An Acad Bras Cienc (2024) 96(1): e20200031 DOI 10.1590/0001-3765202320200031
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

Hamamelis virginiana L. extract presents antimicrobial and antibiofilm effects, absence of cytotoxicity, anti-inflammatory action, and potential to fight infections through the nitric oxide production by macrophages

ISABELA AMÊNDOLA,, DAIANE DE J. VIEGAS, EDUARDO T. FREITAS, JONATAS R. DE OLIVEIRA, JULIANA G. DOS SANTOS, FELIPE E. DE OLIVEIRA, AMANDIO A. LAGAREIRO NETTO, MARIA C. MARCUCCI, LUCIANE D. DE OLIVEIRA & GRAZIELLA N. BACK-BRITO

Abstract: The potential of *H. virginiana* L. was evaluated against *Candida* spp. (*C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, and *C. tropicalis*) and bacteria (*Acinetobacter baumannii*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus mutans*). Effect on murine macrophages (RAW 264.7) was also evaluated with respect to cytotoxicity and production of cytokines (IL-1β and TNF-α) and nitric oxide (NO). The most effective concentrations of the extract were determined by microdilution broth. These concentrations were analyzed on biofilms, after 5 min or 24 h exposure. Cytotoxicity was performed by MTT assay and quantification of cytokines and NO by ELISA and Griess reagent, respectively. The extract acted against the planktonic forms and provided significant reductions of all the microbial biofilms; besides, showed no cytotoxic effect, except at 100 mg/mL, after 24 h exposure. There was cytokine production; however, a modulatory effect was observed in groups exposed to lipopolysaccharide (LPS) from *E. coli*. NO production was similar or higher than the control group. Thus, *H. virginiana* L. extract showed antimicrobial and antibiofilm effects; absence of cytotoxicity for RAW 264.7; anti-inflammatory action; and potential to fight infections through the NO production.

Key words: Antibiofilm activity, anti-inflammatory activity, antimicrobial activity, cytotoxicity, *Hamamelis virginiana* L., nitric oxide.

INTRODUCTION

Hamamelis virginiana L. (*H. virginiana* L.) is a shrub, belonging to the family Hamamelidaceae, native to the region of Virginia, located in the eastern United States of America. Studies have shown that this plant has dozens of phytocomposites, such as flavonoids present in its leaves, as well as catechins, glycosides, tannins, volatile, and fixed oils, choline, free gallic acid, and free hamamelosis, capable of promoting several biological activities such as antiobesity (Boqué et al. 2013), astringent, non-toxic, chemotherapeutic (Dauer et al. 2003a), anti-oxidant (Mitjans et al. 2011), antigenotoxic (Dauer et al. 2003a), antimicrobial (Iauk et al. 2003, Theisen et al. 2014, Mouchrek Junior et al. 2015), and anti-inflammatory activities (Hughes-Formella et al. 1998, Wolff & Kieser 2007).

Scientific investigations with *Candida* spp. are relevant because of the intimate interaction

that these micro-organisms develop with humans, especially in their mucous layers. These yeasts can become highly infectious in cases of immunological weaknesses caused by some medicaments or diseases (Ramage et al. 2009, Silva et al. 2015). Besides, several bacterial species that harmonically inhabit humans have potential to be pathogenic and cause serious infections, including *Acinetobacter baumannii*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus mutans*. They are often isolated from nosocomial infections, and are difficult to treat, due to their ability to develop resistance to the conventional antimicrobials (Gay 2009, Bassyouni et al. 2015, Kavanaugh & Horswill 2016).

It is also necessary checking the cytocompatibility of these natural products, using mammalian cell cultures (Oliveira et al. 2017), such as macrophages (RAW 264.7), which are also cells involved in inflammatory response against microbes, releasing some chemical mediators such as interleukins (IL), tumor necrosis factor (TNF), interferons (INF), and nitric oxide (NO) (Mueller et al. 2010).

The emergence of new cases of resistance presented by some microbial species has been reported constantly; thereby, alternative methods for the control of these micro-organisms have also been studied frequently, including analyses performed with plant products such as extracts, essential oils, and phytocompounds. Additionally, it is also required developing new anti-inflammatory medicaments with controlled toxicity. Thus, the existence of plant products with therapeutic potential increases the importance to analyze them. This study investigated the effect of *H. virginiana* L. extract on some microbial species of medical-dental interest, including yeasts (*C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. tropicalis*) and bacteria (*A. baumannii*, *E. coli*, *E. faecalis*, *K. pneumoniae*, *S. aureus*, and *S. mutans*), both in planktonic cultures and biofilms. And also, in cell cultures of murine macrophages (RAW 264.7), in order to verify the interference of this plant extract in the cell viability. Additionally, the potential anti-inflammatory was also investigated in lipopolysaccharide (LPS)-stimulated RAW 264.7, by checking levels of the cytokines IL-1β and TNF-α. The capability to fight infections was also analyzed by NO quantification produced by the macrophages.

MATERIALS AND METHODS

Plant extract

Leaves of *H. virginiana* L. (Hamameliaceae INCI: *Hamamelis virginiana* Leaf Extract CAS No: 84696-19-5) were extracted in propylene glycol to obtain the glycolic extract, with 100% of purity. This *H. virginiana* L. extract (from Mapric, São Paulo, Brazil) was commercially acquired at 200 mg/mL. The company reported that the extract contains pyrogallol tannins (hamamelitanine), little essential oil, acid saponin, choline, fatty acids, mucilage, and pectin, according to the manufacturer.

Determination of soluble solids content of the plant extract

The plant extract (5 mL) remained at 80°C until complete drying. Then, solid compounds were cooled in a desiccator and the amount of soluble solids in the extract was calculated. The test was performed in triplicate.

Determination of total phenol content of the plant extract

A stock solution of extract diluted in ethanol and then in distilled water was prepared (1:100). An aliquot of 0.2 mL was added in 5 mL of distilled water. To this solution, 0.8 ml of

Folin-Ciocalteau reagent (Merck, Germany) was added. After shaking, 1.2 mL of 20% sodium carbonate-tartrate buffer was added between 1 and 8 min. The solution was kept in a water bath at 20°C for 2 h. Absorbance of the solution was read in a spectrophotometer (760 nm) and the amount of total phenols was determined by a straight line equation using the spreadsheet of calculation (Bankova & Marcucci 2015). The test was performed in triplicate.

Determination of total flavonoid content of the plant extract

A stock solution of extract in methanol was prepared (1:100). An aliquot of 0.2 mL was added in 5 mL of methanol. To this solution, 0.2 mL of aluminum chloride was added and the volume was completed to 10 mL with methanol. The solution was kept in a water bath at 20°C for 30 min. Absorbance of the solution was read in a spectrophotometer (425 nm) and the concentration of total flavonoid expressed in quercitina was determined by a straight-line equation using the spreadsheet of calculation (Bankova & Marcucci 2015). The test was performed in triplicate.

Antioxidant activity

The plant extract was diluted to 0.01% (V/V) and an aliquot was added in 10 mL of ethanol. Ten concentrations of the extract were prepared in ethanol and 2,2-diphenyl-1-picrylhydrazyl (DPPH - Sigma-Aldrich, St. Louis, USA) was added on these solutions. The DPPH radical was used as control (extract-free). After 30 min, the absorbance of the solutions was read in a spectrophotometer (517 nm). Optical density (OD) values were converted to micrograms per milliliter (µg/mL) and the concentration that eliminated 50% of free radicals (EC_{50}) was determined by the s spreadsheet of calculation and least squares method (Veiga et al. 2017). The test was performed in triplicate.

Chromatographic analysis of the plant extract

High-performance liquid chromatography (HPLC) was used to characterize and quantify the content of markers in the plant extract. For this purpose, a chromatograph with a photodiode detector (HPLC-DAD) and an automatic injector (D-7000 Merck-Hitachi) was used. The mobile phase of the chromatography was composed of water-formic acid solution (Merck) diluted in the ratio of 95:5 (solvent A) and methanol HPLC grade (Merck) (solvent B). The flow was 1 mL/min and the linear gradient started with 0% B and ended with 70% B in a running time of 50 min at 280 and 340 nm.

Microbial strains

Reference strains (ATCC - American Type Culture Colection) of *C. albicans* (serotype A – ATCC 36801), *C. dubliniensis* (ATCC MYA 646), *C. glabrata* (ATCC 9030), *C. guilliermondii* (ATCC 6260), *C. krusei* (ATCC 6258), *C. tropicalis* (ATCC13803), *A. baumannii* (ATCC 19606), *E. coli* (ATCC 25922), *E. faecalis* (ATCC 4083), *K. pneumoniae* (ATCC 4352), *S. aureus* (ATCC 6538), and *S. mutans* (ATCC 35688) from the Laboratory of Microbiology and Immunology (ICT - UNESP) were used in this study. The strains were stored at -80ºC in specific freezing media, Yeast Extract Peptone Dextrose broth (YPD - Himedia) with 16% glycerol for yeast and Brain Heart Infusion Broth (BHI - Himedia, Mumbai, India) with 20% glycerol for bacteria. For the tests, *Candida* spp. were reactivated in Sabouraud dextrose agar (SD - Himedia) and bacteria in BHI agar (Himedia) for 24 h at 37°C, with 5% CO_2 for *S. mutans*.

Antimicrobial activity analyzed on planktonic cultures

The broth microdilution test was performed to determine the minimum inhibitory concentration (MIC) of the extract, according to Clinical and Laboratory Standards Institute (CLSI), protocols M27-A2 (2002) and M27-S4 (2012), for yeast, and M7-A6 (2003), for bacteria. After reactivation of the micro-organisms, a stock suspension of sterile saline (NaCl 0.9%) with turbidity adjusted to 10^6 CFU/mL (colony forming units per milliliter) was prepared using a spectrophotometer (Micronal B-582, São Paulo, SP, Brazil). For *Candida* spp., RPMI 1640 broth buffered at pH 7 ± 0.1 with MOPS [3-(N-morpholino) propanesulfonic acid] (Sigma-Aldrich, St. Louis, USA) was used and for the bacteria, Mueller Hinton broth (Himedia). The microdilution was carried out in a 96-well plate, where 100 µL of the culture medium was added in 10 wells and 100 µL of the plant extract only in the first well, performing ten serial dilutions of the extract. Then, 100 µL/well of microbial suspension was added. The final concentration of the bacterial inoculum was 10^5 CFU/mL and the fungal inoculum was between 5 x 10^2 and 2.5 x 10^3 CFU/mL. Culture medium with inoculum was used as negative control and culture medium free of micro-organism as a positive control. This procedure was performed in duplicate with each micro-organism. After incubation (37ºC/24 h), the MIC of the plant extract was determined in the last well with no turbidity. For the determination of minimal fungicidal concentration (MFC) and minimal bactericidal concentration (MBC), drop technique was used (Naghili et al. 2013), which consists of adding a small aliquot of microbial suspension (20 µL) on the surface of the agar in triplicate and keeping it intact without spreading, under 37°C for 24 h. For that, the content of the well correspondent to the MIC and adjacent concentrations were seeded. MFC and MBC were found at the lowest concentration with absence of microbial growth.

Antibiofilm activity

The action against biofilms was verified according to Jesus et al. (2015). After reactivating the micro-organisms on agar (SD or BHI) and Yeast Nitrogen Base (YNB, Himedia) broth for *Candida* spp. or BHI for bacteria, for 24 h, each standard suspension was prepared at 10^7 CFU/mL in sterile saline (NaCl 0.9%). In 96-well plates were added 100 µL/well of the inoculum and initial adhesion of the micro-organisms was allowed for 90 min at 37°C under agitation (75 rpm). Subsequently, the supernatant was discarded, the wells were washed with saline solution to remove non-adherent cells and 200 µL/well of YNB or BHI broth were added. After 24 h of incubation the medium was replaced with fresh medium and the biofilm was formed during 48 h. The biofilms were treated with the most effective concentrations of the plant extract for 5 min or 24 h and saline was used as control (n = 12/experimental group). After washing with saline solution, the biofilms were disaggregated with ultrasonic homogenizer (Sonopuls HD 2200, 50W, Bandelin Electronic, Heinrichstraße, Berlin, Germany) for 30 s under 25% power. Serial dilutions were performed and aliquots of 20 µL were added on SD or BHI agar by means of the drop technique. After incubation (37ºC/48 h), the CFU/mL of each experimental group and the percentage of reduction of the biofilms were determined, in comparison to the control group.

Cell culture

Murine macrophages (RAW 264.7) were obtained from the cell bank of the Paul Ehrlich Technical Scientific Association (APABCAM, Rio de Janeiro, Rio de Janeiro). These cells were grown in tissue culture flasks with Dulbecco's Modified Eagle

Medium (DMEM - Sigma-Aldrich) supplemented with 10% fetal bovine serum (complete medium) and 1% penicillin-streptomycin (Gibco, Grand Island, United States) and incubated at 37°C at an atmospheric humidity of 5% CO_2. Viable cells were quantified by Trypan blue exclusion test (0.4%, Sigma-Aldrich) in automatic counting (Countess, Invitrogen, Paisley, UK).

Cytotoxicity

The cytotoxicity assay was conducted according to Oliveira et al. (2017). In 96-well plate (TPP) 4 x 10^4 viable cells were added per well. After 24 h incubation, the cells were exposed for 5 min or 24 h to different concentrations of *H. virginiana* extract, prepared in DMEM. Extract-free culture medium was used as control (n = 10/experimental group). The cells were then washed with phosphate-buffered saline (PBS, Cultilab, Brazil) and 100 µL/well of MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium solution - Sigma Aldrich] at 0.5 mg/mL PBS were added. After 1 h incubation, protected from light, the wells were washed with PBS, and 100 µL/well of dimethylsulfoxide (DMSO - Sigma Aldrich) were added and remained in contact with the cells for 10 min in the incubator and 10 min under agitation. The optical density (OD) of the wells were obtained with the aid of a spectrophotometer (BioTek, Winooski, VT, USA), at 570 nm, and were converted to percentage of cell viability.

Cytokines quantification

The test was performed according to Oliveira et al. (2017). In a 24-well plate 5 x 10^5 viable cells/mL DMEM were added and incubated for 24 h. The cells were exposed to the most effective concentrations of the plant extract or the extract-free culture medium (n = 10/experimental group). In the groups with exposure to the LPS from *E. coli* (Sigma-Aldrich), 1 µg/mL was added to these solutions. The exposure period was 24 h for all groups. Supernatants were collected and stored at -20°C for further quantification of IL-1β and TNF-α by ELISA, using commercial kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The optical density for the cytokine quantification tests was measured at 450 nm, using a microplate reader (EL808 - BioTek Instruments Inc., Winooski, Vermont, USA) and the values obtained were converted to pg/mL (pg/mL), with the aid of GraphPad Prism 5.0 software.

Nitric oxide production

The analysis was performed according to de Oliveira et al. (2017). In a 96-well plate, 100 µL/well of Griess reagent and cell supernatants were added, resulting in a final content of 200 µL/well. After 10 min under agitation, OD determination of the wells was performed in a microplate reader at 5x70 nm. These values were converted to µM (micromolar), considering the nitrite standard curve (Sigma-Aldrich), using GraphPad Prism 5.0 software.

Statistical analysis

The results were presented in mean values (± standard deviation) and analyzed by a one-way ANOVA and Tukey's test, with significance level of 5% ($P \leq 0.05$). This analysis was performed using GraphPad Prism 5.0 software.

RESULTS

Phytochemical analysis of the plant extract

This verification found: (a) soluble solids content = 1.93 ± 0.08%; (b) total phenol content = 42.39 ± 1.3 µg/mL; (c) total flavonoid content = 3.73 ± 0.04 µg/mL; and (d) concentration that eliminated 50% of free radicals (EC_{50}) = 27.32 µg/mL.

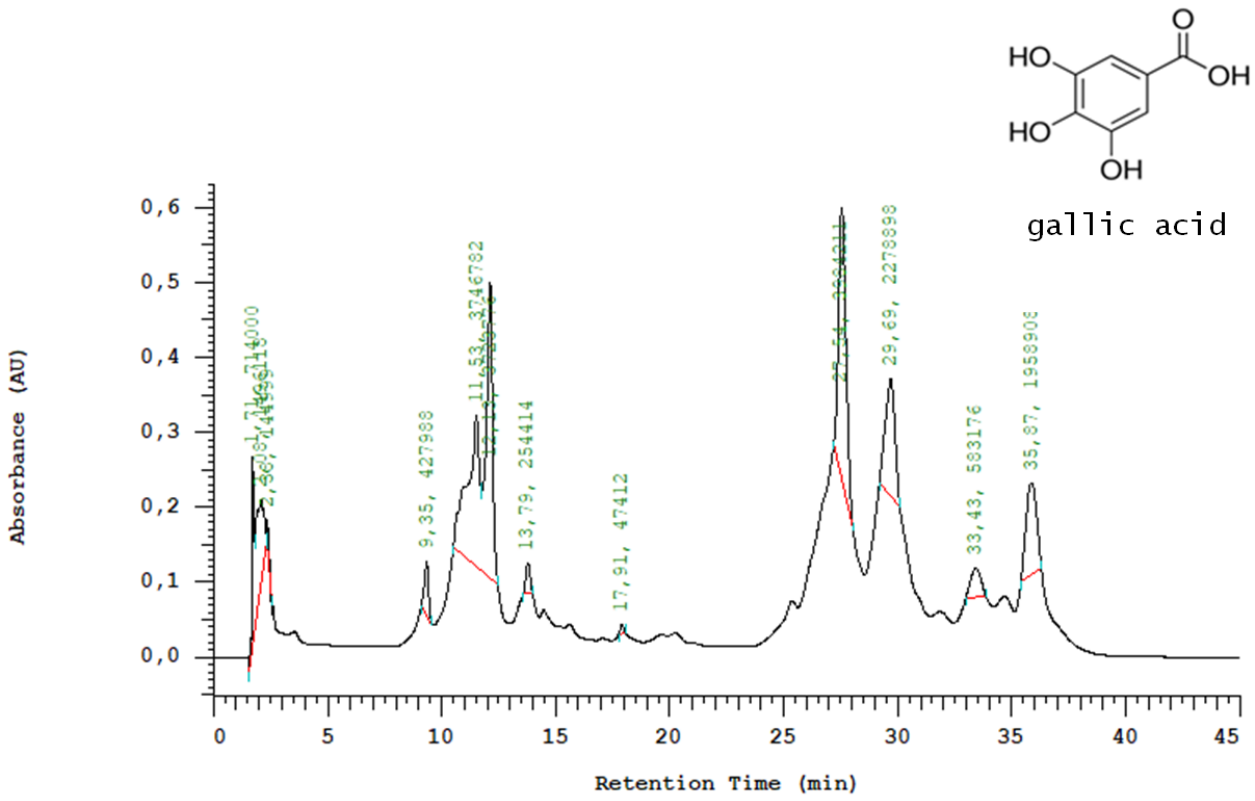

Figure 1. HPLC chromatogram of glycolic extract of *H.virginiana* L. Some derivatives of gallic acid (named gallactoyloses, such as hammamelitannin, according to Duckstein & Stintzing (2011), were found at retention times (tR) of 2.08 (1), 9.35 (2), 11.53 (3), 12.13 (4), 13.79 (5) and 27.54 (6) minutes. Chemical structure of gallic acid is showed enclosed.

HPLC analysis

Chromatographic analysis of the *H. virginiana* L. extract showed the presence of some derivatives of the gallic acid at retention times (Rt) of 2.08, 9.35, 11.53, 12.13, 13.79, and 27.54 min (Figure 1).

Antimicrobial activity on planktonic cultures

H. virginiana L. extract provided effective action against planktonic cultures, showing MIC | MFC (mg/mL) for *C. albicans* (1.56 | 6.25), *C. dubliniensis* (0.78 | 3.12), *C. glabrata* (1.56 | 6.25), *C. guilliermondii* (0.39 | 3.12), *C. krusei* (0.19 | 3.12), and *C. tropicalis* (0.39 | 3.12). The extract also presented MIC | MBC (mg/mL) for *A. baumannii* (3.13 | 12.5), *E. coli* (12.5 | 25), and *K. pneumoniae* (12.5 | 12.5). In addition, concentrations higher than 50 mg/mL were necessary to control *E. faecalis*, *S. aureus*, and *S. mutans* planktonic cultures.

Antibiofilm activity

Candida spp. biofilms exposed for 5 min at different concentrations of *H. virginiana* L. extract showed significant reductions in CFU/mL (Table I). The concentration of 200 mg/mL showed the highest percentage of *C. albicans* biofilm reduction; at 100 mg/mL, the highest reduction occurred in *C. glabrata*, *C. guilliermondii*, and *C. tropicalis*. For *C. dubliniensis*, similar reductions were obtained with concentrations of 50, 100, and 200 mg/mL. Regarding *C. krusei*, concentrations of 25 and 50 mg/mL had an effect similar to 100 mg/mL. After 24 h exposure time, significant reductions of fungal biofilms were also observed. At 25 and 12.5 mg/mL, the highest percentages were similarly demonstrated in *C. albicans* and *C. glabrata* biofilms. For *C. tropicalis* biofilm, the concentration of 12.5 mg/

Table I. Reduction percentage* of microbial biofilms using *H. virginiana* L. extract at different concentrations (mg/mL) in two exposure times.

	Micro-organism	5 min					24 h					
		12.5	25	50	100	200	3.13	6.25	12.5	25	50	100
Yeast	*C. albicans*	-	-	68 ± 2^{0}a	75 ± 25^{a}	100 ± 0^{b}	-	87 ± 15^{A}	97 ± 6^{B}	98 ± 4^{B}	-	-
	C. dubliniensis	-	-	96 ± 1^{2}a	99 ± 1^{a}	100 ± 0^{a}	-	100 ± 0^{A}	100 ± 0^{A}	100 ± 0^{A}	-	-
	C. glabrata	-	85 ± 12^{a}	76 ± 14^{a}	99 ± 0^{b}	-	-	99 ± 1^{A}	100 ± 0^{B}	100 ± 0^{B}	-	-
	C. guilliermondii	-	30 ± 30^{a}	66 ± 26^{b}	93 ± 5^{c}	-	96 ± 9^{A}	97 ± 3^{A}	100 ± 1^{A}	-	-	-
	C. krusei	-	48 ± 17^{a}	72 ± 12^{b}	56 ± 25^{ab}	-	66 ± 50^{A}	75 ± 33^{A}	79 ± 22^{A}	-	-	-
	C. tropicalis	-	84 ± 7^{a}	89 ± 8^{a}	96 ± 4^{b}	-	62 ± 27^{A}	51 ± 28^{A}	89 ± 11^{B}	-	-	
Bacterium	*A. baumannii*	-	98 ± 0^{a}	99 ± 0^{a}	100 ± 0^{b}	-	-	-	92 ± 24^{A}	93 ± 21^{A}	89 ± 21^{A}	-
	E. coli	82 ± 27^{a}	86 ± 22^{a}	75 ± 23^{a}	-	-	-	-	99 ± 1^{A}	99 ± 1^{A}	98 ± 2^{A}	-
	E. faecalis	-	-	54 ± 32^{a}	52 ± 27^{a}	40 ± 13^{a}	-	-	-	57 ± 30^{A}	91 ± 6^{B}	87 ± 18^{B}
	K. pneumoniae	-	-	49 ± 33^{a}	57 ± 27^{a}	51 ± 25^{a}	-	-	82 ± 14^{A}	88 ± 13^{AB}	98 ± 1^{B}	-
	S. aureus	-	-	51 ± 18^{a}	60 ± 18^{a}	65 ± 21^{a}	-	-	-	94 ± 7^{A}	100 ± 0^{B}	100 ± 0^{B}
	S. mutans	-	-	91 ± 4^{a}	79 ± 11^{a}	91 ± 40^{a}	-	-	-	93 ± 5^{A}	92 ± 4^{B}	96 ± 2^{B}

***Compared to the control group (treated with saline solution – 0.9% NaCl). "-" Concentration not evaluated. Different superscript letters indicate statistically significant difference between experimental groups, being lowercase letter for 5 min exposure and uppercase letter for 24 h exposure (One-way ANOVA, Tukey's Test; $P \leq 0.05$; n=12/group).**

mL showed the highest reduction percentage. For the biofilm of *C. guilliermondii* and *C. krusei*, no statistical difference was observed among the reductions provided by exposure to 3.13, 6.25, and 12.5 mg/mL, as well as for *C. dubliniensis* at concentrations of 6.25, 12.5 and 25 mg/mL.

Bacterial biofilms also showed significant reductions after exposure to *H. virginiana* L extract for 5 min (Table I). *A. baumannii* had a higher percentage of reduction after exposure to the concentration of 100 mg/mL. The effect of the concentrations of 50, 100, and 200 mg/mL was similar for biofilms of *E. faecalis*, *K. pneumoniae*, *S. aureus*, and *S. mutans*. On *E. coli,* concentrations of 12.5, 25, and 50 mg/mL presented the same effect. Additionally, after 24 h exposure, concentrations of 12.5, 25, and 50 mg/mL showed similar results on biofilms of *A. baumannii* and *E. coli*. On *E. faecalis*, *S. aureus* and *S. mutans* biofilms, there was a higher reduction at 50 and 100 mg/mL. The concentration of 50 mg/mL was the most effective for *K. pneumoniae* biofilm.

Cytotoxicity analysis

RAW 264.7 exposed for 5 min (Figure 2a) at different concentrations of *H. virginiana* L. extract showed a cell viability percentage similar to the control group (only DMEM). However, after 24 h exposure (Figure 2b), a significant increase in cell viability was observed with concentrations of 3.13, 6.25 and 12.5 mg/mL, compared to the control group. Only at 100 mg/mL it was observed a significant reduction in the viability of the macrophages.

Cytokine quantification

IL-1β levels were significantly decreased in the groups exposed to the LPS and treated with different concentrations of the extract. Significant inhibition of IL-1β was observed in treatments with 25, 50 and 100 mg/mL. TNF-α levels were also significantly decreased in both situations, i.e., stimulated or not by LPS and treated with the plant extract (Table II).

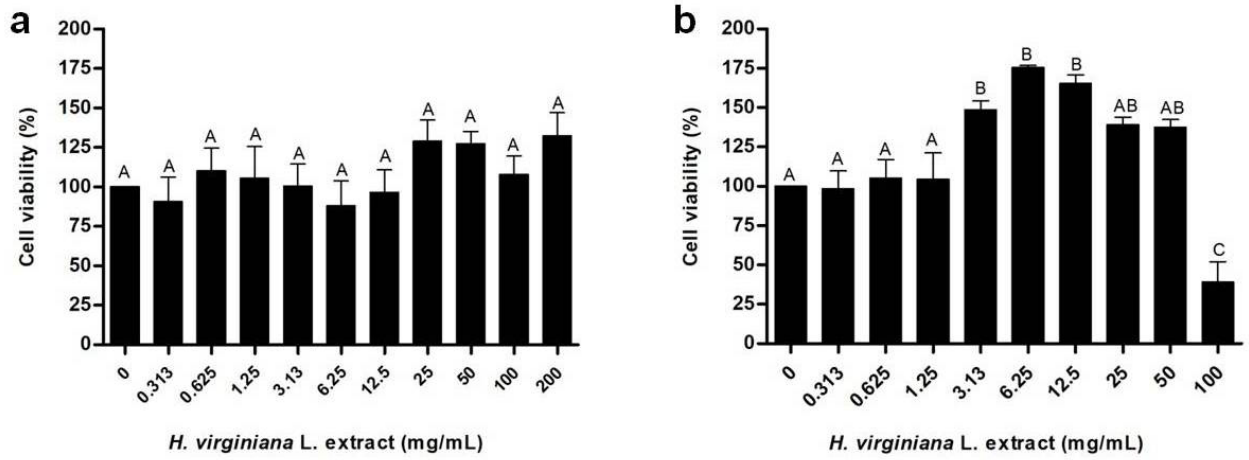

Figure 2. Mean values (± standard deviation) of cell viability percentage presented by murine macrophages (RAW 264.7) after exposure to the *H. virginiana* L. extract for 5 min (a) or 24 h (b), compared to the control group (0 mg/mL). Different letters indicate significant statistical difference (One-way ANOVA, Tukey's Test; $P \leq 0.05$; $n = 10$/group).

Nitric oxide production

A growing stimulus of NO production was observed using *H. virginiana* L. extract at 25, 50, and 100 mg/mL (Figure 3).

Discussion

Observing the results on the chemical analysis, the glycolic extract of *H.virginiana* L., contains a small amount of flavonoids and a more expressive concentration of total phenols. Polymeric proanthocyanidins (flavonoids with high molecular weight) were reported in *H.virginiana* L. (Dauer et al. 2003b), justifying our values of flavonoids and phenols in this study. It was reported that tannins react positively to total phenols assay. These kind of compounds are found in *H.virginiana* L. Its antitumor activity was reported by Sanchez-Tena et al. (2012).

It was reported that hamamelitanine (which are hydrolyzable tannins) are the main constituents of *H.virginiana* L. leaves and not proanthocyanidines, according to Duckstein & Stintzing (2011). Our reports about HPLC chromatogram of glycolic extract of *H.virginiana* L. showed that we have some derivatives of gallic acid (named gallactoyloses) such as hamamelitanine, comparing to the findings of the same authors. In this case we have different retention times but the UV spectra of all selected peaks are the same, indicating that we have similar chemical structures with a basic unit of gallic acid.

The antioxidant activity of the extract is comparable to propolis, reported by Veiga et al. (2017), whereas propolis is an excellent natural antioxidant. So we can conclude that proanthocyanidins and hamamelitanine, among others, are responsible for the pronounced antioxidant activity of *H.virginiana* L. extract.

In the present study, this extract provided growth inhibition and microbicidal effect for planktonic cultures of *Candida* spp. and also for Gram-negative bacteria such as *A. baumannii*, *E. coli*, and *K. pneumoniae*. Among the fungal species, *C. krusei* was the most susceptible to the extract. Regarding the bacterial species, *A. baumannii* had the lowest MIC and MBC. On the other hand, *S. aureus* and *S. mutans* were not susceptible to the concentrations evaluated. The antimicrobial effect of *H. virginiana* L. has

Table II. Production of pro-inflammatory cytokines IL-1β and TNF-α (pg/mL) by RAW 264.7 in the absence or presence of LPS.

H. virginiana L. extract (mg/mL)	IL-1β		TNF-α	
	no LPS	LPS	no LPS	LPS
0	0.14 ± 0.44^{A}	23 ± 2.52^{A}	32.85 ± 6.76^{A}	879.80 ± 219.20^{A}
25	0^{A}	0^{B}	6.32 ± 3.61^{B}	3.37 ± 2.26^{B}
50	0.67 ± 1.15^{AB}	0.02 ± 0.05^{B}	5.22 ± 4.31^{B}	3.52 ± 2.1^{B}
100	1.2 ± 1.1^{B}	0.22 ± 0.34^{B}	2.12 ± 2.98^{B}	0.36 ± 0.44^{B}

Mean values (± standard deviation) of IL-1β and TNF-α (pg/mL) levels released by RAW 264.7 (5×10^5 viable cells/mL) after contact with *H. virginiana* L. extract (mg/mL) at different concentrations for 24 h, both in the absence and presence of LPS (1 µg/mL). Different superscript letters indicate statistically significant difference between experimental groups (*H. virginiana* L. extract). (One-way ANOVA, Tukey's Test; $P \leq 0.05$; $n = 10$/group).

also been reported on periodontopathogenic species, such as *Porphyromonas gingivalis*, *Porphyromonas asaccharolityca*, *Prevotella melaninogenica*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Capnocytophaga gingivalis*, *Veillonella parvula*, *Eikenella corrodens*, *Peptostreptococcus micros*, and *Actinomyces odontolyticus* (Iauk et al. 2003). In addition, one of the main phytocompounds of *H. virginiana* L., hamamelitanine, provided a significant effect on *S. aureus* isolates resistant to different antimicrobials, such as cefazolin, erythromycin, levofloxacin, doxycylin, ciprofloxacin, gentamycin, clindamycin, and chloramphenicol (Bassyouni et al. 2015).

Studies using glycolic extracts from other plant species with final concentration similar to the present study, as well as the methodology, showed effective antimicrobial activity in planktonic cultures, when comparing the groups treated with the control groups (de Oliveira et al. 2019, Jesus et al. 2015), suggesting that screening does not always need to be considered as the main factor for determining the inhibitory action of an extract (Holetz et al. 2002).

The plant extract also provided significant reductions of fungal and bacterial biofilms in both experimental times (5 min and 24 h), with application of different concentrations of the extract (Table I). Application of the two experimental times was used to verify the action of the plant extract in faster treatments, for oral hygiene performed by dentifrice with this vegetal product, and also in longer treatment, for endodontic and periodontal practices. In the future, we intend to elaborate oral hygiene products or medications for dental procedures, constituted by *H. virginiana* L. extract or its phytocompounds.

An example of applying natural products is a study that demonstrates the importance of alternative photosensitizers based on phytocompounds in antimicrobial Photodynamic therapy. *H. virginiana* extract promoted reduction up to 4 logs of *Enterococcus faecalis* with low toxicity about fibroblasts and low risk of tooth pigmentation (Nardini et al. 2019).

The use of membrane added to commercial extract of *H. virginiana* is another example that demonstrates the importance of using therapeutic alternatives for the treatment of infections by resistant microorganisms. The use of the extract allowed the formation of inhibition halo on *S. aureus*, *Pseudomonas aeruginosa* and *C. albicans*, being for the latter, more effective than fluconazole (Solis-Arevalo et al. 2019).

The antibiofilm effect of *H. virginiana* L. extract has been poorly reported and the confirmation of this action may contribute to future studies that aim to find alternative

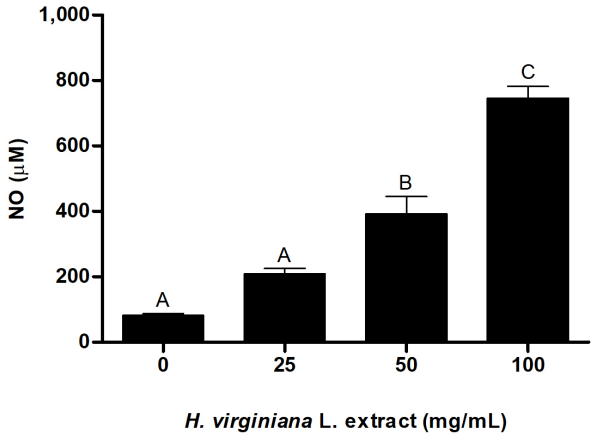

Figure 3. Mean (± standard deviation) of NO production (µM) by murine macrophages (RAW 264.7) after exposure to the *H. virginiana* L. extract for 24 h, compared to the control group (0 mg/mL). Different letters indicate significant statistical difference (One-way ANOVA, Tukey's Test; $P \leq 0.05$; $n = 10$/group).

methods to control micro-organisms responsible for causing infections that start in the oral cavity and may be systemically disseminated to other body organs. In this regard, clinical isolates of *S. aureus* (13 samples of methicillin-resistant and 3 strains susceptible to this antibiotic) and *Staphylococcus epidermidis* (14 resistant isolates) were not form biofilms, when the association between hamamelitanine from *H. virginiana* L. and vancomycin or clindamycin was used. Curiously, these samples were classified with high capacity to form biofilm. Besides, the addition of this phytocompound to vancomycin provided MIC50 and MIC90 < 0.25 µg/mL for biofilms, whereas MIC50 and MIC90 of 4 µg/mL for this antibiotic. In addition, with the association between clindamycin and hamamelitanine, it was observed MIC50 and MIC90 of 4 and 32 µg/mL, respectively (Bassyouni et al. 2015). This fact demonstrates the effectiveness of this phytocompound from H. virginiana L. to control bacterial biofilms.

In another study conducted in mice, it was demonstrated that subcutaneously implanted catheters, and previously soaked with solution of hamamelitanine (MIC, 0.5 x MIC, and 0.25 x MIC), showed significant infection control induced by *S. aureus*, *S. epidermidis* and *A. baumannii*. This phytocompound from *H. virginiana* L. promoted a reduction of biofilm metabolic activity (59%, 36%, and 37%, respectively) and biofilm biomass (48%, 35%, and 32%, respectively). However, no significant effect was demonstrated in *C. albicans* infection (Cobrado et al. 2013).

Although the phytocompound action was performed more frequently, a study with a mouthwash made from *H. virginiana* extract was conducted on dental biofilms of 10 patients. In this study, it was demonstrated a plaque index of 65% in the participants, during 7 days of treatment. After 14 days, this index was 61%, and an index of 59% was observed after 21 days. However, in patients treated with chlorhexidine this index was significantly lower, being 33%, 21%, and 14%, respectively (Mouchrek et al. 2015). Even so, the plant product was capable of acting as an antiplaque agent to control biofilms in patients.

The biocompatibility of plant products is also very important to be analyzed. In the present study, an exposure for 5 min to the *H. virginiana* extract was performed in RAW 264.7. And, all evaluated concentrations showed cell viability percentages similar to the control group, indicating absence of cytotoxicity. In groups exposed for 24 h, decreased cell viability was observed only in treatment with 100 mg/mL. Besides, increased cell viability was found in treatments with 3.13, 6.25, and 12.5 mg/mL, favoring a cell proliferation (Figure 2).

In a study carried out on human keratinocytes, the proliferation of these cells was checked after contact with polysaccharides from *H. virginiana* root at 0.1, 1, 10, and 50 µg/mL. After three days exposure, similar proliferation and cell differentiation were found in groups treated with 10 and 50 µg/mL when compared

to the control group. After 10 days, no influence of the extract was observed with respect to the keratinocytes proliferation. Decreased mitochondrial activity was also observed at 10 and 50 µg/mL compared to the control group. Additionally, tannin fractions presented no cytotoxic effect between 1 and 100 µg/mL (Deters et al. 2001). Some phytochemicals from *H. virginiana* L., such as catechins and hamamelitanine, have been also analyzed on hepatic tissue cultures (Hep G2), and no cytotoxic effect was found at 500 and 166 µg/mL, respectively (Dauer et al. 2003a). With this, it can be observed that products from plant origin may be promising to aid the cell viability, proliferation, and differentiation in adequate doses.

In this study, *H. virginiana* L. extract provided an inhibitory effect for synthesis of pro-inflammatory cytokines (IL-1β and TNF-α) in LPS-stimulated macrophages (RAW 264.7), using different concentrations (Table II). With these outcomes could be indicated the anti-inflammatory potential of the concentrations analyzed. In addition, anti-inflammatory effect of *H. virginiana* L. was also verified in a clinical study conducted with three experimental groups, composed of healthy patients and pretreated with (a) topical lotion containing *H. virginiana* L. 10%, (b) topical lotion with no *H. virginiana* L. and (c) topical lotion with no *H. virginiana* L. and exposed to ultraviolet B light. The results showed that erythema suppression tended to increase throughout the treatment, and topical lotion containing *H. virginiana* L. 10% afforded reduction of inflammatory process in 20% and 27%, after 7 h and 24 h exposure, respectively. These outcomes were significantly higher than those found with other products (Hughes-Formella et al. 1998).

Additionally, *H. virginiana* L. extract at 50 and 100 mg/mL also promoted NO generation by RAW 264.7 after 24 h exposure (Figure 3). The release of this molecule can be effective to control infections due it is present in inflammatory processes to fight invading micro-organisms (Moncada et al. 1991).

In conclusion, *H. virginiana* L. extract showed potential to control several microbial species, both in planktonic cultures and biofilms. It also showed absence of cytotoxicity at the concentrations analyzed, except for 100 mg/mL after 24 h exposure. Anti-inflammatory effect was evidenced by the inhibition of IL-1β and TNF-α in LPS-stimulated RAW 264.7. In addition, this extract could be an effective helper to combat infections, due it was able to induce NO release by macrophages. Briefly, the extract of *H. virginiana* L. presented antimicrobial, antibiofilm, anti-inflammatory activities and potential to control infections.

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How to cite

AMÊNDOLA I, VIEGAS DJ, FREITAS ET, OLIVEIRA JR, SANTOS JG, OLIVEIRA FE, LAGAREIRO NETTO AA, MARACUCCI MC, OLIVEIRA LD & BACK-BRITTO GN. 2023. *Hamamelis virginiana* L. extract presents antimicrobial and antibiofilm effects, absence of cytotoxicity, anti-inflammatory action, and potential to fight infections through the nitric oxide production by macrophages. An Acad Bras Cienc 96: e20200031. DOI 10.1590/0001-3765202320200031.

Manuscript received on January 14, 2020;
accepted for publication on June 15, 2020

ISABELA AMÊNDOLA[1]
https://orcid.org/0000-0002-0010-9757

DAIANE DE J. VIEGAS[1]
https://orcid.org/0000-0002-4829-6395

EDUARDO T. FREITAS[1]
https://orcid.org/0000-0003-4901-8124

JONATAS R. DE OLIVEIRA[2]
https://orcid.org/0000-0003-2398-6506

JULIANA G. DOS SANTOS[1]
https://orcid.org/0000-0002-2531-2102

FELIPE E. DE OLIVEIRA[1]
https://orcid.org/0000-0003-3026-646X

AMANDIO A. LAGAREIRO NETTO[3]
https://orcid.org/0000-0001-7337-9435

MARIA C. MARCUCCI[1]
https://orcid.org/0000-0002-8065-5618

LUCIANE D. DE OLIVEIRA[1]
https://orcid.org/0000-0001-9956-7768

GRAZIELLA N. BACK-BRITO[1]
https://orcid.org/0000-0003-3255-4289

[1]Universidade Estadual Paulista (UNESP), Instituto de Ciência e Tecnologia, Departamento de Biociências e Diagnóstico Bucal, Av. Engenheiro Francisco José Longo, 777, 12245-000 São José dos Campos, SP, Brazil

[2]Universidade Anhembi Morumbi, Escola de Medicina, Av. Deputado Benedito Matarazzo, 4050, Jardim Aquarius, 12230-002 São José dos Campos, SP, Brazil

[3]Universidade Anhanguera, Av. Raimundo Pereira de Magalhães, 3305, 05145-200 São Paulo, SP, Brazil

Correspondence to: **Jônatas R. de Oliveira**
E-mail: jroliveira16@hotmail.com

Author contributions

Design of the study: IA, JRO, LDO, GNBB. Acquisition, analysis, and interpretation of data: IA, DJV, ETF, JGS, AALN. Write of the manuscript: IA, JRO, FEO. Review of the manuscript: MCM, LDO, GNBB.