ACTA
AMAZONICA

http://dx.doi.org/10.1590/1809-4392202201421

ORIGINAL ARTICLE

Anti-herpes activity of the total alkaloid fraction from the branches of *Fusaea longifolia* (Annonaceae)

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ABSTRACT

The herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) occur worldwide. Infections caused by these viruses have great public health importance due to the growing resistance to the first-choice drug, acyclovir, especially in immunosuppressed patients. Alkaloids derived from species of Annonaceae have been reported as antiviral agents against HSV and others viruses. Within this context, we evaluated the antiviral activity of the total alkaloid fraction (TAF) extracted from the branches of *Fusaea longifolia* (Aubl.) Saff. (Annonaceae), a species native to the Amazon region, against the HSV-1 and HSV-2 viruses. The antiviral activity was evaluated through the plate reduction assay and the mode of action was investigated by a set of other assays. The TAF was active against the HSV-2 strain 333 and against the HSV-1 strains KOS and 29R (acyclovir resistant), with selectivity index values (SI = 50% cytotoxic concentration/50% effective concentration) of 5, 4 and 3, respectively. In the preliminary study of the anti-HSV-2 mode of action, TAF showed viral inhibitory effects if added up to 12 h post-infection, had virucidal activity and did not present viral inhibition in pre-treatment. Our results showed that the TAF exhibited anti-HSV activity. Regarding HSV-2, TAF acted after the viral infection and had virucidal activity. A mass spectrometry analysis revealed the presence of nine alkaloids in the TAF that had previously been reported for Annonaceae, including liriodenine, lysicamine and isoboldine, which have been described as potential anti-HSV-1 agents.

KEYWORDS: antiviral, virucidal, liriodenine, lysicamine, isoboldine

Atividade anti-herpes da fração total de alcaloides dos ramos da *Fusaea longifolia* (Annonaceae)

RESUMO

Os vírus herpes simplex tipo 1 (HSV-1) e tipo 2 (HSV-2) têm ampla ocorrência global. As infecções causadas por esses vírus têm grande importância em saúde pública devido à crescente resistência ao fármaco de primeira linha, aciclovir, principalmente em pacientes imunossuprimidos. Alcaloides derivados de espécies de Annonaceae têm sido relatados como agentes antivirais contra o HSV e outros vírus. Neste contexto, nós avaliamos a atividade antiviral da fração alcaloide total (TAF) dos ramos de *Fusaea longifolia* (Aubl.) Saff. (Annonaceae), uma espécie nativa da região amazônica, contra os vírus HSV-1 e HSV-2. A atividade antiviral foi avaliada através do ensaio de redução em placa e o modo de ação foi investigado por um conjunto de ensaios. O TAF foi ativo contra a cepa HSV-2 333 e contra as cepas HSV-1 KOS e 29R (resistente ao aciclovir), com valores de índice de seletividade (IS = 50% concentração citotóxica/50% concentração efetiva) de 5, 4 e 3, respectivamente. No estudo preliminar do modo de ação da atividade anti-HSV-2, o TAF inibiu a replicação viral quando adicionados até 12 h pós-infecção, apresentou atividade virucida e não apresentou inibição viral no pré-tratamento. Nossos resultados mostraram que o TAF exibiu atividade anti-HSV. Em relação ao HSV-2, o TAF atuou após a infecção viral e apresentou atividade virucida. Uma análise do TAF por espectrometria de massas identificou a presença de nove alcaloides, incluindo liriodenina, lisicamina e isoboldina, que já foram descritos como potenciais agentes anti-HSV-1.

PALAVRAS-CHAVE: antiviral, virucida, liriodenina, lisicamina, isoboldina

CITE AS: Dourado, S.H.A.; Santos, C.S.; Ferreira, V.S.; Pontes-Pires, A.F.; Valladão, D.M.S.; Ribeiro, E.B.; Souza, C.A.S.; Silva, F.M.A.; Costa, E.V.; Andrighetti, C.R. 2023. Anti-herpes activity of the total alkaloid fraction from the branches of *Fusaea longifolia* (Annonaceae). *Acta Amazonica* 53: 158-165.

INTRODUCTION

The herpes simplex viruses (HSV) type 1 (HSV-1) and type 2 (HSV-2) are distributed worldwide and produce lifelong infections with frequent reactivations throughout life (James *et al.* 2020). HSV-2 infection is the most common cause of recurrent genital ulcer disease (Looker *et al.* 2020) and is often related to negative psychosocial consequences such as shame, anxiety and depression (Treml *et al.* 2020). HSV-1 normally causes orolabial infection, but has been increasingly implicated as a cause of genital herpes (Looker *et al.* 2020). In 2016, it was estimated that 491 million people aged 15-49 years were infected by HSV-2 and that 3,752 million people were infected by HSV-1, at any site, in 0–49-year-olds. That data corresponds to a global prevalence of 13.2% and 66.6% of the world population, respectively (James *et al.* 2020).

HSV infections have great importance to public health due to the increasing problem of resistance to first-line drugs, acyclovir and its derivatives (Treml *et al.* 2020; Anton-Vazquez *et al.* 2020). Furthermore, epidemiological evidence suggests that HSV-2 infection also increases susceptibility to HIV infection, elevating it even further as a public health concern (Schiffer and Gottlieb 2019; Hopkins *et al.* 2020). Therefore, the search for new antiviral agents, with different mechanisms of action, is a fundamental and necessary issue (Sharifi-Rad, *et al.* 2018; Benassi-Zanqueta *et al*. 2019).

Amazonian species from the Anonnaceae family were described as having promising pharmacological activities, including anti-inflammatory (Silva *et al*. 2021), antimalarial (Nughara *et al.* 2019), anticancer, antimicrobial (Costa *et al.* 2010; Tundis *et al.* 2017; Paz *et al.* 2019; Costa *et al*. 2021) and antiviral (Yu *et al.* 2019; Warowicka *et al.* 2020).

Although the family has been widely investigated from the phytochemical point of view, and is considered a promising source of bioactive alkaloids (Lebouef *et al.* 1982), some Annonaceae species remain unexplored. One example is *Fusaea longifolia* (Aubl.) Saff., which is known in the Amazon region as *araticum*, *ata* or *biritá*, and is still in the early stages of phytochemical investigation (Lebouef *et al*.1982; Tavares *et al.* 2005; Rios *et al.* 2011). *Fusaea longifolia* is used to treat ailments by the Urubu-Kaapor indigenous people (Rios *et al.* 2011), and the essential oil of its aerial parts showed trypanocidal activity against amastigote and trypomastigote forms of *Trypanosoma cruzi* (Bay *et al.* 2019).

In this context, the present study aimed to evaluate the antiviral activity of the total alkaloid fraction from the branches of *F. longifolia* against the HSV-1 and HSV-2 viruses, as well as to perform the dereplication of the alkaloids from this sample.

MATERIAL AND METHODS

Plant material

Branches of *F. longifolia* were collected in the Sinop Forest Park (11°51' 37.4"S, 55°30'56.8"W), Mato Grosso state (Brazil) in September 2013. The botanical material was identified by Prof. Dr. Aline Fernandes Pontes Pires. A voucher specimen collected in July 2013 was deposited in the herbarium of Centro Norte Mato-Grossense (CNMT) at Universidade Federal do Mato Grosso, Campus of Sinop, Mato Grosso, Brazil (voucher # CNMT 6118). The access to genetic heritage was authorized by the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen) (protocol # A9AD5FF).

Alkaloid extraction

The branches were dried in an oven with air circulating at 45 °C for seven days. The ground plant material (52.63 g) was humidified with 5% ammonium hydroxide (NH_4OH) and subjected to exhaustive extraction by maceration with 95% ethanol, ratio of 1:5 (w/v). The extractive solution was concentrated under vacuum to obtain the crude ethanolic extract (CEE). The CEE (5.12 g) was put under mechanical stirring with 3% HCl and then filtered over Celite^R. The obtained filtrate (acid solution) was alkalized with NH_4OH (pH 9-10) and extracted with chloroform until negative to Dragendorff's reagent. The chloroform phase was concentrated under vacuum and provided 1.0361 g of a total alkaloid fraction (TAF) (Tavares *et al.* 2005).

Fractionation of the total alkaloid fraction

Part of the total alkaloid fraction (0.51 g) obtained was solubilized in chloroform and incorporated into 1 g of silica gel 60 (70-230 mesh, Merck) previously treated with a 10% sodium bicarbonate solution (Costa *et al.* 2010). After eliminating the solvent, the TAF was submitted to chromatographic fractionation in a chromatography column (CC) with 15 g of silica gel 60 (70-230 mesh, Merck) previously treated with 10% sodium bicarbonate. The alkaloids were eluted using a polarity gradient with *n*-hexane/dichloromethane/ethyl acetate/methanol), affording 170 fractions (15 mL each). The eluted fractions were analyzed and pooled according to the chromatographic profile obtained by thin layer chromatography (TLC), after examination under ultraviolet (UV) light (254 and 365 nm) and revealed with Dragendorff's reagent, affording seven groups: A (57.0 mg), B (18.6 mg), C (13.7 mg), D (29.9 mg), E (33.1 mg), F (92.1 mg) and G (134.4 mg). The groups were purified using preparative TLC eluted with chloroform:methanol (95:05, *v/v*) and UV light at 254 nm and Dragendorff's reagent for visualization. Sample recovery was performed using dichloromethane, chloroform, methanol and/or mixtures of these solvents.

Mass spectrometry analysis

The APCI-MS and APCI-MS/MS analyses were performed as following: the stock solutions of the samples A to G were prepared with methanol (HPLC grade) at 1.0 mg mL^{-1}, diluted to 10.0 μg mL^{-1}, and analyzed using direct infusion via an instrument syringe pump (10.0 μL min^{-1}) in a TSQ Quantum Access mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization source (APCI) and programmed to operate in positive acquisition mode (Lima *et al*. 2020).

Cells and viruses for *in vitro* assay

All biological assays were performed using Vero E6 cells, cultivated in Leibovitz medium (L-15), supplemented with 5% fetal bovine serum (FBS) and 1% PSA (10,000 IU mL^{-1} of penicillin G, 10,000 μg mL^{-1} of streptomycin and 25 μg mL^{-1} of amphotericin B), and maintained at 37 °C. The following viruses were used: Herpes simplex virus type 2 (HSV-2), 333 strain, from the Department of Clinical Virology, University of Goteborg, Sweden, and the Herpes simplex virus type 1 (HSV-1) KOS and 29R strains, which are acyclovir (AVC) sensitive and resistant, respectively, from the Laboratory of Applied Virology at the Federal University of Santa Catarina, Brazil. The Viruses were propagated in Vero E6 cells. Viral stocks were stored at -80°C and titrated based on plaque forming units (PFU) counted by plaque assay as previously described by Burleson *et al*. (1992).

Cytotoxicity assay

Cytotoxicity was determined by the MTT [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] assay (Mosmann 1983), with minor modifications. The Vero E6 cells (1.8 $x10^5$ cells mL^{-1}) were cultured in 96-well microplates at 37 °C for 24 h until confluence of the cell monolayer. Several concentrations of the TAF (200 μg mL^{-1} to 0.78 μg mL^{-1}) or acyclovir (225.21 μg mL^{-1} to 0.88 μg mL^{-1}), 1:2 ratio were added to the cells and incubated at 37 °C, for 48 h. After that period, the supernatants were removed and 50 μL of MTT solution (1mg mL^{-1}) was added per well for 4 h at 37 °C. Then the supernatant was removed and 100 μl of dimethyl sulfoxide (DMSO) was added in each well. All experiments were performed in duplicate with three plates. The absorbances were read in a spectrophotometer (Biolisa Reader R792, Bioclin, China) at 495 nm. The percentages of cell viability (X%), in relation to the untreated cell control were obtained by the following formula: X% = sample Abs x 100/untreated cell control Abs, where Abs corresponds to the absorbances. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced 50% of cell viability when compared to the untreated cell control.

Viral plaque reduction assay

This test was performed according to Kuo *et al.* (2001), with some modifications. Briefly, confluent cell monolayers (1.8 $x10^5$ cells mL^{-1}) in 24-well plates were infected with approximately 50 PFU of each virus strain for 1 h at 37 ºC. The viral suspension was removed and treated with different concentrations of the TAF (50.00 to 3.12 μg mL^{-1}, ratio 1:2) or acyclovir (positive control) (50.00 μg mL^{-1} to 0.05 μg mL^{-1}, ratio 1:10), prepared in a solution of L-15 medium (2X) containing 1.5% carboxymethylcellulose (CMC). After 72 hours or 48 hours for HSV-1 and HSV-2, respectively, the medium was removed, cells were fixed and stained with naphthol blue-black and plaques were counted. Experiments were performed in duplicate with three plates. The EC_{50} was calculated as the effective concentration that inhibited the formation of viral plaques by 50% when compared to untreated controls. The selectivity index ($SI=CC_{50}/EC_{50}$) was calculated.

Pre-treatment

This assay was performed according to Bertol *et al.* (2011). The Vero E6 cells confluent layer was treated with different concentrations of TAF (50 to 3.12 μg mL^{-1}, ratio 1:2) and incubated for 3 h at 37 ºC. After this period, the samples were removed, the cell monolayers were washed with PBS and infected with 50 PFU of HSV-2 for 1 h at 37 ºC. After this period, the medium was removed, the infected cells were overlaid with L-15 containing 1.5% CMC, incubated at 37 ºC for 48 h, and treated as described earlier for the plaque reduction assay. Experiments were performed in duplicate with three plates.

Virucidal activity

This test was performed according to Kuo *et al.* (2001), with some modifications. Mixtures of equal volumes of TAF at different concentrations (100 to 6.25 μg mL^{-1}, ratio 1:2) and 50 PFU of HSV-2 were co-incubated for 30 minutes at 37 °C. After this time, the residual infectivity was determined by plaque reduction assay as described above. Untreated viral suspensions were used as infection controls. Experiments were performed in duplicate with three plates. EC_{50} and SI values were calculated as described above.

TAF effect on viral adsorption

This assay was performed according to Bertol *et al.* (2011) with some modifications. Confluent Vero E6 cells were prechilled at 4 ºC for 30 minutes and exposed to a mixture of equal volumes of TAF at different concentrations (100 to 6.25 μg mL^{-1}, ratio 1:2) and 50 PFU of HSV-2 were incubated at 4 ºC for 2 hours. Viral controls were treated with culture medium only. Unabsorbed virus and remaining samples were removed by washing with cold PBS and cells were overlaid with L-15 containing 1.5% CMC, incubated at 37 ºC for 48

h, and treated as described earlier for plaque reduction assay. One of the viral controls was washed with a citrate buffer (pH = 3.0), because, at this pH, the viral particles adsorbed on the plasma membranes are destabilized, thus promoting virus release and elimination. Experiments were performed in duplicate with three plates.

Time-of-addition effect on viral replication cycle

This assay was performed according to Bertol *et al.* (2011) with some modifications. Vero E6 cells (1.8 x10^5 cells mL^{-1}) were infected with 50 PFU of HSV-2 and incubated for 1h at 37 ºC. The TAF was prepared at the maximum non-toxic concentration (50 µg mL^{-1}) and was added at different times: simultaneously with the viral suspension (time zero) and at intervals of 1, 2, 4, 6, 8, 10, 12, 18 and 24 h post- infection. After 48 h of incubation and cells were treated as described earlier for plaque reduction assay. Experiments were performed in triplicate with three plates.

Data analysis

The dates expressed as mean values ± standard deviation. CC_{50} and EC_{50} values were estimated from concentration effect curves after non-linear regression analysis by using Microsoft Excel®. Student´s *t* test ($p < 0.05$) was carried out to compare SI values of TAF and acyclovir (positive control) for each virus separately, using GraphPad Prism software (version 5.0 for Windows).

RESULTS

Antiviral evaluation of TAF fractions

The MTT assay to determine TAF cytotoxicity resulted in a CC_{50} of 72.9 ± 0.6 µg mL^{-1} (Table 1) and a maximum nontoxic concentration of 50.0 µg mL^{-1}, which was used in the antiviral activity assays. Acyclovir was not cytotoxic at the maximum concentration tested of 225.21 µg mL^{-1}.

The TAF showed antiherpes activity against the HSV-2 333 strain and the HSV-1 KOS and 29R strains, in non-cytotoxic concentration, with EC_{50} values of 15.31 ± 2.16 µg mL^{-1}, 16.9 ± 3.48 µg mL^{-1}, and 20.97 ± 4.42 µg mL^{-1}, respectively (Table 1). Acyclovir did not inhibit HSV-1 (strain 29R) replication and showed EC_{50} values of 1.76 ± 0.40 µg mL^{-1} and 1.13 ± 0.12 µg mL^{-1} against HSV-2 (strain 333) and HSV-1 (strain KOS), respectively. For a sample to present antiviral activity, the selectivity index (SI = CC_{50} /ÆEC_{50}) must be greater than 4 (Amoros 1992). For this reason, we continued the study of the TAF's antiherpes mechanism against HSV-2 strain 333 (Table 1). However, it is important to mention that the fraction showed antiviral activity against the acyclovir-resistant strain and, therefore, it may have a different mechanism of action than this drug.

In the pretreatment assay, no inhibition of HSV-2 replication was observed. In the virucidal assay, the complete inactivation of HSV-2 infectivity was observed, with a EC_{50} of 3.13 ± 18.86 µg mL^{-1} and a SI of 23. In the viral adsorption assay, the TAF showed 53.71 ± 14.10% of viral inhibition at the highest concentration tested (50.0 µg mL^{-1}), not being effective in other concentrations. In the time-of-addition test, the TAF (50.0 µg mL^{-1}) inhibited the replication of the HSV-2 virus when the fraction was added up to 12 h post-infection and decreased thereafter (Figure 1).

Mass spectrometry analysis

In positive mode, the mass spectra (Supplementary Material, Figure S1) of the fractions A-G displayed base peaks at *m/z* 276 (fraction A), 292 (fraction B), 322 (fraction C), 306 (fraction D), 296 (fraction E), 282 (fraction F), and 328 (fraction G), all even values, an indicative of protonated alkaloids (Lima *et al.* 2020).

The MS^2 spectra of the ions at *m/z* 276, 292, 322, and 306 306 (Supplementary Material, Figures S2-S5) presented key fragmentation patterns commonly observed in oxoaporphine

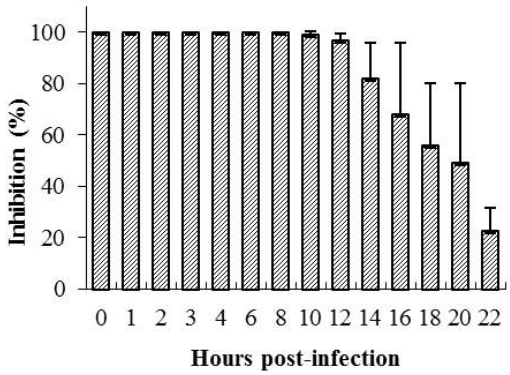

Figure 1. Effect of time-of-addition of the total alkaloid fraction (TAF) from *Fusaea longifolia* (50.0 µg mL^{-1}) on HSV-2 (333 strain) replication determined by plaque reduction assay. Results are plotted as % of viral inhibition in comparison to the untreated controls. Columns are the mean and bars the standard deviation of three independent experiments performed in duplicate.

Table 1. Cytotoxicity and anti-herpes activity of the total alkaloid fraction (TAF) of branches from *Fusaea longifolia*. CC_{50} = 50% cytotoxic concentration for Vero E6 cells (µg mL^{-1}); EC_{50} = 50% effective concentration that inhibited viral replication (µg mL^{-1}); Selective index (SI = CC_{50}/EC_{50}). NA = non active. Values are the mean ± standard deviation of three independent experiments. [a,b] Statistically significant inhibitory effect as related to acyclovir ($p < 0.05$) for each virus separately.

		HSV-2 333 strain		HSV-1 KOS strain		HSV-1 29R strain	
Sample	**CC_{50}**	**EC_{50}**	**SI**	**EC_{50}**	**SI**	**EC_{50}**	**SI**
TAF	72.90 ± 0.60	15.31 ± 2.16	5 [a]	16.9 ± 3.48	4[a]	20.97 ± 4.42	3
Acyclovir	> 225.21	1.76 ± 0.40	127[b]	1.13 ± 0.12	200[b]	NA	

alkaloids, such as carbon monoxide (CO) (28 Da) and a methyl radical (·CH_3) (15 Da) loss (Silva *et al.* 2017). At *m/z* 276, two subsequent losses of 28 Da (*m/z* 276 → *m/z* 248 and *m/z* 248 → *m/z* 220) were observed (Table 2). This fragmentation pattern is consistent with the oxoaporphine alkaloid liriodenine (Figure 2 [1]) (Silva *et al.* 2017; Lima *et al*. 2020). At *m/z* 292, 322, and 306, we observed an initial loss of 15 Da (*m/z* 292 → *m/z* 277; *m/z* 322 → *m/z* 307; *m/z* 306 → *m/z* 291), and subsequent losses of 29, 15 and 28 Da, respectively. The fragmentation patterns observed for the protonated molecules at *m/z* 292 and 322 were consistent with the oxoaporphine alkaloids lysicamine [2] and *O*-methylmoschatoline [3], respectively, while at *m/z* 306 it was consistent with the oxoaporphine alkaloids oxoputerine [4] (Silva *et al.* 2017).

The MS^2 spectra of the ions at *m/z* 296, 282, and 328 (Supplementary Material, Figure S6-S8) presented key fragmentation patterns commonly observed in aporphine alkaloids (Lima *et al.* 2020). The MS^2 spectrum of the protonated molecule at *m/z* 296 presented an initial loss of 17 Da (NH_3) (*m/z* 296 → 279), followed by subsequent losses of 30 Da (CH_2O) (*m/z* 279 → 249) and 28 Da (CO) (*m/z* 249 → 221), consistent with the aporphine alkaloid puterine [5] (Lima *et al.* 2020). At *m/z* 282, we observed an initial loss of 17 Da (*m/z* 282 → 265), followed by competitive losses of 15 Da (*m/z* 265 → 250) and 31 Da (·OCH_3) (*m/z* 265 → 234), consistent with the aporphine alkaloid nornuciferine [6] (Lima *et al.* 2020). At *m/z* 328, we observed an initial loss of 31 Da (CH_3NH_2) (*m/z* 328 → 297), followed by subsequent losses of 32 Da (CH_3OH) (*m/z* 297 → 265) and 28 Da (CO) (*m/z* 265 → 237), consistent with the aporphine alkaloid isoboldine [7] (Lima *et al.* 2020).

The ions at *m/z* 312 and 342 present in fractions F and C, respectively, were tentatively identified using fragmentation pattern analysis (Supplementary Material, Figure S9 and S10) and comparison with the literature. The MS^2 spectrum of the ion at *m/z* 312 presented an initial loss of 17 Da (*m/z* 312 → 295), followed by competitive losses of 15 Da (*m/z* 295 → 280) and 31 Da (*m/z* 295 → 244), consistent with the aporphine alkaloid *O*-methylisopiline [8] (Lima *et al.* 2020). Finally, at *m/z* 342, there was a high mass loss, commonly observed in tetrahydroprotoberberine alkaloids, with a base peak at *m/z* 178, which is in accordance with the structure of isocorypalmine [9] (Lima *et al.* 2020).

Table 2. Mass spectrometry data of the alkaloids tentatively identified in fractions A-G of the *Fusaea longifolia* branch extract. Class: Oxoaporphine (O), aporphine (A) or tetrahydroprotoberberine (T) alkaloid. MS/MS: Main product ions obtained in QqQ-MS/MS analysis.

Compounds	Class	$[M+H]^+$	MS/MS
Liriodenine (1)	O	276	248, 220
Lysicamine (2)	O	292	277, 248
O-Methylmoschatoline (3)	O	322	307, 292
Oxoputerine (4)	O	306	291, 263
Puterine (5)	A	296	279, 249, 221
Nornuciferine (6)	A	282	265, 250, 234
Isoboldine (7)	A	328	297, 265, 237
O-methylisopiline (8)	A	312	295, 280, 264
Isocorypalmine (9)	T	342	178, 165, 151

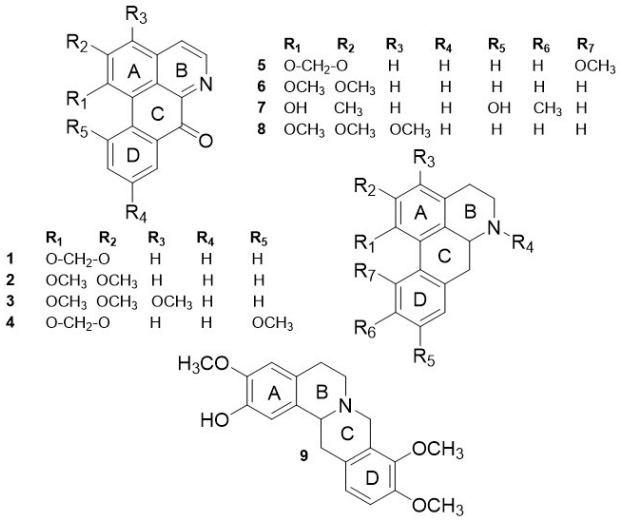

Figure 2. Structures of oxoaporphine and aporphine alkaloids present in the fractions A–G of *Fusaea longifolia* extract. Credit: F.M.A. Silva.

DISCUSSION

Cytotoxicity assessment prior to performing antiviral evaluations is important because it allows to determine that an observed reduction in virus infectivity is due to an effect of the samples on the virus, and not to a toxic effect on the host cells (Vlietinck *et al.* 1997; Schnitzler 2019). It also allows the determination of the maximum noncytotoxic concentration (MNTC) of a sample, which is used in antiviral assays (Pritchett *et al.* 2014; Schnitzler 2019). The MTT assay is one of the most widely exploited approaches for assessing cell viability and drug cytotoxicity (Stockert *et al.* 2018).

The *F. longifolia* TAF showed antiviral activity against both HSV-1 strains [KOS and 29R (acyclovir resistant)], as well as against the HSV-2 (strain 333) presenting SI values of 4, 3 and 5, respectively. The effect of TAF was considerably less potent then that of acyclovir against HSV-1 KOS and HSV-2 333, however, it showed antiherpes activity against HSV-1 29R, implying that the target of this fraction is probably different from those of acyclovir.

Although the TAF has shown activity against the HSV-1 strains that are sensitive (KOS) and resistant (29R) to acyclovir, we decided to study the antiviral mechanism of the TAF against HSV-2, which presented the highest selectivity index (SI) of 5. In antiviral activity studies, samples must be selective against the virus and not against the cells. The

SI represents the relationship between the 50% cytotoxic concentration and the 50% effective concentration (Amoros *et al.* 1992), and is a widely accepted criterion used to express the in vitro efficacy of a sample in terms of inhibiting virus replication (Pritchett *et al.* 2014).

Antiviral agents can be virucidal, causing an irreversible inactivation of the viral particle (virion) before it reaches the target cell, or they can interfere in the different stages of virus replication, from virus adsorption to the host cell until its release, or with virus binding, modifying cell membrane components, when incubated with host cells, before viral infection (Santos *et al.* 2013; Schnitzler 2019). Agents with virucidal capacity can damage the virion protein capsid, alter viral integrity, or penetrate into the virion and destroy the viral genome (Galabov 2007). Infections with HSV-2, and also with HSV-1, result in recurrent genital lesions (Looker *et al.* 2020). Thus, a strategy for the treatment and transmission prevention of these human viral infections is the development of topical formulations with compounds that can permanently inactivate viral particles (Ekblad *et al.* 2010; Mishra *et al.* 2018).

The synthesis of HSV viral gene products is carried out in three sequential phases, immediate (α), early (β) and late (γ). In the immediate phase, 2-4 hours after infection, the transcriptional machinery is activated and the proteins ICP4, ICP22, ICP27 and ICP47 are produced, which are fundamental for the production of all encoded viral proteins (Santos *et al.* 2013). In the early phase, which reaches its peak between 5-7 hours after infection, enzymes for viral replication are produced, e.g. thymidine kinase and viral DNA polymerase. In the late phase, until 12 hours after infection, are synthetized the structural components of the virions. The whole HSV cycle in cell lines takes about 18 to 20 hours (Santos *et al.* 2013). For these reasons, the prospection of the antiviral mechanism of action tested the TAF at different stages of the HSV-2 viral infection.

The pre-treatment of Vero E6 cells tested the effect of TAF on cells before they were infected with HSV-2, demonstrating no effect on virus replication, and therefore showing that the TAF has no *in vitro* prophylactic effect against HSV-2 infection. Yet, the TAF caused complete inactivation of HSV-2 infectivity at the lower EC_{50} value (< 3.1 ± 18.9 μg.mL^{-1} and SI > 23), i.e., the TAF showed direct action on viral particles, and significantly decreased their infectious capacity, maintaining 76 ± 6% of HSV-2 inhibition at the lowest concentration tested (3.1 μg mL^{-1}). The adsorption assay assessed whether the TAF was able to inhibit virus binding to the host cell membrane, one of the early steps of viral infection, with only 53.7 ± 14.1% of viral inhibition at the highest concentration tested (50 μg mL^{-1}) and no effectiveness in other concentrations.

The time-of-addition assay, which evaluated the antiviral activity in different stages of HSV-2 replication, the TAF inhibited almost 100% HSV-2 replication between 0 and 12 h post-infection, decreasing thereafter. These findings suggested that, the alkaloid(s) present in the TAF can interfere in the three sequential phases of the HSV-2 infection: immediate, early and late. New studies are necessary to investigate the effect of TAF on HSV-2 gene expression in all these stages.

Annonaceae alkaloids have already been described as having an antiviral activity (Padma *et al.* 1998; Yu *et al.* 2019). Of the aporphine alkaloids found in the TAF, isoboldine and nuciferine (Boustie *et al.* 1998) were active *in vitro* against the human poliovirus, with an SI > 14. Nuciferine was suggested as a prototype for the development of anti-HIV drugs (Kashiwada *et al.* 2005). Liriodenine, lysicamine and isoboldine were described as having antiviral activities against the HSV-1 virus strain H 29S, with SI of 6.9, 4.8 and 3.5, respectively (Montanha *et al.* 1995).

We showed that the TAF is active against the HSV-1 strain KOS (SI = 4), and the HSV-1 strain 29R (resistant to acyclovir) (SI = 3). This is the first report on the pharmacological activity of *F. longifolia* and the presence of the alkaloids liriodenine, lysicamine and isoboldine in its TAF, which are likely linked to its antiviral activity against the HSV-1 and HSV-2 viruses. Further studies should identify which alkaloids are involved in the antiviral activity and and their mechanisms of action and/or potential synergism.

CONCLUSIONS

The total alkaloid fraction (TAF) of *Fusaea longifolia* branches showed antiviral activity against the HSV-2 virus (strain 333) and the HSV-1 virus (strains KOS and 29R, the latter being acyclovir resistant). Against HSV-2, the TAF acted after the viral infection, with virucidal activity. Mass spectrometry identified the presence of nine alkaloids in the TAF, including liriodenine, lysicamine and isoboldine, which have already been described as having anti-HSV-1 activity.

ACKNOWLEDGMENTS

This study was partially supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grants # 481824/2013-8 and # 304577/2020-0) and CNPq/Institutos Nacionais de Ciência e Tecnologia (INCT) (grant # 465357/2014-8). C.S.S. and V.S.F. are grateful to CNPq for their undergraduate scholarship. The authors are also grateful to Central Analítica/Centro de Apoio Multidisciplinar /Universidade Federal do Amazonas (CA/CAM/UFAM) for the MS analysis, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Code 001, Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM), and Universidade Federal do Amazonas (UFAM) for financial support and fellowships.

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RECEIVED: 04/05/2022
ACCEPTED: 17/11/2022
ASSOCIATE EDITOR: Jorge Mauricio David

SUPPLEMENTARY MATERIAL (only available in the electronic version)

Dourado *et al*. Anti-herpes activity of the total alkaloid fraction from the branches of *Fusaea longifolia* (Annonaceae)

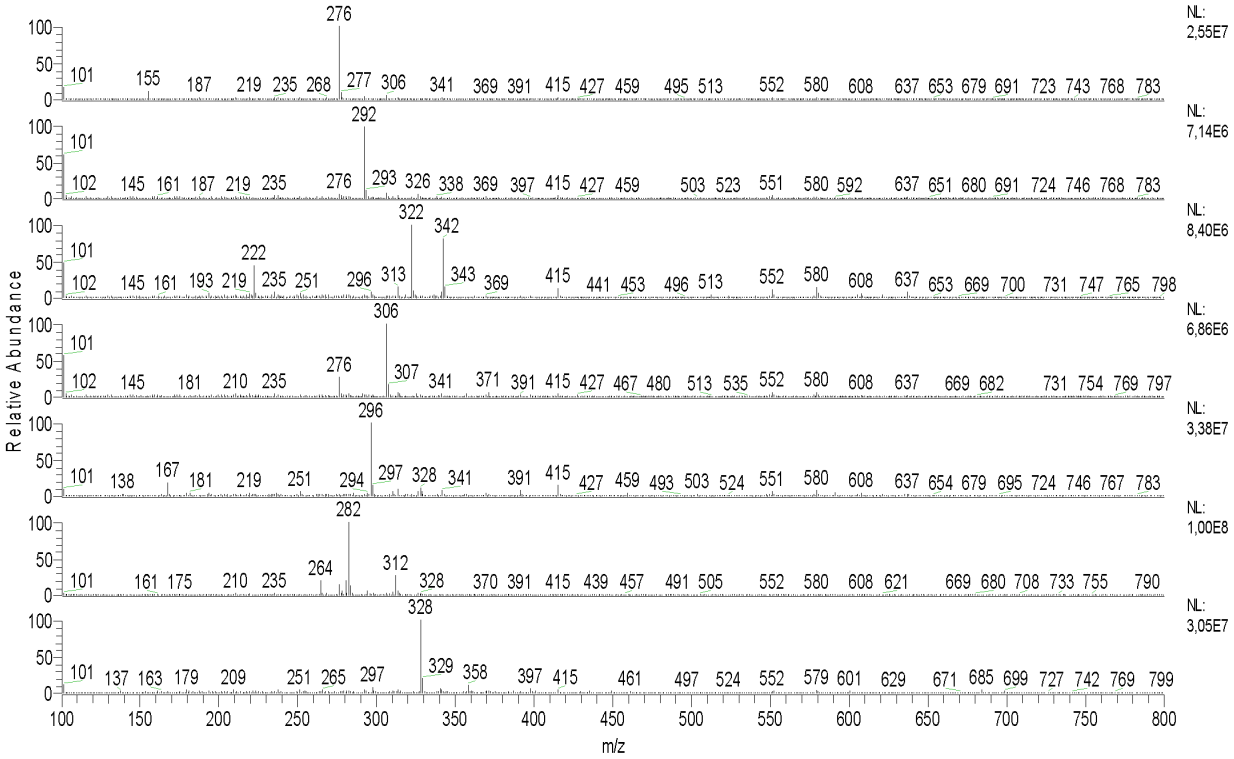

Figure S1. APCI-MS spectra of fractions A-G obtained from total alkaloid fraction (TAF) of branches from *Fusaea longifolia* (Aubl.) Saff. from Sinop, Mato Grosso, Brazil.

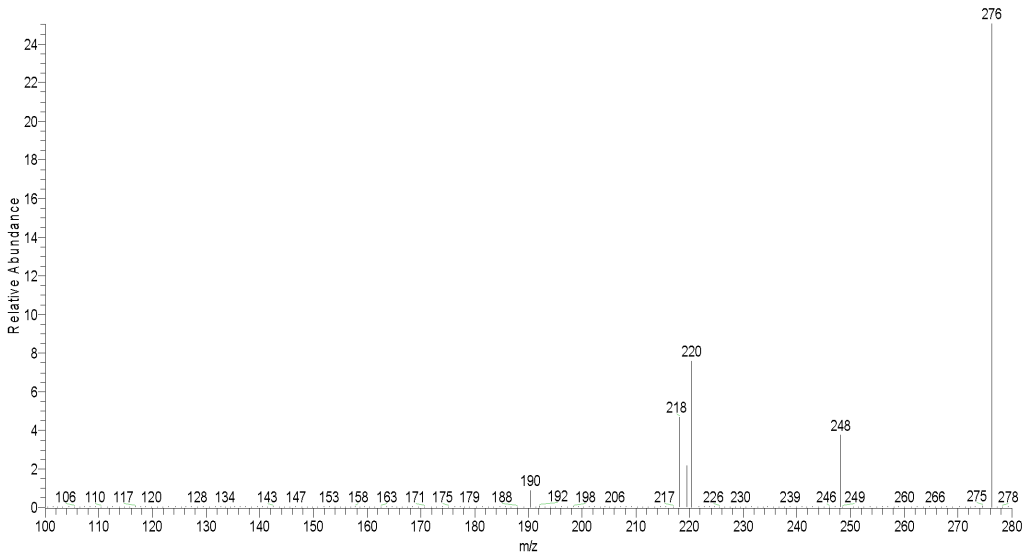

Figure S2. MS/MS spectrum of the ion at m/z 276 present in the fraction A of total alkaloid fraction (TAF) of branches from *Fusaea longifolia* from Sinop, Mato Grosso, Brazil.

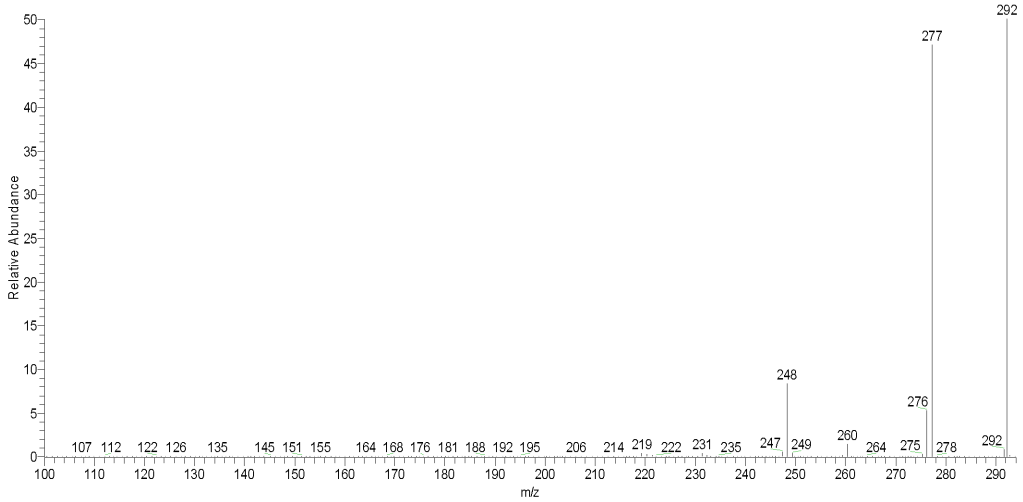

Figure S3. MS/MS spectrum of the ion at m/z 292 present in the fraction B of total alkaloid fraction (TAF) of branches from *Fusaea longifolia* from Sinop, Mato Grosso, Brazil.

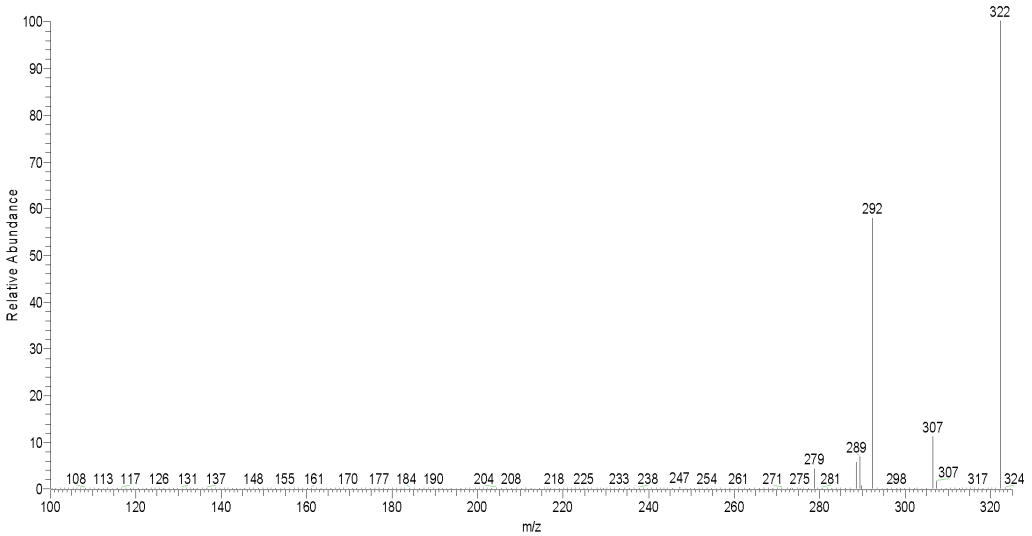

Figure S4. MS/MS spectrum of the ion at m/z 322 present in the fraction C of total alkaloid fraction (TAF) of branches from *Fusaea longifolia* from Sinop, Mato Grosso, Brazil.

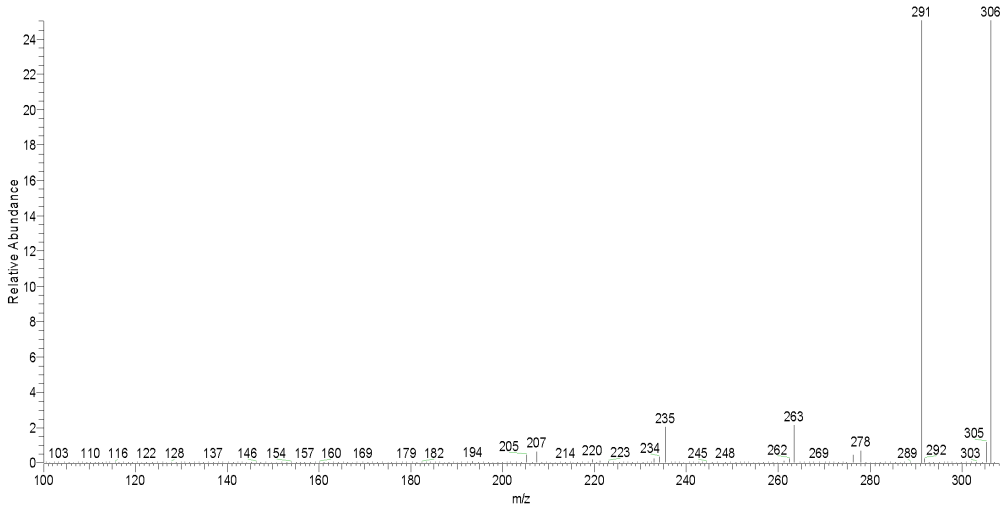

Figure S5. MS/MS spectrum of the ion at m/z 306 present in the fraction D of total alkaloid fraction (TAF) of branches from *Fusaea longifolia* from Sinop, Mato Grosso, Brazil.

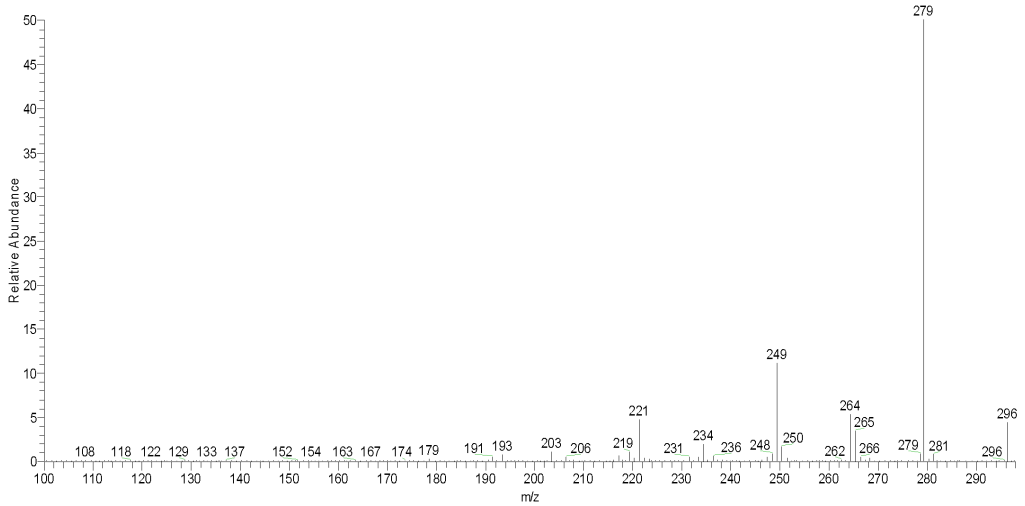

Figure S6. MS/MS spectrum of the ion at m/z 296 present in the fraction E of total alkaloid fraction (TAF) of branches from *Fusaea longifolia* from Sinop, Mato Grosso, Brazil.

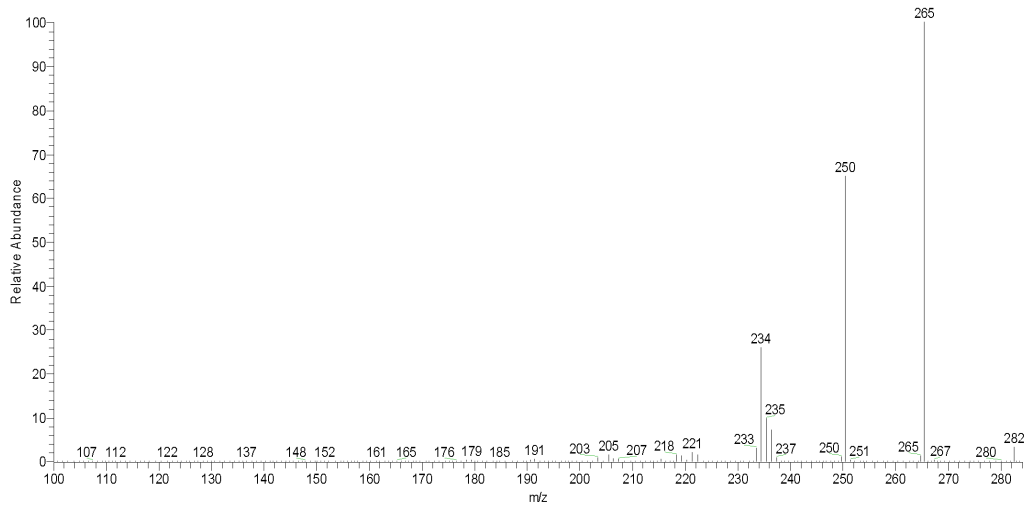

Figure S7. MS/MS spectrum of the ion at m/z 282 present in the fraction F of total alkaloid fraction (TAF) of branches from *Fusaea longifolia* from Sinop, Mato Grosso, Brazil.

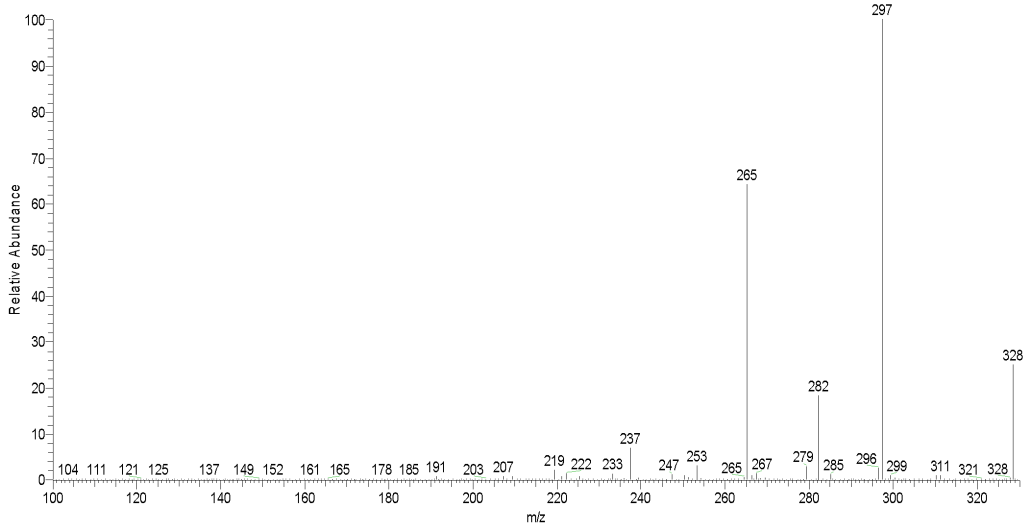

Figure S8. MS/MS spectrum of the ion at m/z 328 present in the fraction G of total alkaloid fraction (TAF) of branches from *Fusaea longifolia* from Sinop, Mato Grosso, Brazil.

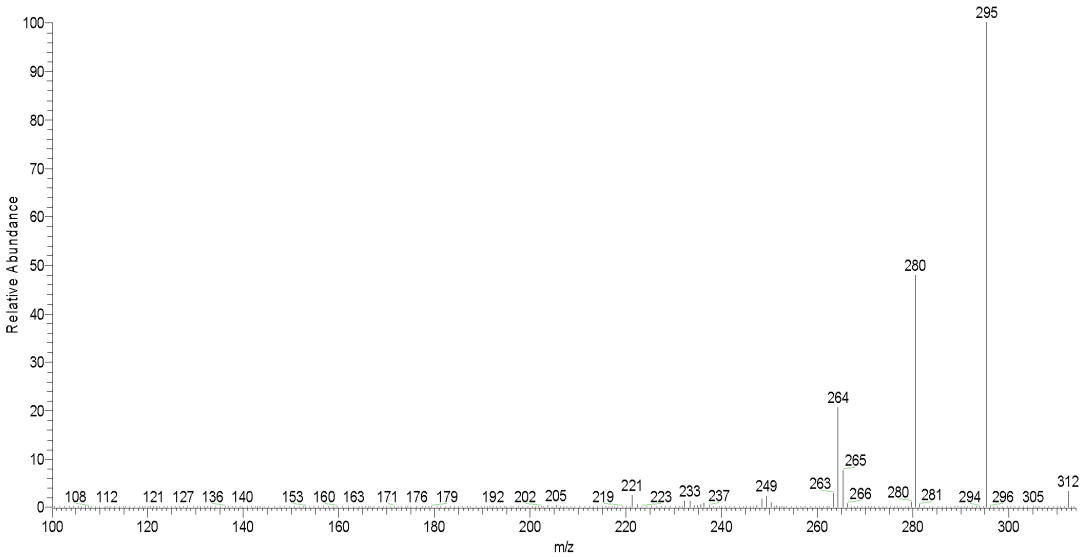

Figure S9. MS/MS spectrum of the ion at m/z 312 present in the fraction F of total alkaloid fraction (TAF) of branches from *Fusaea longifolia* from Sinop, Mato Grosso, Brazil.

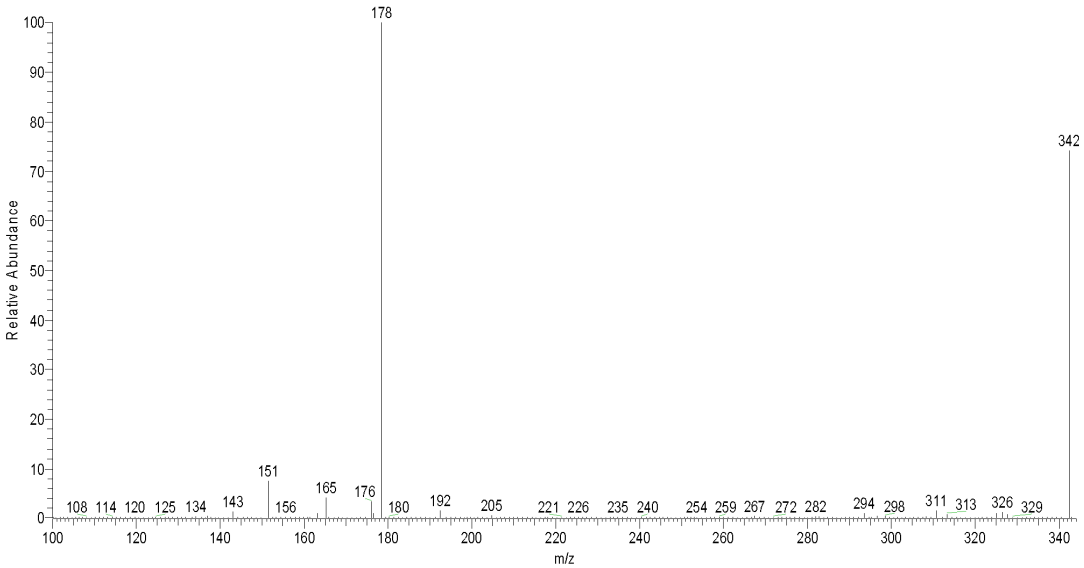

Figure S10. MS/MS spectrum of the ion at m/z 342 present in the fraction C of total alkaloid fraction (TAF) of branches from *Fusaea longifolia* from Sinop, Mato Grosso, Brazil.