M. javanica*.

Materials and Methods

Plant material

Ten species of *Ficus* were collected from stretches of the Atlantic Forest located in Viçosa-MG. The plant materials were processed for the separation of leaves and branches, followed by fresh weight determination. The samples were submitted to the dehydration process in a ventilated oven for about 72 h, at 40°C. Subsequently, the dried samples were pulverized in a knife mill. The dry weight was determined and the samples were stored at –80°C. Specimens of the collected plant materials are deposited in the UFV herbarium and the overall information is available at the Species Link platform, under the VIC numbers described in Table 1 (http://splink.cria.org.br/).

Metabolite extraction from leaves and branches

The pulverized plant tissues were submitted to extraction in a percolator, at room temperature, from 150 g dried tissues in 1.5 l of ethanol 100%. The extracts generated from the percolator were immediately concentrated, using a rotary evaporator under vacuum, at 40°C and then lyophilized and kept at –80°C. The yield of the crude extracts obtained was calculated, as described in Table 2.

Preparation of extracts for biological assays

Stock solutions (3 mg · ml^{-1}) were prepared from 30 mg of the lyophilized ethanolic extracts in 10 ml autoclaved deionized water containing 2.5% (v/v) DMSO (dimethyl sulfoxide) and Tween 20, 2.5%. Then, they were vortexed for 60 s and kept in an ultrasonic bath, at room temperature for 1 h. After solubilization,

Table 1. *Ficus* species obtained from the Brazilian Atlantic Forest. The geographic coordinates and the VIC numbers (Herbarium codes of Universidade Federal de Viçosar – UFV) are indicated

Ficus species	VIC	Geographic coordinates	
F. adhatodifolia	31644	20°45'40.2"(S)	42°52'14.1"(W)
F. arpazusa	40259	20°48'05.7"(S)	42°51'39.5"(W)
F. benjamina	31702	20°45'37.8"(S)	42° 51'54.1"(W)
F. carica L.	31703	20°45'44.1"(S)	42°51'07.5"(W)
F. elástica	22434	20°45'19.1"(S)	42°52'25.5"(W)
F. eximia	22435	20°48'02.1"(S)	42°51'52.0"(W)
F. glabra	37043	20°46'03.6"(S)	42°51'53.0"(W)
F. gomelleira	31667	20°46'25.0"(S)	42°52'29.8"(W)
F. mexiae	32923	20°45'14.0"(S)	42°52'54.8"(W)
F. microcarpa	31464	20°45'27.3"(S)	42°52'30.0"(W)

Table 2. Yield data of plant material and extracts of *Ficus* spp.

Ficus species	Parts of plant	Fresh vegetable material[1] [g]	Dehydrated vegetable material[2] [g]	Yield [%]	Plant drug[3] [g]	Extract [g]	Yield [%]
F. adhatodifolia	L	4,810	1,120	23.28	150	8.06	5.37
	B	3,210	645	20.37	150	3.45	3.50
F. arpazusa	L	1,375	360	26.18	150	9.25	6.17
	B	1,485	460	31.00	150	3.90	2.60
F. benjamina	L	1,916	1,110	57.94	150	11.01	7.40
	B	1,800	955	53.05	150	3.72	2.50
F. carica	L	2,075	460	22.17	150	17.41	26.11
	B	1,015	205	20.20	150	7.82	11.73
F. elastica	L	3,770	1,125	29.84	150	9.35	6.24
	B	2,130	715	33.57	150	10.65	7.10
F. eximia	L	5,170	930	18.00	150	5.64	3.76
	B	2,605	840	32.24	150	1.57	1.04
F. glabra	L	2,500	695	27.80	150	5.83	3.88
	B	1,800	530	29.40	150	3.92	2.60
F. gomelleira	L	2,395	878	36.67	150	15.56	10.40
	B	2,660	911	34.25	150	6.90	4.60
F. mexiae	L	2,955	925	31.30	150	3.93	4.93
	B	2,960	915	30.91	150	4.53	4.50
F. microcarpa	L	4,045	1,170	28.90	150	17.56	7.02
	B	1,348	540	40.05	150	34.26	13.70

L – leaf; B – branch; [1]total plant material; [2]total plant material after drying; [3]dry plant material used for extractions

the stock solutions were kept at –80°C. For the biological assays, the extracts were centrifuged at 14,000 g, for 10 min, and the supernatants were filtered through a PVDF 0.22 µm membrane (millipore filter) and then diluted to obtain metabolite concentrations of 500, 1,000 and 1,500 µg · ml^{-1}.

Production of eggs and juveniles of second stage (J2) *M. javanica*

Meloidogyne javanica eggs were obtained from the roots of infected tomato plants from the Laboratory of Biological Control of Phytonematodes (BIOAGRO, UFV). Suspensions of 5,000 eggs per 15 ml of water were prepared to be applied on the soil around the roots of 20-day-old tomato seedlings of *Solanum lycopersum*. The plants were kept in 2 l pots containing a mixture of clayey soil, sand and commercial substrate at a proportion of 1 : 1 : 2 (v/v), previously treated with Basamid® fumigant. Inoculated tomatoes were kept in the greenhouse for 60 days and the eggs produced were extracted from the roots using the centrifugal flotation method in sucrose solution (2.5%), according to Jenkins (1964). This inoculation procedure was repeated every 45 days for the periodic production of *M. javanica* eggs used in the *in vitro* assays.

To obtain the second-stage juveniles (J2), a suspension containing eggs was carefully poured into a filter paper system over a sieve, which allowed constant contact with sterilized water (hatch chamber). The hatching chamber was then kept under the controlled temperature of 28°C, for 48 h. The juveniles retained in a 500 mesh sieve were collected by gently washing with sterilized water.

In vitro activity inhibition assays

Evaluation of egg hatching and juvenile movements in the presence of the metabolite extracts from the 10 *Ficus* species were determined *in vitro* in 96-well plates. One hundred microliters of sterilized deionized water containing 30 eggs or juveniles J2 were added to each well. Then, an aliquot of 100 µl of each metabolite extract was added at the following concentrations: 500, 1,000 and 1,500 µg · ml^{-1}. The negative controls were composed of pure water (H_2O), water + DMSO + Tween 20

(1.25%) (WDT) and, as a positive control, the commercial nematicide Rugby 200 was used at a final concentration of 500 µg · ml^{-1}. The plates were wrapped with PVC and aluminum films and kept at 28°C for the entire evaluation period. Four replicates were used for each treatment and the experiment was performed twice. The experimental design considered for statistical analysis was completely randomized.

Paralysis activity assays

The J2 paralysis activity was evaluated at 24, 48 and 72 h after the addition of the extracts. The plates were evaluated using an inverted optical microscope (EVOS FL), 10X magnification. The J2s were separated into two distinct categories: with motility (M) and without motility (I). Juveniles without motility presented a stiff body, stretched as a straight line and were considered paralyzed. The percentage of paralyzed J2s (J2P) was determined by the following equation:

$$\text{J2P} = \frac{\text{number of J2P}}{\text{J2 total number}} \times 100\ [\%].$$

Evaluation of egg-hatching inhibition

In this assay, the eggs were used in different embryonic stages. The evaluations were performed after 2, 4, 6 and 8 days of exposure of the eggs to the plant extract. Cumulative percentage values were obtained by direct counting of hatched eggs in each treatment, according to the formula below:

$$\text{Cumulative percentage of hatching eggs} = \left(\text{total eggs} - \frac{\text{hatched eggs}}{\text{total eggs}}\right) \times 100\ [\%].$$

Metabolic profiling by GC/MS

Aliquots of 50 mg of lyophilized extracts were solubilized in 1.5 ml of cold extraction solution (1 : 2.5 : 1 water, methanol, chloroform) containing 60 µl of ribitol (0.2 mg · ml^{-1} stock in water) as an internal standard. Metabolic extraction and derivatization were performed following Lima *et al.* (2019).

Raw GC/MS data were processed and converted for the CDF format (Computable Document Format) automatically analyzed by TargetSearch algorithms as described by Lima *et al.* (2019). The following parameters were used: massRange of 85–500 Da, IntThreshold 50, TopMasses 10, thresh of 0.05. A filtered identification table, for a quality factor index over 600, containing the identification compounds and their intensities, was used as input data for processing and statistical analysis by MetaboAnalyst platform (http://www.metaboanalyst.ca/).

Raw data converted to the CDF format (NetCDF) were also used to compare the GC/MS Total Ion Chromatograms (TICs) from the 10 *Ficus* species by XCMS platform (https://xcmsonline.scripps.edu). This approach made it possible to compare the TICs that were not identified by TagertSearch approach.

Data processing and statistical analysis

The percentages of paralyzed J2 were corrected by data from H_2O controls, to eliminate the natural death/paralysis, according to the Schneider Orelli's formula (Puntener 1981):

$$\text{Correction} = \frac{\text{\% of paralysis in treatment} - \text{\% of paralysis in control}}{100 - \text{\% of mortality in control}} \times 100\ [\%].$$

The values obtained were initially analyzed by analysis of variance (ANOVA) for the assessment of the effect of the incubation times. When the interaction was non-significant, averages were calculated in both experiments. Thus, the mean values of eight replicates (four from each experiment) were submitted to linear regression analysis, according to the Probit Analysis method, proposed by Finney (1971), to estimate the concentration that causes the paralysis of 50% of J2s in solution (EC_{50}). The 95% confidence interval was adopted, given $p < 0.05$. In general, the same statistical approach described above was adopted for the egg hatch inhibition assays. However, data from eggs hatched in solution were represented as reduction percentages relative to the number of J2s recovered from the H_2O control, according to Abbott's formula:

$$\text{Correction} = \left(1 - \frac{\text{J2 in the treatment}}{\text{J2 in the control}}\right) \times 100\ [\%].$$

In this case, the mean values of eight replicates (concentration and immersion period) were used to estimate the concentration that reduces the number of J2s in solution (EC_{50}) in 50%.

Results

Mortality of J2 *Meloidogyne javanica* by *Ficus* extracts

The paralysis activities of *M. javanica* juveniles caused by extracts from leaves and branches of 10 species of *Ficus* were determined and the EC_{50} values were calculated (Table 3) to assess which genotypes or tissues showed inhibitory activities. After 48 h, only the branch extracts from *F. carica* showed significant effects on the

Table 3. EC_{50} values of the paralysis assays of *Meloidogyne javanica* J2 in the presence of *Ficus* extracts after 24–72 h

Ficus species	Plant material	Incubation times [h]											
		24				48				72			
		EC_{50} [µg · ml]	R^2	$b_{EC_{50}}$	95%CI	EC_{50} [µg · ml]	R^2	$b_{EC_{50}}$	95%CI	EC_{50} [µg · ml]	R^2	$b_{EC_{50}}$	95%CI
F. adhatodifolia	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. arpazusa	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500			–	>1,500			–
F. benjamina	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. carica	L	>1,500	–	–	–	>1,500	–	–	–	1,202.26	0.99	0.82	1,201.86–1,203.50
	B	1,548.82	0.9969	1.09	1,548.50–1,550.70	371.54	0.83	2.02	362.07–385.05	134.9	0.97	1.64	133.09–140.00
F. elastica	L	>1,500	–	–	–	>1,500	–	–	–	1,548.82	0.86	2.54	1,538.30–1,564.41
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. eximia	L	>1,500	–	–	–	>1,500	–	–	–	1,479.11	0.87	2.24	1,470.19–1,492.52
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. gomelleira	L	>1,500	–	–	–	>1,500	–	–	–	>1,500			–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. glabra	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. mexiae	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. microcarpa	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–

L – leaf; B – branches; EC_{50} – effective concentration; $b_{EC_{50}}$ – angular coefficient; R^2 – coefficient of determination; 95%CI – confidence interval, p = 95%

motility of J2s (EC_{50} = 371.6 µg · ml^{-1}), causing paralysis of movement. After 72 h of incubation, the branch extracts of this species were also the most effective (EC_{50} = 134.90 µg · ml^{-1}), as well as their leaf extracts (EC_{50} = 1,202.26 µg · ml^{-1}). The extracts from *F. elastica* leaves also presented inhibitory activity (EC_{50} = 1,479.11 µg · ml^{-1}), but were less effective (Table 3).

In the presence of *Ficus* spp. extracts, especially for 1,500 µg · ml^{-1} *F. carica* branches, J2 motility was altered 24 h after exposure. There was a rate of 25.43 ± 1.86% of paralyzed J2s, which was higher than the rates of the negative controls (H_2O and WDT were only 4.27 ± 0.38% and 6.17 ± 0.40%, respectively). For the doses of 1,000 µg · ml^{-1}, only treatments based on the extract from *F. carica* branches had almost 20% reduction (19.26 ± 0.16%), which was not observed in any treatment in which 500 µg · ml^{-1} were applied. The positive control, Rugby 200, showed 100% of J2 paralysis in solution for all conditions (Table 4).

After 48 h, *F. carica* extract resulted in the highest paralysis percentage among all treatments and tested concentrations. At 1,500 µg · ml^{-1}, the extract from the branch was the most effective and induced the paralysis of 87.37 ± 3.30% of J2s, followed by 46.81 ± 2.41% obtained by the extract from its leaves. With the same concentrations, leaf extracts from *F. elastica* and *F. gomelleira* showed paralysis percentages slightly above 30% and from *F. arpazusa*, *F. eximiae* and *F. glabra,* above 20% (Table 4). At 1,000 µg · ml^{-1}, J2 paralysis for the *F. carica* extract was the highest of all the treatments (88.97 ± 4.40%), as observed for 1,500 µg · ml^{-1}. Other inhibitions were also exhibited by the leaf extract, also from *F. carica* (39.30 ± 0.76%), followed by the leaves from *F. gomelleira* (28.57 ± 0.63%). The extract from the *F. carica* branches was also the most effective at 500 µg · ml^{-1} (60.86 ± 6.33%), followed by the extract from the leaves of the same species (37.24 ± 1.06%) and the leaves of *F. gomelleira* (23.11 ± 1.35%) (Table 4).

Ficus carica extracts caused the greatest inhibition of the J2 *M. javanica* motility at 72 h (Fig. 1). At 1,500 µg · ml^{-1}, the extract from its branches induced 96.48 ± 5.00% paralysis, while the negative controls obtained only 13.40 ± 1.26% (H_2O) and 16.23 ± 2.75% (WDT). The inhibition was also high for 1,000 µg · ml^{-1} (92.40 ± 5.46%) and at 500 µg · ml^{-1} (84.86 ± 6.12%). Leaf extracts were also effective: 55.24 ± 0.03% (1,500 µg · ml^{-1}), 54.19 ± 6.61% (1,000 µg · ml^{-1}) and 44.93 ± 1.27% (500 µg · ml^{-1}).

Higher concentrations of the extracts also produced inhibitory effects on other genotypes, such as the leaves and branches of *F. elastic* (60.63 ± 1.70% and 41.18 ± 2.65%, respectively), *F. eximiae* (48.83 ± 2.89% and 38.94 ± 2.13%), at 1,500 µg · ml^{-1}, and leaves of *F. eximiae*, *F. gomelleira*, *F. mexiae* and *F. glabra* (48.83 ± 2.88%, 46.03 ± 2.15%, 42.24 ± 4.05%, 40.30 ± 2.47%) (Table 4).

Inhibition of *Meloidogyne javanica* egg-hatching by *Ficus* spp.

The highest inhibitions of *M. javanica* egg-hatching were obtained by *F. carica* leaf and branch extracts (EC_{50} = 469.51 µg · ml^{-1} and EC_{50} = 562.34 µg · ml^{-1}, respectively), after 2 days of exposure. However, this effect was also observed, to a lesser extent for *F. elastic* (EC_{50} = 1,512.03 µg · ml^{-1}), *F. glabra* (EC_{50} = 1,348.96 µg · ml^{-1}) and *F. glabra* leaf extracts (EC_{50} = 1,412.54 µg · ml^{-1}) (Table 5). After 4 days, *F. carica* branch extracts showed an inhibitory activity slightly higher than that of its leaves (EC_{50} = 588.84 µg · ml^{-1} and EC_{50} = 501.19 µg · ml^{-1}, respectively), which were followed only by *F. elastica* (EC_{50} = 1,258.93 µg · ml^{-1}) (Table 5). This difference between the inhibition observed for branches and leaves of *F. carica* increased after 6 days [EC_{50} = 436.52 and 616.60 µg · ml^{-1}, respectively (Table 5)]. After 8 days, the leaf extract of *F. carica* exhibited the highest efficiency among all treatments (EC_{50} = 501.19 µg · ml^{-1}), especially for branch extracts (EC_{50} = 630.96 µg · ml^{-1}). *Ficus elastica* leaf extract also showed efficacy (EC_{50} = 1,412.54 µg · ml^{-1}), but its activity was significantly lower than that observed for *F. carica* (Table 5).

The cumulative percentages of eggs hatched in the presence of *Ficus* spp. extracts are presented in Table 5. After 2 days, the values of 2.44 ± 2.82% and 2.53 ± 3.04% were verified for branches and leaves of *F. carica* (1,500 µg · ml^{-1}), respectively, while the negative controls H_2O and WDT obtained, respectively, 16.73 ± 2.86% and 15.88 ± 0.36%.

On the fourth day, only 2.50 ± 2.26% of the eggs hatched in the presence of the extract of *F. carica,* at 1,500 µg · ml^{-1}, which was close to the value observed for 1,000 µg · ml^{-1} (5.35 ± 3.72%) and lower than that obtained at 500 µg · ml^{-1} (11.40 ± 7.36%). All of these were significantly different than the controls H_2O (26.28 ± 9.97%) and WDT (23.4 ± 5.14%). For extracts from other genotypes, inhibition of egg-hatching was observed using extracts from leaves and branches of *F. elastica* (11.96 ± 2.62% and 16.81 ± 4.85%), *F. arpazusa* (15.60 ± 3.60) an *F. gomolleira* (11.96 ± 4.24%) (Table 6).

After 6 days the percentages of eggs hatched were 4.89 ± 3.30% and 6.97 ± 3.27%, in the presence of *F. carica* (1,500 µg · ml^{-1}) branch and leaf extracts, respectively. These inhibitory effects were also significant with 1,000 µg · ml^{-1} and 500 µg · ml^{-1}, when compared with the controls. Some inhibition was observed for *F. gomelleira* and *F. elastica*, but to a lesser extent (Table 6). Although controls showed reduced hatching 8 days after the start of the assays, the inhibition of *F. carica* extracts reached 90% (16 ± 4.40% of hatched eggs) for both branches and leaves. Inhibition percentages were also significant even for low concentrations of *F. carica* extracts, which indicates their efficiency in

Table 4. Paralysis activity (%) of *Ficus* spp. extracts on second stage juveniles (J2s) of *Meloidogyne javanica*

Ficus species	Plant material	Incubation time [h]																	
		24						48						72					
		H_2O[a]	H_2O + + DMSO[b]	R[c]	1,500 [µg · ml^{-1}]	1,000 [µg · ml^{-1}]	500 [µg · ml^{-1}]	H_2O[a]	H_2O + + DMSO[b]	R[c]	1,500 [µg · ml^{-1}]	1,000 [µg · ml^{-1}]	500 [µg · ml^{-1}]	H_2O[a]	H_2O + + DMSO[b]	R[c]	1,500 [µg · ml^{-1}]	1,000 [µg · ml^{-1}]	500 [µg · ml^{-1}]
F. adhatodifolia	L	0.00	3.44	100	2.88	3.27	2.12	5.49	3.44	100	12.38	8.44	8.26	10.98	8.62	100	35.46	16.37	14.97
	B				5.39	2.23	0.79				10.71	8.73	7.91				26.53	13.54	13.33
F. arpazusa	L	2.08	2.27	100	8.99	8.60	6.80	6.54	9.30	100	21.22	17.98	17.82	13.39	16.23	100	39.24	21.98	19.62
	B				9.14	8.13	7.82				18.28	15.94	12.81				27.21	20.90	17.27
F. benjamina	L	1.47	3.39	100	5.37	3.83	2.40	7.53	8.58	100	14.49	10.46	8.89	9.00	13.42	100	27.05	24.24	23.87
	B				6.76	5.38	2.29				14.48	10.73	8.44				24.60	21.80	21.58
F. carica	L	1.51	4.00	100	15.92	12.47	8.72	8.24	8.00	100	46.81	39.30	37.24	11.62	8.00	100	55.23	54.19	44.93
	B				25.43	19.26	12.35				87.37	88.97	60.85				96.48	92.39	84.86
F. elastica	L	3.99	3.33	100	13.04	7.51	5.41	3.99	8.09	100	32.70	19.55	16.77	9.71	9.76	100	60.63	29.94	21.26
	B				8.329	8.39	5.90				18.74	17.56	13.52				41.18	23.93	20.80
F. eximia	L	2.17	5.08	100	9.607	6.97	5.91	3.78	5.08	100	24.95	19.23	14.01	3.78	5.08	100	48.83	29.63	20.10
	B				6.524	5.41	5.05				15.28	14.70	12.03				38.94	23.74	17.52
F. gomelleira	L	4.27	6.16	100	14.91	11.28	9.97	4.27	6.16	100	32.78	28.57	23.11	6.27	7.63	100	46.03	38.05	27.42
	B				11.64	10.24	8.95				23.61	21.74	18.69				37.04	31.03	27.01
F. glabra	L	0.00	0.00	100	10.51	5.44	1.39	0.00	0.00	100	21.22	10.32	7.43	3.51	4.00	100	40.30	30.74	17.69
	B				6.233	5.50	3.49				16.39	10.71	6.94				26.11	25.39	18.66
F. mexiae	L	2.00	2.50	100	6.869	6.35	6.10	2.00	4.16	100	14.50	9.75	7.51	4.38	4.16	100	42.24	22.54	14.08
	B				5.609	4.77	4.55				10.64	9.32	7.60				29.43	17.78	9.81
F. microcarpa	L	0.00	1.51	100	4.84	2.83	2.74	0.00	1.51	100	15.40	8.56	6.35	1.61	1.51	100	32.62	21.45	14.80
	B				5.04	3.92	1.86				11.87	12.16	10.91				27.18	19.06	14.43

L – leaf; B – branch; [a, b]negatives controls; [c]positive control RUGBY 200; DMSO – dimethyl sulfoxide

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Table 5. EC_{50} values of the inhibition of the egg hatching of *Meloidogyne javanica* in the presence of *Ficus* extracts after 8 days

Ficus species	Plant material	Incubation time [days]															
		2				4				6				8			
		EC_{50} [µg · ml^{-1}]	R^2	$b_{EC_{50}}$	95%CI	EC_{50} [µg · ml^{-1}]	R^2	$b_{EC_{50}}$	95%CI	EC_{50} [µg · ml^{-1}]	R^2	$b_{EC_{50}}$	95%CI	EC_{50} [µg · ml^{-1}]	R^2	$b_{EC_{50}}$	95%CI
F. adhatodifolia	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. arpazusa	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. benjamina	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. carica	L	469.51	0.93	2.01	464.62–478.42	588.84	0.94	1.65	585.37–595.62	616.6	0.98	1.50	615.15–621.06	>1,500	0.97	1.40	499.60–505.56
	B	562.34	0.95	2.54	557.31–572.45	501.19	0.99	2.40	501.11–501.27	436.52	0.99	1.68	434.99–438.34	630.96	0.94	1.96	626.85–638.99
F. elastica	L	1,512. 03	0.99	2.70	1,510.01–1,515.48	1,258.93	0.99	2.90	1,256.60–1,262.39	1,380.38	0.9	2.16	1,373.50–1391.58	1,412.54	0.98	3.18	1,411.48–1,419.96
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. eximia	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. gomelleira	L	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
	B	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–	>1,500	–	–	–
F. glabra	L	1,412.54	0.99	2.14	1,411.89–1,416.17	>1500	–	–	–	>1500	–	–	–	>1,500	–	–	–
	B	1,348.96	0.96	1.90	1,345.87–1,355.84	>1500	–	–	–	>1500	–	–	–	>1,500	–	–	–
F. mexiae	L	>1500	–	–	–	>1500	–	–	–	>1500	–	–	–	>1,500	–	–	–
	B	>1500	–	–	–	>1500	–	–	–	>1500	–	–	–	>1,500		–	–
F. microcarpa	L	>1500	–	–	–	>1500	–	–	–	>1500	–	–	–	>1,500		–	–
	B	>1500	–	–	–	>1500	–	–	–	>1500	–	–	–	>1500		–	–

L – Leaf; B – branches; EC_{50} – effective concentration; $b_{EC_{50}}$ – angular coefficient; R^2 – coefficient of determination; 95%CI – confidence interval; p = 95%

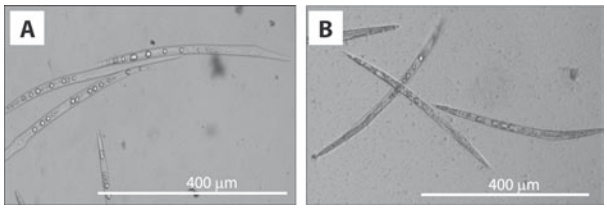

Fig. 1. Analysis by optical microscopy of the *Meloidogyne javanica* J2 paralyzed by the presence of *Ficus carica* branch extracts (1,000 µg · ml^{-1}) after 72 h

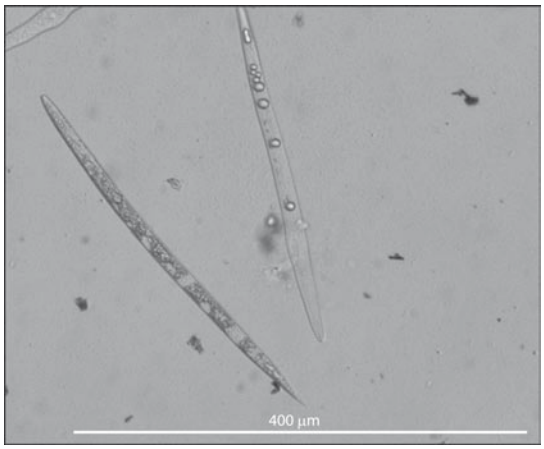

Fig. 2. Detailed morphological arrangement of J2 in solution considered paralyzed during the all evaluations

egg-hatch control. In addition to the high inhibition of egg-hatching, most J2s that managed to exit the eggs were observed paralyzed close to the empty eggs only in the presence of the *F. carica* branch extracts (Fig. 2 and Fig. 3).

Metabolomic profiling of *Ficus* spp. by GC/MS

The aqueous extracts used in the biological assays were derivatized and subjected to GC/MS analysis for the identification of compounds related to the inhibitory effects on the nematodes. The most intense peaks identified by TargetSearch for leaf and branch tissues were used for cluster analysis by principal component analysis (PCA) and partial least squares-discriminant analysis (PSL-DA), by means of the MetaboAnalyst platform. For both branches and leaves, the profiles of the species *F. carica* differed from those of the other species (Figs. 4, 5), with greater proximity to *F. elastica* (F5). The intensities of each identified metabolite were also used for the ANOVA to verify some molecules specifically abundant in *F. carica*.

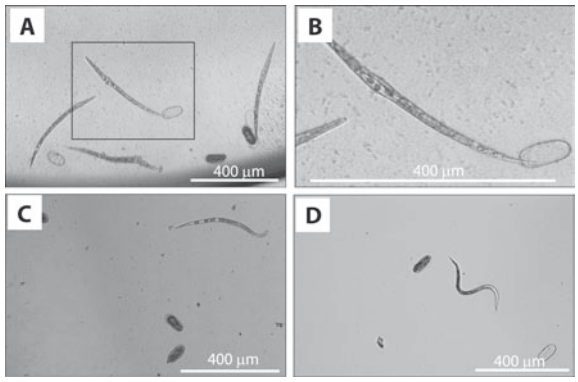

Fig. 3. Analysis by optical microscopy of the *Meloidagyne javanica* J2 after migration from the hatched eggs in the presence of the *Ficus* extracts. A – *F. carica* branch extracts (1,000 µg · ml^{-1} after 48 h); B – *F. carica* branch extracts (1,000 µg · ml^{-1} after 48 h) with an enlarged image; C – H_2O control after 72 h; D – *F. elastica* leaf extracts (1,500 µg · ml^{-1} after 72 h)

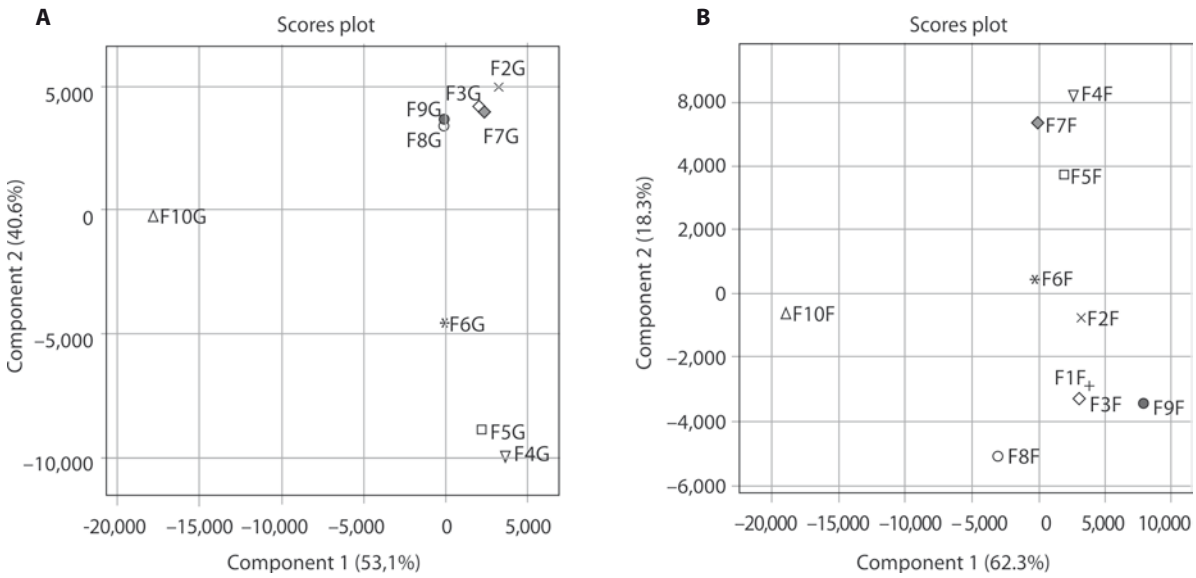

Fig. 4. Principal component analysis (PCA) of the compounds identified by GC/MS from *Ficus* extracts: from branches (A) and from leaves (B). F1 – *F. adhatodifolia*; F2 – *F. arpazusa*; F3 – *F. benjamina*; F4 – *F. carica*; F5 – *F. elastica;* F6 – *F. eximia*; F7 – *F. glabra*; F8 – *F. gomelleira*; F9 – *F. mexiae*; F10 – *F. microcarpa*; plant material from branches (G) and from leaves (F)

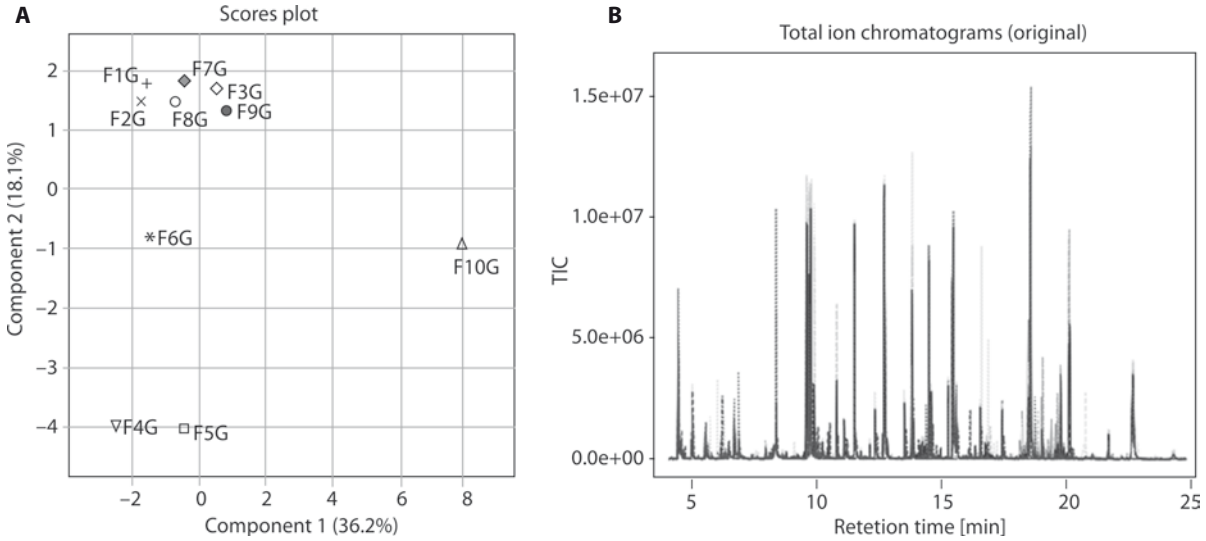

Fig. 5. Principal component analysis (PCA) (A) and chromatogram alignments of TIC (total ion chromatogram) by XCMS (B) of the profiles generated by GC/MS from *Ficus* extracts. F1 – *F. adhatodifolia*; F2 – *F. arpazusa*; F3 – *F. benjamina*; F4 – *F. carica*; F5 – *F. elastica*; F6 – *F. eximia*; F7 – *F. glabra*; F8 – *F. gomelleira*; F9 – *F. mexiae*; F10 – *F. microcarpa*; plant material from branches (G)

Chromatograms of the ions generated by GC/MS, without processing for identification, were converted to CDF format and submitted to cluster analysis using the XCMS platform. In this approach, the peaks present in the chromatograms are used for the alignment of all samples, regardless of whether they were identified or not by TargetSearch. It was observed again that the species *F. carica* presented different profiles in relation to the other species (Fig. 4), with greater proximity to the *F. elastica* (F5) species.

The intensities of each ion chromatographic peak were also used for ANOVA to verify some molecules very abundant in *F. carica*. Two chromatographic peaks showed great abundance only for the *F. carica* extracts (Fig. 6). Chromatographic peaks 1370 and 3183 (Fig. 5) were identified against the EI (Electron Impact) NIST14 (National Institute of Standards and Technology) spectra library, as turanose and oxalic acid, respectively (Fig. 7).

Discussion

The ability to penetrate the host root is critical for the survival of nematodes in the environment. Therefore, motility is a key process to reach the infection sites and start the parasitic cycle (Bellafiore *et al.* 2008; Curtis 2008; Ntalli *et al.* 2016). Thus, the paralysis of juveniles of the second stage (J2), similarly to the inhibition of egg hatching, limits infection in the host. Therefore, these parameters are good indicators of the cytotoxic potential of plant extracts. Under the experimental conditions established, among the 10 species evaluated,

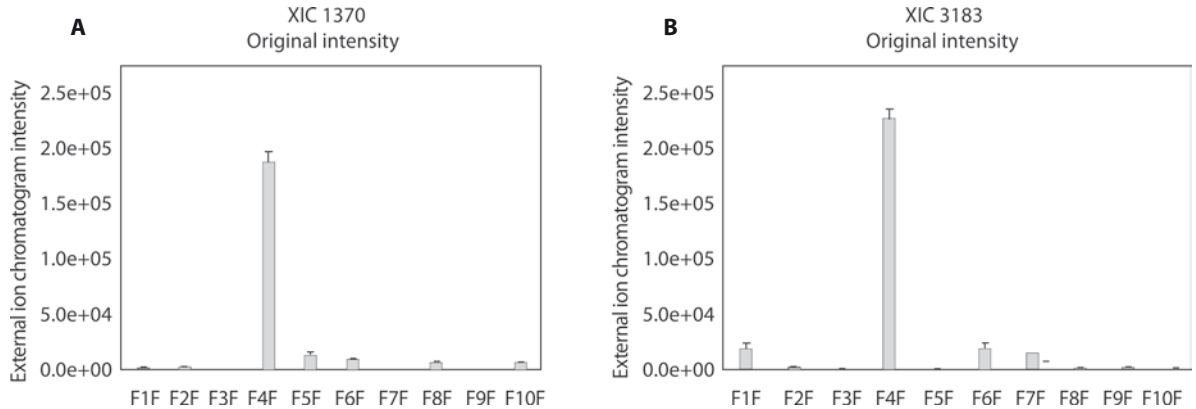

Fig. 6. Intensities of the chromatogram extracted of the ions (XIC) 1370 (RT = 16.70 min) (A) and 3183 (RT = 4.2 min) (B) present in high abundance in *Ficus carica* branch extracts. F1 – *F. adhatodifolia*; F2 – *F. arpazusa*; F3 – *F. benjamina*; F4 – *F. carica*; F5 – *F. elastica;* F6 – *F. eximia*; F7 – *F. glabra*; F8 – *F. gomelleira*; F9 – *F. mexiae*; F10 – *F. microcarpa*; F – leaves

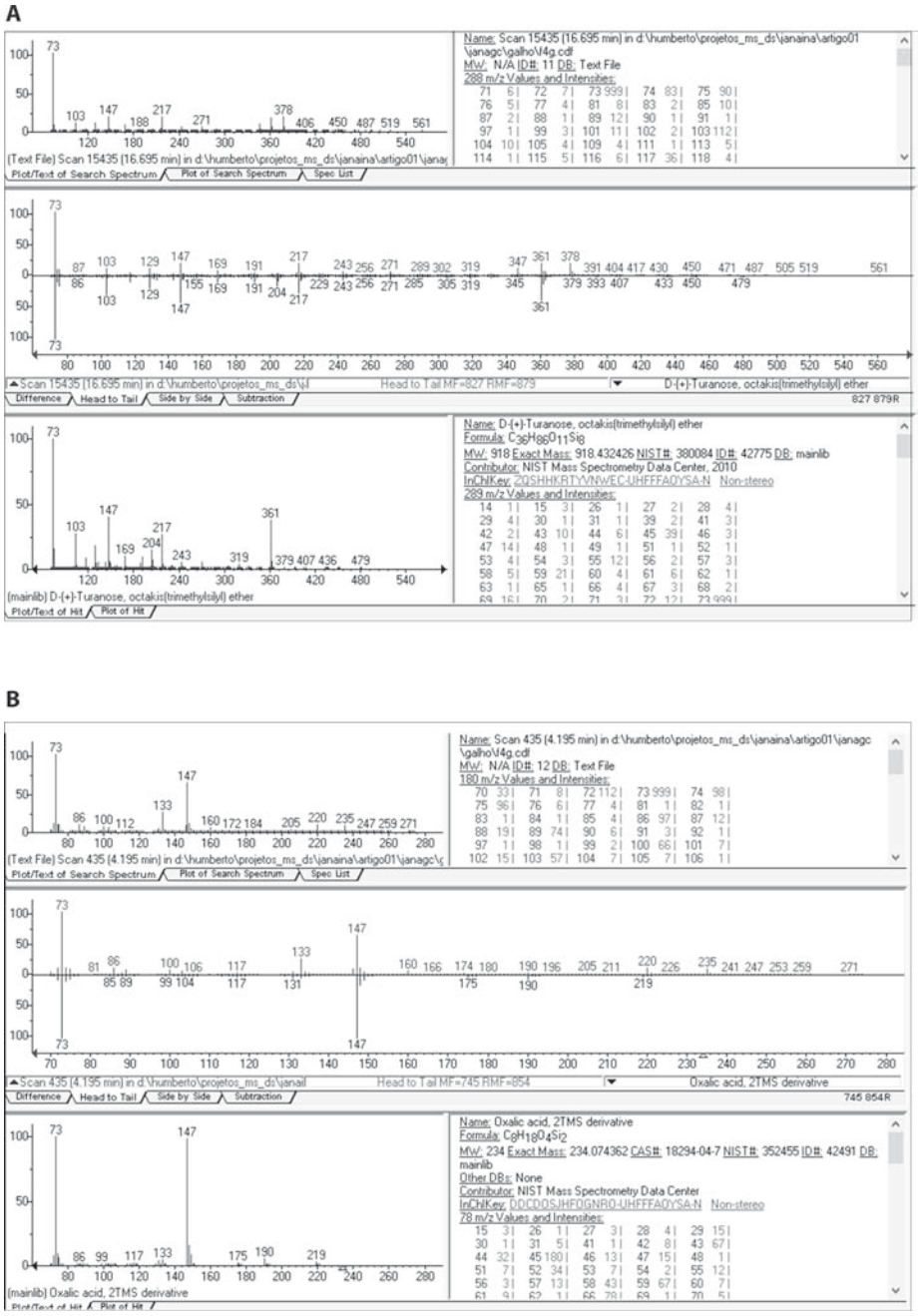

Fig. 7. Identification of the compounds of the XICs 1370 (A) and 3183 (B) against the EI library NIST14

only three species, *F. carica*, *F. elastica* and *F. eximia* reached a paralysis rate of at least 50% in J2s, which indicates the presence of active compounds. However, the effects were much more effective when *F. carica* branch extracts were used (EC_{50} = 134.90 µg · ml^{-1}). This effect on the nematode was similar to that observed by Caboni *et al.* (2015), for the suppression of motility evaluated for *M. incognita*, *M. hapla* and *M. arenaria,* when using a pure compound (Psoralen) identified in the methanolic extracts of *Petrolselinum crispum* (EC_{50} = 147 ± 88 µg · ml^{-1}).

The morphological alterations observed in J2s treated with the extract of *F. carica* branches can be related to specific compounds that affect the developmental process of the nematode. Yang *et al.* (2015) also reported morphological variations of *M. javanica* J2s after 72 h of treatment with *Camellia* seed cake, but they were less pronounced than *F. carica*. The percentage of immobile J2s indicated that the action of *F. carica* branch extract started after 24 h (25% paralyzed), despite the increase after 48 and 72 h. Liu *et al.* (2011) observed nematicidal action up to 30%, just after 72 h.

In relation to the eggs of *M. javanica*, the *F. carica* extracts also presented a stronger hatching inhibition effect than that obtained by other plant species. However, the activity level of the leaf extract was slightly

higher than that of the branches. Both achieved significant hatching inhibition after 2 days and increased over 8 days. These results were similar to those found by Yang *et al.* (2015) and Ntalli *et al.* (2016) (1,000 µg · ml^{-1} of 2,4-decadienal for *M. incognita* eggs). The lowest cumulative percentage of eggs hatched after 8 days was observed for the extracts of the leaves and branches of *F. carica*. Figure 8 illustrates the superior inhibition performance over time by extracts from *F. carica*, when compared to the extracts from *F. elastica* and *F. adhatodifolia*.

Optical microscopy images revealed the paralysis of juveniles in the second stage, mainly for *F. carica* branch extracts, which has not been reported by any other similar studies. This indicates that the compounds that inhibit egg hatching can also promote paralysis in

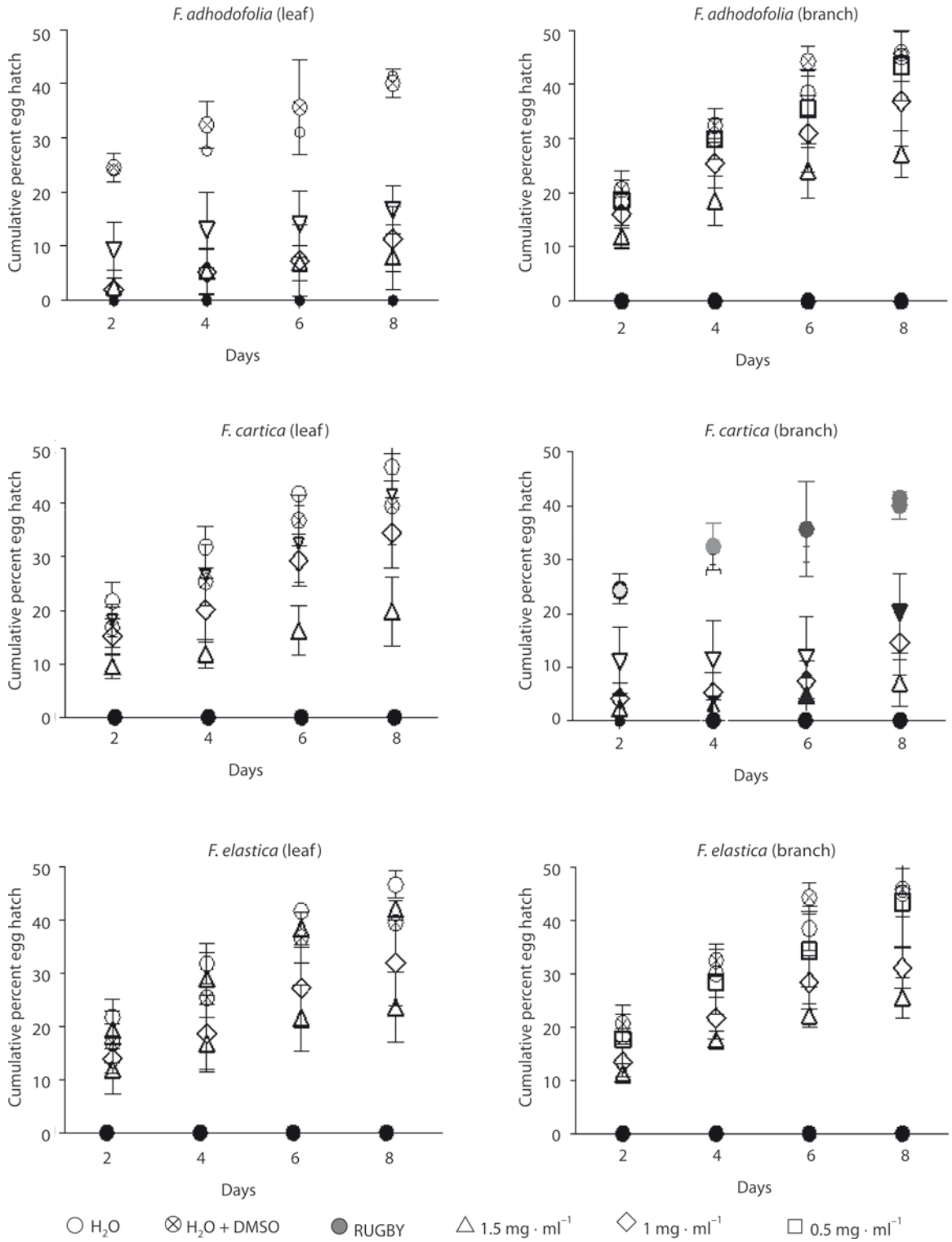

Fig. 8. Cumulative percentage of hatching eggs over the time of *Meloidogyne javanica* in the presence of extracts from *Ficus carica*, *F. elastica*, *F. adhatodifolia*

J2s. *Ficus carica* extract possibly contains new active molecules against *M. javanica* not described yet.

Ntalli *et al.* (2016) and Caboni *et al.* (2013) identified compounds that were active in both J2 paralysis and egg hatching when combined in the same solution. Thus, in complex samples, such as plant extracts, the antagonistic effect may be the result of a synergistic mechanism between different compounds. The qualitative profile of leaves and branches of *Ficus* species revealed the presence of different classes of secondary metabolites, including essential oils, saponins, tannins, triterpenes, anthraquinones, coumarins and flavonoids. Alkaloids were detected only in *F. carica*. However, the nematotoxic effect on the J2s and eggs of the genus *Meloidogyne* may be associated with chemical compounds of classes such as alkaloids, flavonoids, saponins, coumarins, aldehydes, amides, benzamides and ketones, which may act either isolatedly or in combination (Caboni *et al.* 2015; Ntalli *et al.* 2016; Lu *et. al.* 2017; Lahm *et al.* 2017).

The highest activity of J2 paralysis was observed only for *F. carica* branch extracts. Thus, the activity observed may be attributed to the performance of compounds of specific classes that may be absent or present at different levels in different species. In fact, cluster analysis by PCA and PSL-DA of the metabolic profiles by GC/MS indicated significant differences between species, mainly for *F. carica*. Phylogenetic analyses of the genus *Ficus* (Ronsted *et al.* 2008) indicated that this species presents a greater evolutionary distance than the species used in this study. Comparisons of the identified metabolite content for the 10 species did not allow the assignment of a putative compound for the inhibitory activities observed. However, the alignment and comparison of the chromatographic peaks generated by GC/MS allowed for the identification of two highly abundant metabolites in *F. carica* (turanose and oxalic acid).

Another important factor for the inhibitory effect may be pH variation in the samples. According to Fleming *et al.* (2017), medium pH is a determinant of the nematostatic activity of key molecules. Thus, the phytonematode behavior could be affected by pH and can be attracted or repelled by chemical and environmental stimuli, through a highly sensitive chemosensory system used to detect and infect possible host plants (Xiang and Laurence 2016; Fleming *et al.* 2017). The samples of *F. carica* branches were more acidic than those of the leaves. This observation justifies the high values of oxalic acid found in these samples. There are reports in the literature (Seo and Kim 2014; Fleming *et al.* 2017) of the nematicidal potential of acid compounds, such as salicylic acid, p-coumaric acid and cinnamic acid, among other organic acids, either isolated or in mixtures. In addition to the high potential of *F. carica* extracts for application in the control of *M. javanica*, these compounds were considered nontoxic to NIH3T3 lineage animal cells at 100 $\mu g \cdot ml^{-1}$. Thus, the non-toxicity of *Ficus* spp. to cells of animal lineage is a good indicator of safety for the use of these extracts as sources of active biomolecules for the control of phytonematodes.

Conclusions

The *in vitro* phytochemical screening of 10 species of the genus *Ficus* spp., a species native to the Brazilian Atlantic Forest, showed that *F. carica* is active in the control of *M. javanica*, which indicates the presence of nematotoxic compounds in its chemical composition. *Ficus carica* branch extracts significantly reduced the motility of J2 and the hatching of nematode eggs. Differences in efficacy between the analyzed species are supported by the variation in the composition of the metabolites of the ethanolic extracts. *Ficus carica* also presented profiles of metabolites generated by GC/MS distinct to other plant species and allowed for the identification of putative compounds for the inhibitory activity. Therefore, bioactive molecules present in *F. carica* constitute an alternative to be explored for the control of *M. javanica*.

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