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RESEARCH ARTICLE

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Anti-Zika Virus Activity of Plant Extracts Containing Polyphenols and Triterpenes on Vero CCL-81 and Human Neuroblastoma SH-SY5Y Cells

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Zika virus (ZIKV) infection is a global threat associated to neurological disorders in adults and microcephaly in children born to infected mothers. No vaccine or drug is available against ZIKV. We herein report the anti-ZIKV activity of 36 plant extracts containing polyphenols and/or triterpenes. ZIKV-infected Vero CCL-81 cells were treated with samples at non-cytotoxic concentrations, determined by MTT and LDH assays. One third of the extracts elicited concentration-dependent anti-ZIKV effect, with viral loads reduction from 0.4 to 3.8 log units. The 12 active extracts were tested on ZIKV-infected SH-SY5Y cells and significant reductions of viral loads (in log units) were induced by *Maytenus ilicifolia* (4.5 log), *Terminalia phaeocarpa* (3.7 log), *Maytenus rigida* (1.7 log) and *Echinodorus grandiflorus* (1.7 log) extracts. Median cytotoxic concentration (CC_{50}) of these extracts in Vero cells were higher than in SH-SY5Y lineage. *M. ilicifolia* ($IC_{50} = 16.8 \pm 10.3$ μg/mL, SI = 3.4) and *T. phaeocarpa* ($IC_{50} = 22.0 \pm 6.8$ μg/mL, SI = 4.8) were the most active extracts. UPLC-ESI-MS/MS analysis of *M. ilicifolia* extract led to the identification of 7 triterpenes, of which lupeol and a mixture of friedelin/friedelinol showed no activity against ZIKV. The composition of *T. phaeocarpa* extract comprises phenolic acids, ellagitannins and flavonoids, as recently reported by us. In conclusion, the anti-ZIKV activity of 12 plant extracts is here described for the first time and polyphenols and triterpenes were identified as the probable bioactive constituents of *T. phaeocarpa* and *M. ilicifolia*, respectively.

Keywords: chemosystematic approach, *Terminalia phaeocarpa*, *Maytenus ilicifolia*, *Zika virus* inhibition, SH-SY5Y cells.

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Introduction

Zika virus (ZIKV) is a mosquito-borne Flavivirus transmitted to subjects through the bite of infected female

Aedes mosquitoes like *Aedes aegypti* and *Aedes albopictus*, and other routes such as sexual contact, blood transfusion, and vertical transmission during pregnancy.[1] This infection gained prominence in 2015 when a massive ZIKV epidemic started in Northeast Brazil, followed by Rio de Janeiro, São Paulo, and other Brazilian states.[2] The current incidence rate of ZIKV in Brazil is estimated as 2.8 cases/100,000 inhabitants.[3] The virus continues to circulate in the country, but the ongoing pandemic of SARS-CoV-2 probably causes a delay or subnotification of the current number of cases.

The World Health Organization (WHO) declared the ZIKV infection as a public health emergency of global relevance in 2016, due to its association with the occurrence of severe outcomes in humans, such as the congenital Zika syndrome. In this condition, children born to mothers infected during pregnancy present several nervous system commitments, of which microcephaly is the utmost severe manifestation.[4] A serious disorder caused by ZIKV in adults is the Guillain-Barré syndrome, characterized by peripheral neuropathy associated with progressive paralysis and other neurological complications.[5]

There is currently neither a drug available to treat ZIKV infection nor a vaccine to prevent it, despite the relevant impact of this virus on human health, causing severe social and economic burden, and its probable expansion in the forthcoming years.[6] Plants may offer a repository of bioactive compounds against ZIKV and only a few publications have addressed this source so far. Brazil is considered to host the largest plant biodiversity on Earth, but the country also holds the sad record of having the highest number of ZIKV infections globally, and a whole generation of children born with microcephaly. These facts have motivated us to explore Brazilian plants in the search for species active against ZIKV. Thus, we adopted a chemosystematic approach for selecting plants containing polyphenols, triterpenes, and steroids, and tested *in vitro* 36 extracts in CCL-81 Vero cells infected with ZIKV, along with some isolated compounds. Furthermore, the active extracts had their effect on ZIKV replication assayed in neuronal SH-SY5Y cells infected with ZIKV. The two most active extracts had their chemical composition investigated by UPLC-ESI-MS/MS analysis.

Results and Discussion

Thirty plant species belonging to seven distinct families were selected for study, of which 36 extracts were obtained from different anatomical parts (*Table 1*). The occurrence of triterpenes, steroids, or polyphenols – classes of compounds previously reported to possess anti-ZIKV activity, as discussed throughout the manuscript – was the chemosystematic criteria adopted for plant selection. Besides, we prioritized plants with reported anti-inflammatory activity, since the antiviral effect of some natural products has been assigned to the anti-inflammatory responses induced in the host cells.[7,8] Eight compounds and a mixture of triterpenes isolated from the tested plant species, or selected by the same approach, were also assayed for their anti-ZIKV activity.

Cell viability assay is an important step to define non-toxic concentrations of samples for antiviral testing. It is particularly challenging for extracts, since some constituents may interfere chemically with the test system generating false positive results. The cytotoxicity of the samples was herein evaluated in Vero CCL-81 cells both by the MTT and the lactate dehydrogenase (LDH) assays. The MTT assay disclosed 13 out of 36 extracts as cytotoxic (cell viability $< 70\%$) at 90 μg/mL, but indicated absence of toxicity at 30 μg/mL (*Figure S1*, available as *Supporting Information*).

The MTT assay may produce false positive results for plant extracts or compounds with intrinsic reducing potential[9] and the LDH assay was used to confirm the results. Seven cytotoxic extracts in the MTT assay did not show toxicity in the LDH test (*Figure S2*). This assay is less sensitive than the MTT test to detect early cytotoxic events,[10] what may explain the observed differences, in addition to possible false positive results for MTT (*Figure S1* and S2). Six *Baccharis* species and *M. ilicifolia* were cytotoxic in both assays, confirming previous toxicity data reported for some of them.[11,12] Regarding the compounds, lupeol, ouratein D and *trans*-aconitic acid were cytotoxic at 10 μM in the MTT assay, but not in the LDH test (*Figure S3A* and S3B). Six extracts showed cell viability above 100% in the MTT assay (*Figure S1*), probably due to the interference of phenolic compounds capable of reducing MTT into formazan crystals by a chemical reaction independent of the cell.[9] These extracts showed no cytotoxicity in the sulforhodamine B assay (*Figure S4*).

The effect of the extracts and compounds on ZIKV replication was initially tested on Vero CCL-81 cells. The viability of cells infected by ZIKV with different MOIs was assessed by the MTT (*Figure 1*A) and LDH assays (*Figure 1B*). There were significant differences in cell viability after ZIKV infection with MOIs of 0.3, 1, and 3 in the presence of 0.3% v/v DMSO, when

Table 1. Plant extracts assayed *in vitro* on CCL-81 Vero cells infected with ZIKV.

Family	Species	Excicate number	Part used	Extract number
Alismataceae	*Echinodorus grandiflorus* (Cham. & Schltdl.) Micheli.	BHCB107791	leaves	1
Apocynaceae	*Hancornia speciosa* Gomes	BHCB165298	steams	2
			leaves	3
			leaves	4
Asteraceae	*Baccharis altimontana* G. Heiden et al.	ECT5981	aerial parts	5
	Baccharis brevifolia DC.	ECT6259	leaves	6
	Baccharis calvescens DC.	ECT5549	leaves	7
			stems	8
	Baccharis dracunculifolia DC.	RB777684	leaves	9
	Baccharis hemiptera G. Heiden et al.	ECT5982	aerial parts	10
	Baccharis imbricata Malag.	ECT5912	leaves	11
	Baccharis intermixta Gardner	ECT0355	leaves	12
	Baccharis magnifica G. Heiden, Leoni & J.N.Nakaj.	ECT5983	stems	13
	Baccharis myriocephala DC.	ECT6257	aerial parts	14
	Baccharis opuntioides Mart. ex Baker	ECT5987	aerial parts	15
	Baccharis parvidentata Malag.	ECT5984	leaves	16
	Baccharis platypoda DC.	ECT5974	leaves	17
	Baccharis reticularia DC.	ECT6262	leaves	18
	Baccharis retusa DC.	ECT6260	leaves	19
	Baccharis serrulata (Lam.) Pers.	ECT6213	aerial parts	20
	Symphyopappus brasiliensis (Gardner) R.M.King & H.Rob.	ECT5975	leaves	21
	Symphyopappus lymansmithii B.L.Rob.	ECT 5970	leaves	22
Celastraceae	*Maytenus acanthophylla* Reissek	HUESB7813	leaves	23
			leaves	24
	Maytenus ilicifolia Mart. ex Reissek	FUEL21881	leaves	25
			leaves	26
	Maytenus rigida Mart.	HUESB7056	roots	27
	Maytenus truncata Reissek	HUESB9311	leaves	28
Combretacae	*Terminalia phaeocarpa* Eichler	BHCB201083	leaves	29
Erytroxylaceae	*Erythroxylum deciduum* A. St.-Hil	BHCB115735	leaves	30
	Erythroxylum tortuosum Mart.	BHCB111065	leaves	31
Malvaceae	*Sida glaziovii* K. Schum.	PAMG45844	leaves	32
Ochnaceae	*Ouratea castaneifolia* DC.	BHCB50394	stems	33
			leaves	34
	Ouratea spectabilis Mart. ex Engl.	BHCB48940	stems	35
			leaves	36

All extracts were prepared by percolation with EtOH 96 °GL, except 3 (percolation with acetone/water 7:3), 4 (percolation with AcOEt/MeOH 1:1), 23 (decoction with water), 25 (percolation with DCM) and 26 (percolation with MeOH). BHCB = Herbarium of the Universidade Federal de Minas Gerais; ECT = Herbarium of the Empresa Brasileira de Pesquisa Agropecuária, Clima Temperado, Rio Grande do Sul; FUEL = Herbarium of the Universidade Federal de Londrina, Brazil; HUESB = Herbarium of the Universidade Estadual do Sudoeste da Bahia, Brazil; PAMG = Herbarium of the Empresa de Pesquisa Agropecuária de Minas Gerais, Brazil; RB = Herbarium of the Jardim Botânico do Rio de Janeiro, Brazil.

compared to the control cells. However, no significant differences in the viral loads were observed between the treatments, except for the groups ZIKV MOI of 3 and ZIKV + DMSO MOI of 3. The MOI of 1, which induces ~75% of cell viability, was selected for the antiviral assays (*Figure 1C*).

The extracts and a mixture of friedelin/friedelinol were tested at 3, 10, and 30 μg/mL on Vero CCL-81 cells infected with ZIKV and after treatment cell viability was measured by the MTT and LDH assays (*Figure 2* and *3*, respectively). AH-D peptide, whose antiviral activity has been demonstrated by *in vitro* and *in vivo* assays, was used as a positive control.[13] Twelve out of the 36 extracts (*Figure 4*) reduced viral loads of treated cells in a concentration-dependent manner (expressed as reduction of log units related to the initial inoculum): *B. calvescens* (2.0 log), *B. imbricata* (1.0 log), *B. magnifica* (0.4 log), *B. opuntioides* (0.4 log), *E. grandiflorus* (3.8 log), *M. acanthophylla* (1.1 log), *M. ilicifolia* (2.4 log, dichloromethane extract; 2.0 log,

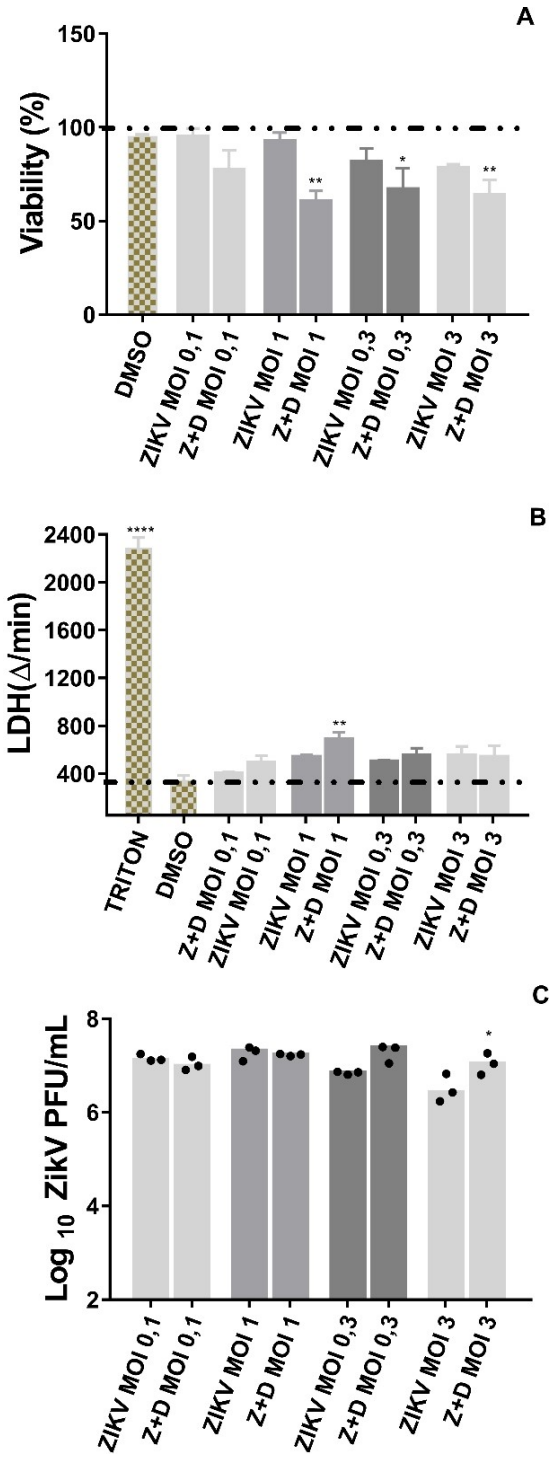

Figure 1. Standardization of ZIKV MOI in Vero CCL-81 cells. Cell toxicity was assayed by the MTT (A) and LDH (B) methods. The bars represent mean values ± standard error of the mean (SEM), where * $P < 0.05$, ** $P < 0.01$ versus cell control group. Viral load (C) was determined by the viral lysis plate reduction test. The bars represent median values, where * $p < 0.05$ for ZIKV MOI 3 versus Z + D MOI 3. Dashed line = cell control, without ZIKV. Z + D = ZIKV + DMSO.

methanol extract), *M. rigida* (2.4 log), *M. truncata* (2.5 log), *O. spectabilis* (1.1 log), and *T. phaeocarpa* (1.8 log). These extracts did not affect cell viability of infected and treated cells, in comparison to the control group (ZIKV + DMSO) (*Figure 2* and *3*). Besides, the significant reduction of the viral loads of the treated cells shows that the above-mentioned extracts exerted antiviral effect (*Figure 4*).

As far as we know, there are no previous reports on plant screening against ZIKV and the chemosystematic approach adopted herein resulted in nearly 30% of active extracts. The relevance of this finding can be evaluated by comparison with other antiviral screenings based on ethnopharmacological approaches for plant selection. For instance, 13% and 11% of the tested extracts were active against Herpes simplex virus type 1[14] and type 2,[15] whereas 40% were found to be active against Dengue virus serotypes 2 or 3.[16] Therefore, the chemosystematic approach seems to be a suited approach for selecting plants active against ZIKV, since similar or higher percentage of active extracts were here identified.

The anti-ZIKV activity of the above-mentioned species is here described for the first time. It is possible to infer that the activity of *M. rigida*, *M. truncata* and *M. ilicifolia* can be attributed to triterpenes, which are abundantly found in *Maytenus* species.[17] Polyphenols can also be the antiviral compounds of the active species, since their anti-ZIKV effect has been previously reported. The aqueous extract of *Psiloxylon mauritianum* aerial parts inhibited ZIKV infection in Vero cells at 100 μg/mL, decreasing by 2-log units the production of infectious viral particles.[18] This extract is rich in phenolic compounds such as gallic acid, quercetin and kaempferol. The extract of *Doratoxylon apetalum* aerial parts inhibited ZIKV infection in A549 cells in a concentration dependent manner and promoted a 5-log reduction of viral progeny at 100 μg/mL.[19] In its turn, the 70% hydroethanolic extract of *Phyllanthus phillyreifolius* leaves promoted a 3-log reduction of ZIKV progeny at 250 μg/mL.[20] The 12 active extracts disclosed in the present study were not cytotoxic and showed significant antiviral effect, with ZIKV load reduction ranging from 0.4 to 3.8 log units, when tested at lower concentrations than the above-cited literature data.

We also tested the anti-ZIKV activity of eight compounds. None of them inhibited ZIKV replication at the tested concentrations, and the viral load of the treated and infected group remained like the control group (ZIKV + DMSO) (*Figure S5C*). Although mansoin A and ouratein D were inactive in the present work,

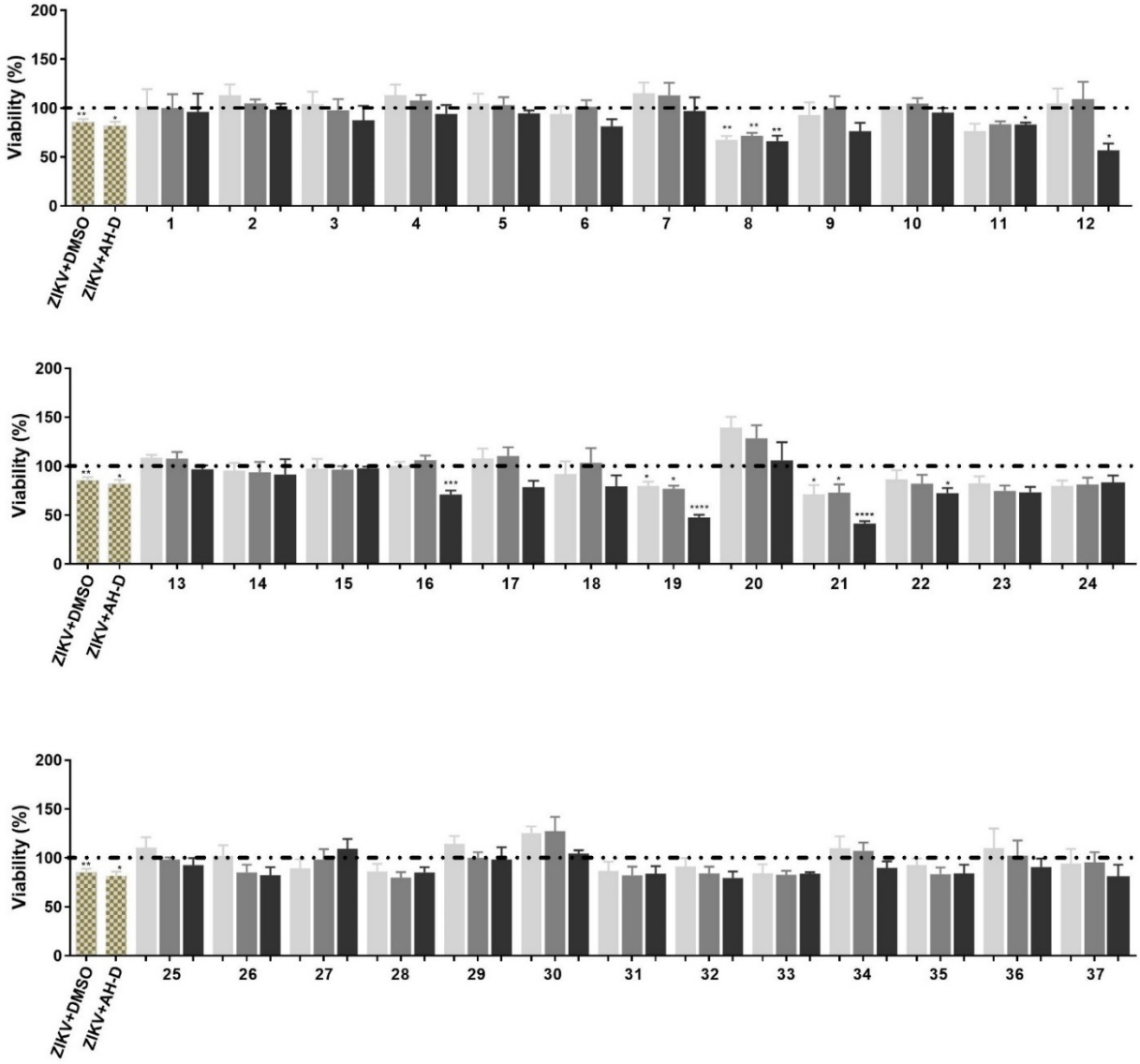

Figure 2. Effect of extracts on the viability of ZIKV-infected Vero CCL-81 cells, assayed by the MTT method. See *Table 1* for identification of the extracts. Sample 37 is a mixture of friedelin and friedelinol (M1). The extracts and M1 were tested at 3 μg/mL (light gray bars), 10 μg/mL (medium gray bars), and 30 μg/mL (black bars). The bars represent mean values ± SEM, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ versus cell control group. Triton was employed as the positive control and DMSO as the negative control. Dashed line = cell control, without extract and ZIKV.

some other polyphenols have been reported to be active against ZIKV, including houtuinoid B and tetra-acetylhoutuinoid B,[21] quercetin-3-β-*O*-D-glycoside,[22] isoquercitrin,[23] rutin and catechin,[24] resveratrol,[25] naringenin,[26] ellagic acid,[27] delfinidine, epigallocatechin gallate, and pinocembrine, among others.[28,29] Terpenes have been also described to possess anti-ZIKV activity. Six terpenoids isolated from the hexane extract of *Stillingia loranthacea* root bark exhibited significant antiviral effects against ZIKV, evaluated in Vero cells.[30] It is also possible to infer that triterpenes different from lupeol, friedelin and friedelinol are the bioactive compounds found in the dichloromethane extract of *M. ilicifolia*, since these constituents were inactive, or that they act synergistically with other constituents of the extract.

The 12 active extracts in ZIKV-infected Vero CCL-81 cells were further tested in the human neuroblastoma cell lines SH-SY5Y for their anti-ZIKV activity. ZIKV efficiently infects SH-SY5Y cell cultures, and the effects can be correlated with the neuropathological outcomes of ZIKV in infected human adults.[31] The

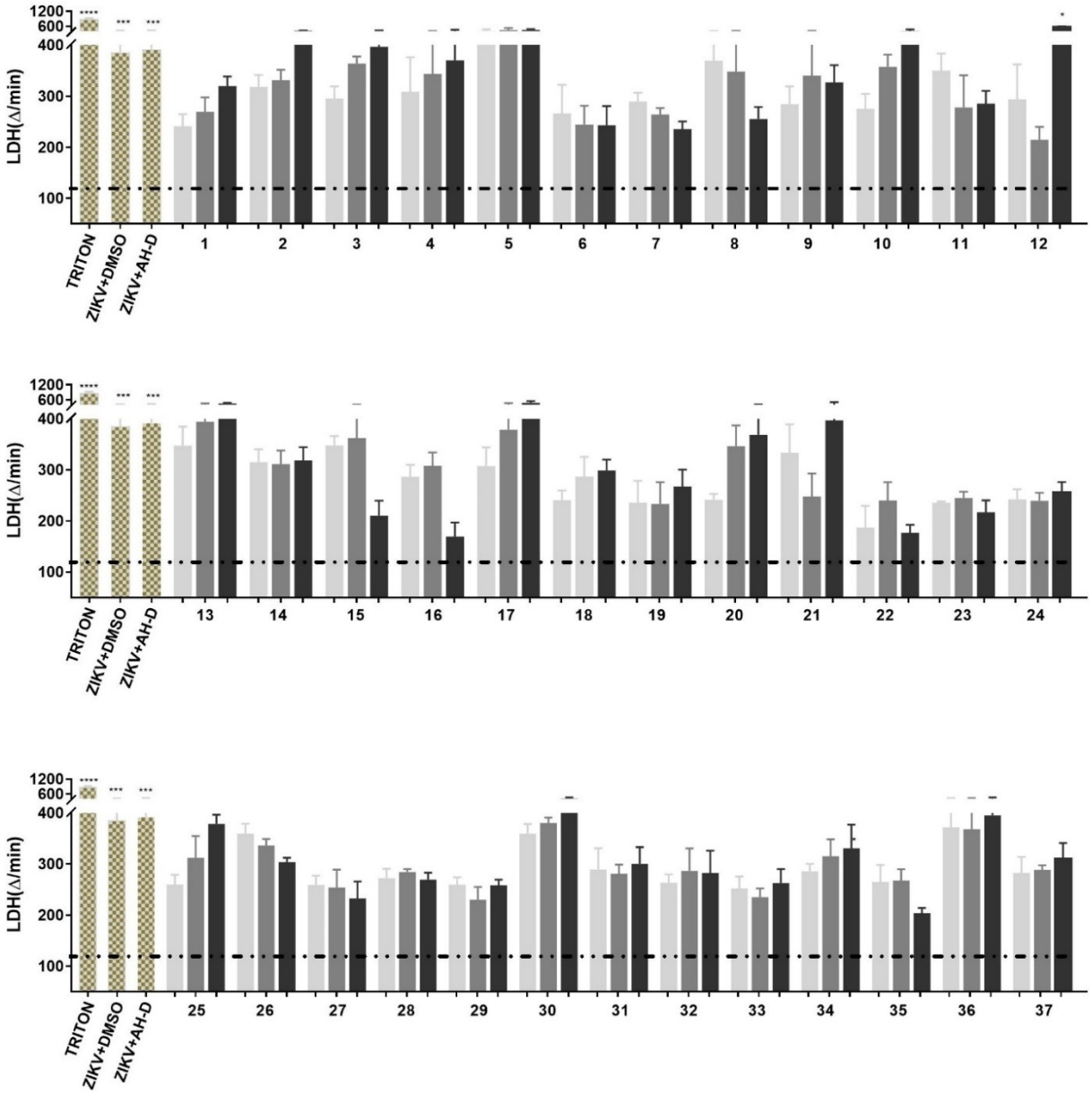

Figure 3. Effect of extracts on the viability of ZIKV-infected Vero CCL-81 cells, assayed by the LDH method. See *Table 1* for identification of the extracts. Sample 37 is a mixture of friedelin and friedelinol (M1). The extracts and M1 were tested at 3 μg/mL (light gray bars), 10 μg/mL (medium gray bars), and 30 μg/mL (black bars). The bars represent mean values ± SEM, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ versus cell control group. Triton was employed as the positive control and DMSO as the negative control. Dashed line = cell control, without extract and ZIKV.

cytotoxicity of the extracts was tested by the MTT and LDH assays and none of them reduced cell viability, except *B. imbricata* at 30 μg/mL (*Figure S6*).

SH-SY5Y cells infected with ZIKV in the MOI of 0.001 resulted in cell viability around 60% by the MTT assay, with no statistical difference among the viral loads of the groups (*Figure S7*), and this MOI was selected to perform the *in vitro* anti-ZIKV tests. This MOI value is significantly lower than the MOIs of 1 and 10 previously reported for SH-SY5Y cells.[31,32] The difference is probably due to the higher virulence of the ZIKV clinical isolate used herein, in comparison to the virus lines employed in the aforementioned studies.

The extracts had their anti-ZIKV activity assayed in SH-SY5Y cells at 3, 10, and 30 μg/mL, except for *B.*

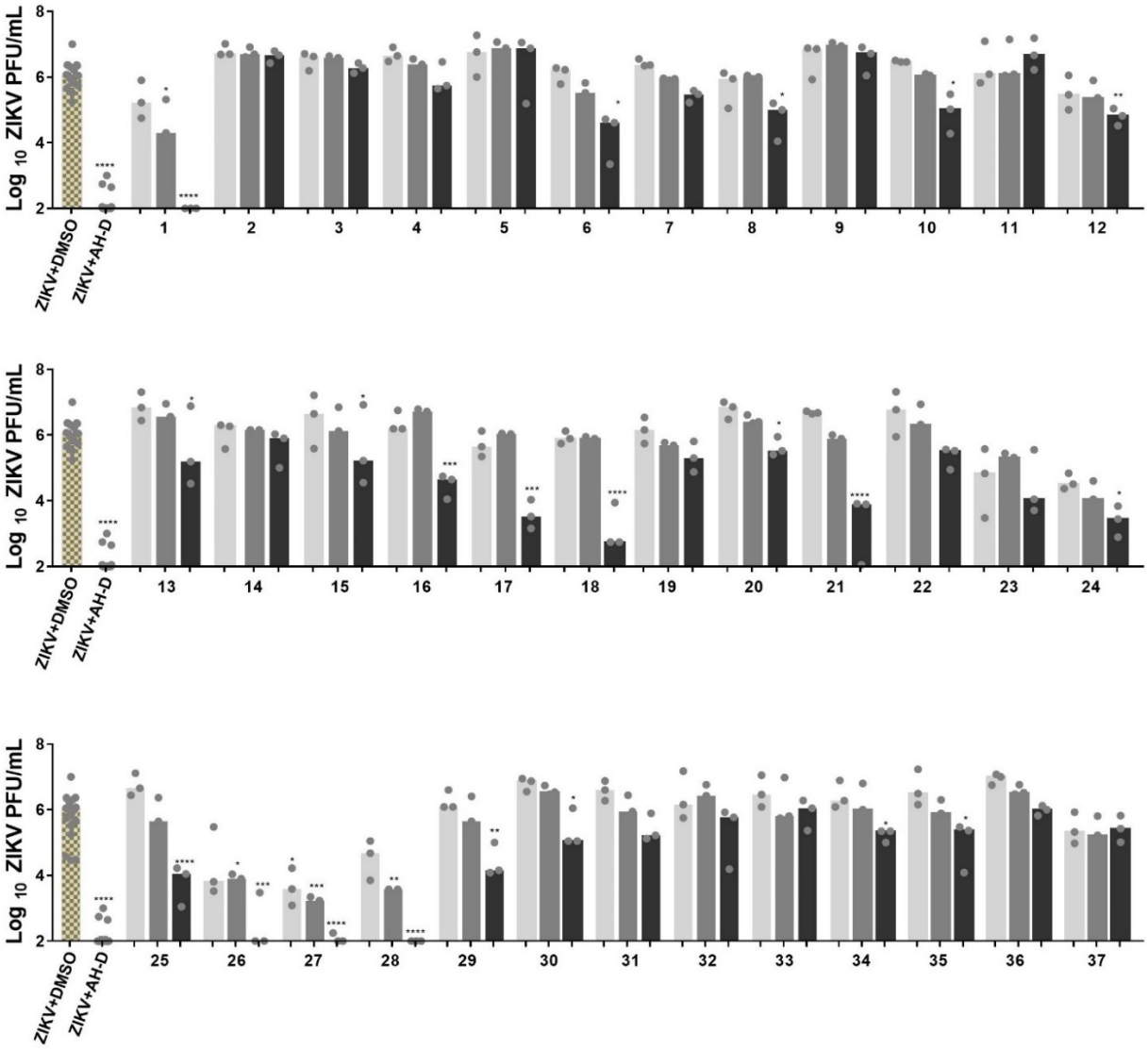

Figure 4. Effect of the tested extracts on the viral loads of ZIKV-infected Vero CCL-81 cells. See *Table 1* for identification of the extracts. The extracts were tested at 3 μg/mL (light gray bars), 10 μg/mL (medium gray bars), and 30 μg/mL (black bars). The bars represent median values. Data were compared by Tukey's test (ZIKV + treated versus ZIKV + DMSO), where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. AH-D was employed as the positive control and DMSO as the negative control.

imbricata, tested at 0.3, 3, and 10 μg/mL. The results of the MTT and LDH cytotoxicity assays are depicted in *Figure 5A* and *5B*, respectively. Significant reductions of viral loads (expressed in log units) were induced by the extracts of *E. grandiflorus* (1.7 log), *M. ilicifolia* (dichloromethane extract; 4.5 log), *M. rigida* (1.7 log), and *T. phaeocarpa* (3.7 log), when tested at 30 μg/mL (*Figure 6*), while cell viability of infected and treated cells was similar to the control group ZIKV + DMSO (*Figure 5A* and *5B*). Furthermore, the extracts of *M. ilicifolia* and *T. phaeocarpa* reduced viral loads in a concentration-dependent manner.

To the best of our knowledge, there are no previous reports on the activity of plant extracts against ZIKV-infected SH-SY5Y cells, thus highlighting the relevance of the data herein presented. There are few reports on the activity of natural products against ZIKV-infected SH-SY5Y cells, comprising isoquercitrin (assayed at 100 μM),[23] an omega-3-polyunsaturated fatty acid (assayed at 12.5 μM),[32] and the bacterial metabolite bafilomycin A1 (assayed at 10 nM).[33] Additionally, some natural products have been reported to exert anti-ZIKV activity in other neuronal cells, including chloroquine, assayed at 50 μM and 12.5 μM,

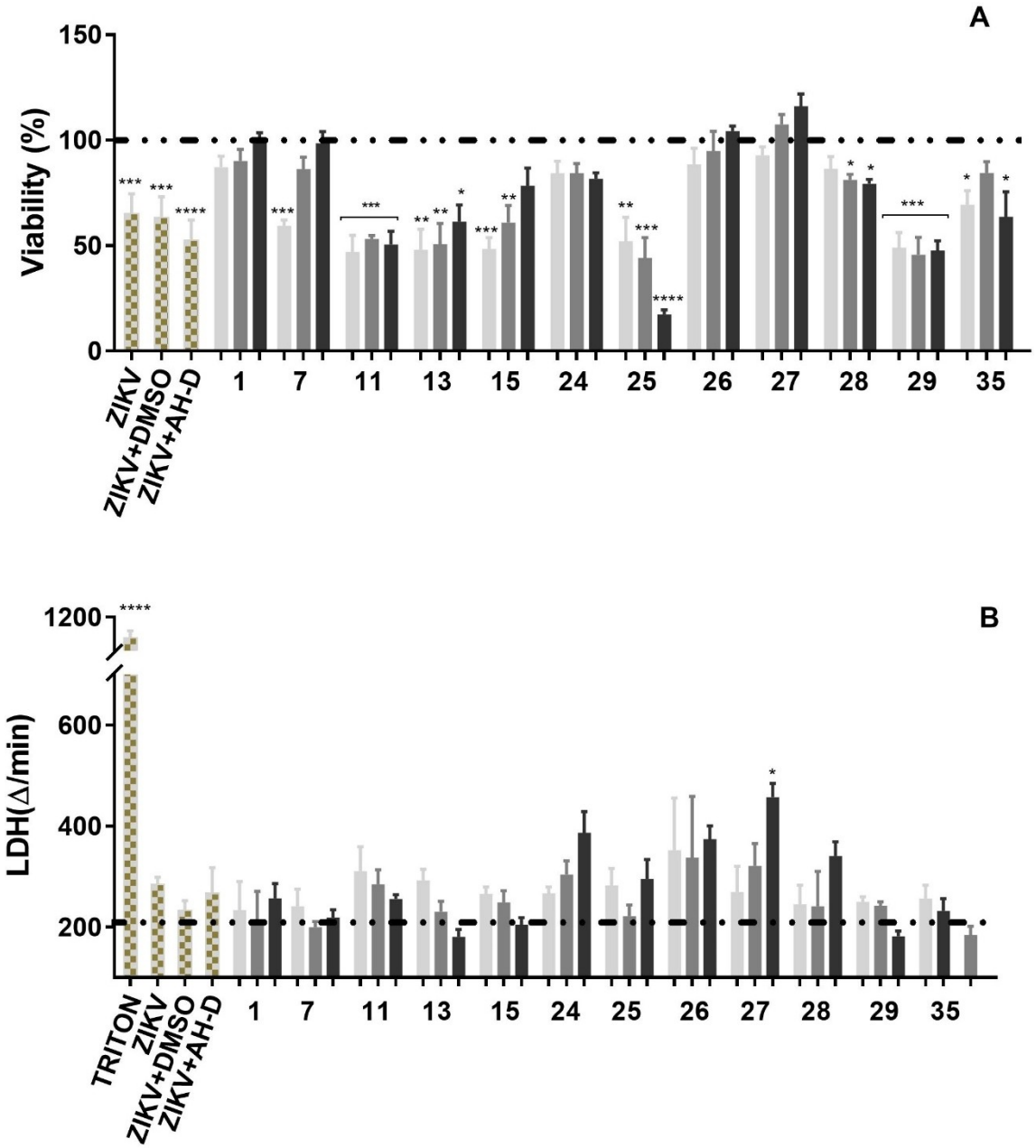

Figure 5. Effect of extracts on the viability of ZIKV-infected SH-SY5Y cells, assayed by the MTT (A) and LDH (B) methods. See *Table 1* for identification of the extracts. The extracts were tested at 3 μg/mL (light gray bars), 10 μg/mL (medium gray bars), and 30 μg/mL (black bars). The bars represent mean values ± SEM, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ versus cell control group. Triton was employed as the positive control and DMSO as the negative control. Dashed line = cell control, without extract and ZIKV.

respectively in human fetal neuronal stem cells[34] and in mouse neurospheres,[35] in addition to betulinic acid (assayed at 50 μM) in human neural progenitor cells.[36]

The extracts of *M. rigida*, *E. grandiflorus*, *M. ilicifolia* (dichloromethane), and *T. phaeocarpa* had their 50% cytotoxic concentration (CC_{50}) determined in Vero CCL-81 and SH-SY5Y cells by the MTT assay. The CC_{50} values obtained in Vero CCL-81 cells were significantly higher than those found for the SH-SY5Y lineage (extracts 1, 25, 27 and 29; *Table 2*).

Despite being two immortalized cell lines, SH-SY5Y cells are neuronal cells, more sensitive to xenobiotics, and potentially more responsive to cell damage, thus

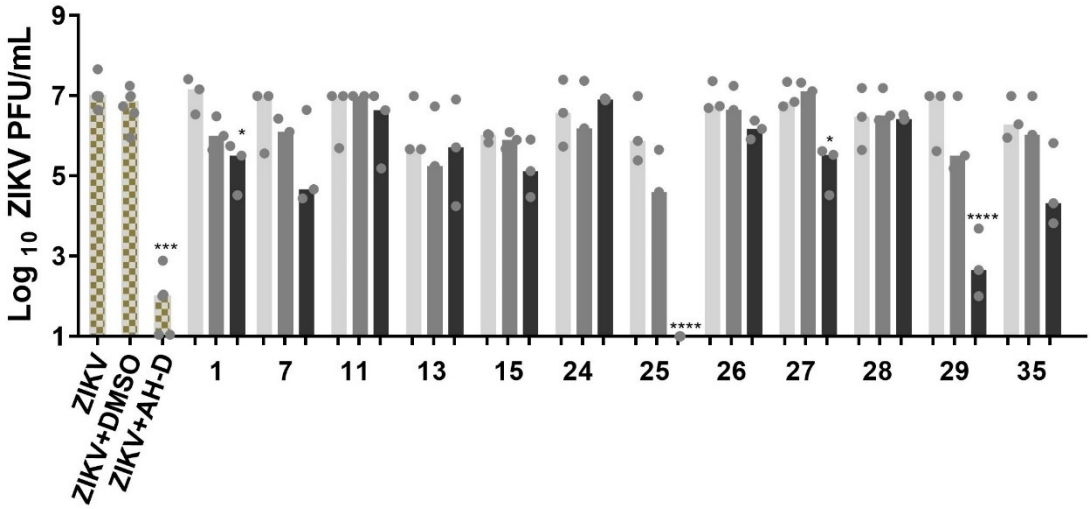

Figure 6. Effect of selected extracts on the viral loads of ZIKV-infected SH-SY5Y cells. See *Table 1* for identification of the extracts. The extracts were tested at 3 μg/mL (light gray bars), 10 μg/mL (medium gray bars), and 30 μg/mL (black bars), except for 11 (tested at 0.3, 3 and 10 μg/mL, respectively). The bars represent median values. Data were compared by Tukey's test (ZIKV + treated versus ZIKV + DMSO), where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. AH-D was employed as the positive control and DMSO as the negative control.

Table 2. CC_{50} of the most active extracts against ZIKV on Vero CCL-81 and SH-SY5Y cell lines.

Plant species	Extract number	Part of the plant	Extract	CC_{50} (μg/mL, mean ± sd) Vero CCL-81	SH-SY5Y
Echinodorus grandiflorus	1	Leaves	Ethanolic	398.1 ± 26.4	96.8 ± 17.3
Maytenus ilicifolia	25	Leaves	Dichloromethane	158.9 ± 17.3	57.2 ± 16.7
Maytenus rigida	27	Roots	Ethanolic	> 480	107.1 ± 19.3
Terminalia phaeocarpa	29	Leaves	Ethanolic	> 480	104.8 ± 28.9

explaining the observed difference. The 50% inhibitory concentration (IC_{50}) of *M. ilicifolia* (dichloromethane) and *T. phaeocarpa* extracts were determined in SH-SY5Y cells, as well as their selective index ($SI = CC_{50}/IC_{50}$) since they elicited more significant reduction of viral load. IC_{50} values of 16.8 ± 10.3 μg/mL and 22.0 ± 6.8 μg/mL were obtained, respectively for *M. ilicifolia* and *T. phaeocarpa* extracts, with corresponding SI of 3.4 and 4.8. SI values greater than 4 are recommended for an antiviral compound[37] and therefore both extracts should be considered promising sources of antiviral constituents, since their fractionation is expected to afford compounds with higher selective indexes.

The chemical composition of the two most active species – *T. phaeocarpa* and *M. ilicifolia* – has been previously investigated. The Brazilian endemic species *T. phaeocarpa* is traditionally used as antidiabetic and anti-inflammatory.[38] By using UPLC-ESI-MS/MS analyses, we recently identified 38 phenolic compounds in the ethanol extract of *T. phaeocarpa* leaves herein tested.[39] The identified constituents were mainly phenolic acids, ellagitannins and flavonoids, classes of compounds widely distributed in *Terminalia* species. Some of them have had their antiviral activity reported against different viruses, like the hydrolysable tannins casuarinin (from *Terminalia arjuna*) and chebulagic acid.[40] Furthermore, several flavonoids and hydrolysable tannins isolated from *Terminalia catappa* leaves (chebulinic acid, cyanidin, procyanidin, apigenin 8-C-(2′-galloyl)-β-D-glycoside, apigenin 6-C-(2′-galloyl)-β-D-glycoside, punicalin, and punicalagin) showed anti-HIV activity.[41] It is feasible to suppose that the polyphenolic compounds found in *T. phaeocarpa* extract may be responsible for the anti-ZIKV activity elicited by the extract.

M. ilicifolia is traditionally used in Brazil to treat gastric ulcer and other gastrointestinal problems.[42] Its chemical composition comprises mainly flavonoids, triterpenes, and steroids.[17] The chemical composition of the dichloromethane extract from *M. ilicifolia* leaves was here investigated by UPLC-ESI-MS/MS aiming to

detect triterpenes and steroids. The compounds were identified based on their MS/MS fragmentation pattern and comparison with reference compounds and literature data.[43] The obtained data disclosed friedelin, friedelinol, amyrin, lupeol, betulinic acid and ursolic acid as constituents of the extract (*Table 3*).

Lupeol has been described as active against Dengue virus serotype 2 in Vero cells.[44] In its turn, betulinic acid was active against ZIKV in human neural progenitor cells[36] and against Dengue virus serotype 2 in Huh7, BHK-21, HepG2, HEK293T and Vero cell lines.[45] β-amyrin has been reported to possess antiviral activity against influenza A and Herpes simplex virus,[46] whereas ursolic acid was shown to elicit potent antiviral effect against Herpes simplex virus, human immunodeficiency virus, and human hepatitis C virus.[47,48] Therefore, it is also feasible to assume that triterpenes might account for the anti-ZIKV activity elicited by the dichloromethane extract of *M. ilicifolia*. The phytochemical study of *M. ilicifolia* and *T. phaeocarpa* will be carried out in the future to isolate and unambiguously identify their anti-ZIKV constituents.

Conclusion

The chemosystematic approach was shown to be suited for selecting plant species with anti-ZIKV activity, since approximately 30% of tested extracts were active in ZIKV-infected Vero CCL-81 cells. SH-SY5Y neuronal cells infected with ZIKV were less responsive to treatment than the Vero CCL-81 lineage. The extracts of *M. ilicifolia* (dichloromethane), *T. phaeocarpa*, *M. rigida* and *E. grandiflorus* showed significant anti-ZIKV activity in SH-SY5Y neuronal cells. These extracts reduced viral load and cell death by maintaining or increasing cell viability. It is possible to infer that polyphenols and triterpenes are the bioactive constituents, respectively of *T. phaeocarpa* and *M. ilicifolia*, based on their occurrence in the species and the antiviral activity reported for some of them.

Experimental Section

Selection of Plant Species

A chemosystematic approach was adopted for selecting plants for study. A survey was carried out in Pubmed, Scopus, and SciFinder databases to identify plant species or plant genera for which the occurrence of polyphenols, triterpenes and steroids have been reported, and whose extracts were available in our laboratory extract collection. Collection data for the selected species are listed in *Table 4*.

Preparation of Extracts

The tested extracts were available at the extract collection of the Phytochemistry Laboratory, Faculty of Pharmacy, UFMG, Brazil. In brief, each plant material was dried in a ventilated oven, at < 50 °C. Portions (10 g) of the dried and powdered material were extracted by percolation (3× 50 mL) with ethanol 96 °GL, except **3** (percolation with acetone/water 7:3), **4** (percolation with AcOEt/MeOH 1:1), **23** (decoction with water; 10 g to 300 mL water), **25** (percolation with DCM) and **26** (percolation with MeOH) (*Table 1*). Solvent was removed under reduced pressure in a rotatory evaporator at < 50 °C. For the aqueous extract, water was removed by lyophilization. The crude extracts were kept in a desiccator, under vacuum, to eliminate the residual solvent. The tested extracts are listed in *Table 1*, along with the exsiccate numbers of the plants.

Chemicals

LC-MS grade acetonitrile and methanol were purchased from Merck and DMSO from Synth. Deionized water was obtained from a Milli-Q system (Millipore). Mansoin A (C1), ouratein D (C2), dihydroisocumarin (C3), lupeol (C6), dulcitol (C7), and L-(+)-bornesitol (C8), along with a mixture friedelin/friedelinol (M1) were previously obtained by our

Table 3. Compounds putatively identified in *M. ilicifolia* dichloromethane extract by UPLC-DAD-ESI-MS/MS using MRM in positive ionization mode.

Compound	Collision energy (V)	Retention time (min)	$[M+H]^+$ (*m/z*)	Fragment ion (*m/z*)
Ursolic acid	20	10.01	457	203; 191
Betulinic acid	15	12.20	457	393; 287
Friedelin	32	19.72	427	409; 121; 109
Friedelinol	32	11.03	429	411; 109
Lupeol	32	18.86	427	409; 137; 109
Amyrin	32	22.53	427	409; 149.1; 134.9; 109

Table 4. Collection data of plants species evaluated in the anti-ZIKV screening.

Plant species	Place of collection	Date	Collector	Identification
Baccharis altimontana G. Heiden et al.	Caparaó National Park (ES/MG)	07/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis brevifolia DC.	Serra da Calçada (MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis calvescens DC.	Serra da Calçada (MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis dracunculifolia DC.	Serra do Cipó (MG)	02/2011	Gustavo Heiden	Gustavo Heiden (Embrapa)
Baccharis hemiptera G. Heiden et al.	Caparaó National Park (ES/MG)	07/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis imbricata Malag.	Serra da Calçada (MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis intermixta Gardner	Serra do Cipó (MG)	06/2010	Gustavo Heiden	Gustavo Heiden (Embrapa)
Baccharis magnifica G. Heiden, Leoni & J. N. Nakaj.	Caparaó National Park (ES/MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis myriocephala DC.	Serra da Calçada (MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis opuntioides Mart. ex Baker	Serra da Calçada (MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis parvidentata Malag.	Serra da Calçada (MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis platypoda DC.	Serra da Calçada (MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis reticularia DC.	Serra da Calçada (MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Baccharis retusa DC.	Serra da Calçada (MG)	06/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Echinodorus grandiflorus (Cham. & Schltdl.) Micheli.	Telêmaco Borba (PR)	11/2006	Loana A. P. S. Johansson	João Renato Stehmann (UFMG)
Erythroxylum deciduum A. St.-Hil	Floresta Estadual do Uaimii, Ouro Preto (MG)	10/2007	João Renato Stehmann	João Renato Stehmann (UFMG)
Erythroxylum tortuosum Mart.	Parque da Aeronáutica, Lagoa Santa (MG)	09/2007	João Renato Stehmann	João Renato Stehmann (UFMG)
Hancornia speciosa Gomes	Purchased from Catedral Laboratory (MG)	06/2010	Purchased from Catedral Laboratory (MG)	João Renato Stehmann (UFMG)
Maytenus acanthophylla Reissek	Jequié, Bahia (rural area)	09/2016	Djalma Menezes de Oliveira	Rita Maria de Carvalho Okano (UESB)
Maytenus ilicifolia Mart. ex Reissek	Santa Catarina	06/1999	Paulo G. Ribeiro	Paulo G. Ribeiro (IAPAR)
Maytenus rigida Mart.	Jequié, Bahia (rural area)	09/2016	Djalma Menezes de Oliveira	Rita Maria de Carvalho Okano (UESB)
Maytenus truncata Reissek	Jequié, Bahia (rural area)	09/2016	Djalma Menezes de Oliveira	Rita Maria de Carvalho Okano (UESB)
Ouratea castaneifolia DC.	UFMG campus, Belo Horizonte (MG)	08/1999	Júlio A. Lombardi	Júlio A. Lombardi (UFMG)
Ouratea spectabilis Mart. ex Engl.	Perdizes (MG)	09/1999	Júlio A. Lombardi	Júlio A. Lombardi (UFMG)
Sida glaziovii K. Schum.	Prudente de Morais	11/1998	Mitzi Brandão	Mitzi Brandão (EPAMIG)
Symphyopappus brasiliensis (Gardner) R. M. King & H. Rob.	Caparaó National Park (ES/MG)	07/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Symphyopappus lymansmithii B. L. Rob.	Caparaó National Park (ES/MG)	07/2018	Geraldo W. Fernandes	Gustavo Heiden (Embrapa)
Terminalia phaeocarpa Eichler	UFMG campus, Belo Horizonte (MG)	01/2017	João Renato Stehmann	João Renato Stehmann (UFMG)

Embrapa = Empresa Brasileira de Pesquisa Agropecuária; EPAMIG = Empresa de Pesquisa Agropecuária de Minas Gerais; IAPAR = Instituto Agronômico do Paraná; UFMG = Universidade Federal de Minas Gerais; UESB = Universidade Estadual do Sudoeste da Bahia.

group as detailed in the *Supporting Information* (*Figure S8*). All isolated compounds presented purity > 95%, as attested by chromatographic and NMR analyses. *trans*-Aconitic acid (C4) and *cis*-aconitic acid (C5) were purchased from Sigma.

Cells and Virus Cultures

Vero cells ATCC CCL-81 were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and antibiotics, and SH-SY5Y cells ATCC CRL-2266 were grown in DMEM/F-12 medium supplemented with non-essential amino acids (1%), sodium pyruvate (1 mM), L-glutamine (2 mM), 10% FBS, both kept at 37 °C in a 5% CO_2 atmosphere. A clinical isolate of ZIKV (HS-2015-BA-01; access number www.ncbi.nlm.nih.gov/nuccore/KX520666) was kindly provided by Prof. Sílvia Sardi, Universidade Federal da Bahia, and used in the assays. It was propagated in *Aedes albopictus* C6/36 cells (Rio de Janeiro Cell Bank BCRJ 0343) and kept in a BOD incubator, at 28 °C, in the presence of L-15 medium supplemented with 2% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C and 5% CO_2, for 7 days. Cell supernatant obtained from the infected cultures was centrifuged at 644 *g* for 10 min to remove cell debris from the cell culture and concentrated using centrifugal filters Amicon (Ultracel®-100 K).

Sample Preparation for Testing

Stock solutions of extracts and M1, stored at −20 °C until the day of the test, were prepared by diluting 2.0 mg of each sample in 100 μL DMSO (20 mg/mL). Compounds C1-C8 were diluted in DMSO to produce the stock solutions (500 μM). They were diluted with RPMI to obtain the working solutions, whose maxima DMSO concentration was 0.3% v/v.

Viability Assays

Cell's viability was assessed by the MTT and LDH assays, performed with cells from three different cell passages, each one carried out as experimental triplicates ($n = 9$). The MTT assay was undertaken as previously described,[49] employing DMSO as negative control. Details of the assay are available in the *Supporting Information*. Cell viability was calculated from the ratio between the absorbances of treated and untreated cells. Samples that gave cell viability higher than 80% were considered non-toxic for the Vero CCL-81 cell lines. CC_{50} values were determined for the extracts that showed antiviral activity by non-linear regression analysis of the concentration-response curves. Data were analyzed using the GraphPad Prism 5.0 software (GraphPad Software, Inc). CC_{50} was expressed as the mean ± standard deviation of three different experiments carried out in triplicate ($n = 9$).

The lactate-dehydrogenase (LDH) assay was carried out using the Bioclin LDH Kit and Triton as the positive control. To perform the assay, an aliquot (4 μL) of the supernatant from each well was transferred to a 96-well microtiter plate and 200 μL of the LDH reagent (reagent 1: buffered substrate and reagent 2: coenzyme, in proportion 1:5) was added. The absorbance was measured at 340 nm in a microplate reader immediately after adding the LDH reagent, and at 1, 3 and 4 min later. Cell viability was calculated using the following equation (Bioclin LDH Kit): $\Delta/\text{min} = [(\text{Abs. } 1^{st} \text{ reading - Abs. } 2^{nd} \text{ reading}) + (\text{Abs. } 3^{rd} \text{ reading - Abs. } 4^{th} \text{ reading})]/2 \times 20794.34$, where the number is a correction factor given by the kit manufacturer.

The cytotoxicity of some selected extracts was assessed by the sulforhodamine B (SRB) assay,[50] with modifications, described in the *Supporting Information*. Cell viability was calculated as described above for the MTT method.

ZIKV Inhibition Assay

The anti-ZIKV activity of the extract and compounds was determined using a viral plaque reduction assay on Vero CCL-81 cells for all samples and on SH-SY5Y cells for selected samples. DMSO (0.3% v/v solution) and AH-D (100 μM solution in DMSO 0.3% v/v solution) were employed respectively as the negative and the positive controls. This peptide was kindly provided by Prof. Nanjoom Cho from Nanyang Technological University, Singapore. All antiviral assays were performed in triplicate, with three repetitions each ($n = 9$).

The following groups were used in the assays: cells plus medium; cells infected with ZIKV (MOI 1 and 0.001 respectively for Vero CCL-81 and SH-SY5Y cells); cells infected with ZIKV and treated with the vehicle (DMSO 0.3% v/v); and cells treated with the sample solutions and infected with ZIKV. The cells were distributed in a 96-well microtiter plate, at a density of 1×10^5 cells/well and incubated at 37 °C with 5% CO_2 for 24 h. Medium was removed, and cells were immediately infected with 100 μL of ZIKV (MOI 1 or 0.001). Then, plates were incubated at 37 °C with 5% CO_2 for 1 h to allow viral adsorption. In the sequence, the medium containing ZIKV was removed, and the cells were treated with 100 μL of the sample solutions, followed by the addition of 100 μL of RPMI medium supplemented with 2% FBS and incubation for 48 h at 37 °C with 5% CO_2. After this period, 100 μL of the

supernatant was collected and immediately stored at –80 °C to perform the viral lysis plate reduction test. Cell pellet was used to assess cell viability by the LDH and MTT assays.

To assess the ability of samples to reduce the number of ZIKV plaque forming units (PFU), Vero CCL-81 cells were cultured in 24-well plates (5×10^5 cells/well) until reaching 90% confluence. Then, 300 µL of each serial dilution of the supernatants (collected in the above-described antiviral assay) prepared in RPMI without FBS supplementation was added to the wells, whereas 300 µL of RPMI medium without FBS was added as the cell control wells. After 1 h adsorption at 37 °C, with manual homogenization every 10 min, supernatant was removed and 1.0 mL RPMI containing carboxymethyl cellulose (CMC) 1.6% v/v was added to each well, followed by incubation at 37 °C with 5% CO_2. After 4 days, medium was removed and cells fixed with 10% v/v formaldehyde solution for 2 h. After discarding the supernatant, cell monolayer was stained with a violet crystal solution (10% v/v buffered formaldehyde and 1% w/v violet crystal). ZIKV lysis plates were counted, and viral loads expressed as number of PFU/mL. IC_{50} values on SH-SY5Y cells were calculated using the GraphPad Prism 5.0 software for the extracts that showed significant reduction in viral loads. The selectivity index (SI) was calculated as the ratio between the median cytotoxic concentration and the median inhibitory concentration ($SI = CC_{50}/IC_{50}$).

Statistical Analysis

Results of the MTT and LDH assays are presented as mean ± standard error of mean (SEM), while viral loads are expressed as medians. CC_{50} data were compared by unidirectional ANOVA followed by Dunnett's test (cell control *versus* ZIKV + DMSO and/or ZIKV + treated; cell control *versus* DMSO and/or treated) or by multiple comparisons by Tukey's test (ZIKV + treated *versus* ZIKV + DMSO). *P* alues less than 0.05 ($p < 0.05$) were considered significant. GraphPad Prism version 5 was employed for data tabulation and analysis.

Chemical Characterization of M. ilicifolia *Active Extract*

The chemical composition of *M. ilicifolia* dichloromethane extract was characterized by UPLC-DAD-ESI-MS/MS using electrospray ionization (ESI) source. Sample preparation, equipment, and the protocol used in the analyses are available as *Supporting Information*. The compounds were identified by multiple reaction monitoring (MRM) in the positive ionization mode, using precursor-to-product ion transitions previously described for the reference compounds.[43]

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Author Contribution Statement

R.S.P., V.V.C., G.L.M.G., P.R.V.C., R.M.P., M.B.S.J., Y.O., G.H., G.W.F., D.M.O., D.G.S., M.M.T., F.C.B. participated in the design of the study and drafted the manuscript. R.S.P, P.R.V.C., R.M.P., M.B.S.J., Y.O., G.H., G.W.F., D.M.O., F.C.B. contributed with the selection the plants species and collection of plants species. R.S.P, P.R.V.C., R.M.P., D.M.O., F.C.B. performed the obtaining of extracts and isolated compounds. R.SP. and G.L.M.G. performed the experiments of viability assays and antiviral activities. R.S.P., V.V.C., P.R.V.C. carried out the statistical analyses. R.S.P, P.R.V.C., R.M.P., F.C.B. performed the chemical characterization of *M. ilicifolia* active extract. V.V.C., P.R.V.C., D.G.S., M.M.T., G.W.F., R.M.P., F.C.B critically revised the manuscript. All authors gave final approval for publication.

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