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RESEARCH ARTICLE

PHYTOCHEMICAL, CYTOTOXICITY, AND ANTIHERPESVIRAL COMPARISON BETWEEN THREE *LANTANA* SPECIES

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ABSTRACT

The *Lantana* L. genus, Verbenaceae, is a pan tropical flowering plant. Three species naturally growing in Mata Atlantica biome were chosen for this study: *L.camara*, *L . macrophylla a*and *L.undulata*. In folk medicine, these species are indicated to treat bronchitis and mucous secretions. Although *L. camara* is a well-known plant, little is known about *L. macrophylla* and *L. undulata*. The objective of this study was to perform chemical analysis, cytotoxic on Vero cells and antiherpesviral activity of three *Lantana* species. Phytochemical prospection demonstrated the presence of condensed tanins in all of three species; saponins were found only in *L. camara* and flavonones, flavonols and xantones, flavones and cathechins were seen only in *L. undulata*. The presence of oleanolic and ursolic acids in *L. macrophylla*, lantanolic acid in *L. camara* was suggested by thin layer chromatography technique. The content of condensed tannins was statiscally higher in *L. macrophylla* and *L. undulata* compared to *L. camara*. The cytotoxic effect to 50% of the cell culture (CE_{50}) and maximal cytotoxic concentration (CMNC) were stablished using tetrazolium technique and morphological evaluation. *L. camara* (CC_{50}=208.4 µg.mL^{-1}, CMNC=250 µg.mL^{-1}) and *L. macrophylla* (CC_{50}=2851 µg.mL^{-1}; CMNC=125 µg.mL^{-1}) were less toxic than *L. undulata* (CC_{50}=5.7 µg.mL^{-1}; CMNC=2.5 µg.mL^{-1}), and *L. macrophylla* showed the best results for antiviral (83%) and virucidal activity (99.7%). Taking together, results demonstrate the biological potential of *Lantana* species occurring in Mata Atlantica biome with emphasis to antiherpesvirus action.

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INTRODUCTION

The *Lantana* Linnaeus genus belongs to Verbenaceae family and is characterized by a perennial flowering plant. Native from tropical and subtropical America, the occurrence of this plant is reported in about 50 countries with around 150species depicted (Ghisalberti, 2000). In Brazil, the genus *Lantana* is widely distributed and can be found in almost all regions. Until now, among21 species described, 11 are endemic (Silva and Salimena, 2016).

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The three species chosen for this study were *L. camara* Linn which is world widely distributed and *L. macrophylla* Schauer and *L. undulata* Schrank which are endemic in this country (Salimena and Mulgura, 2016; Silva and Salimena, 2016). Popularly, the three species have similar use. *Lantana camara* Linn known bythe names of "arch man", "common lantana"; "cambará", "camará-de-chumbo", "camará-de-espinho" and "erva-chumbinho"is indicated to treat lung disease, asthma, bronchitis, catarrhal cough and rheumatism (Liberherbarum, 2015a). *Lantana macrophylla* Schaueror "cambará-de-folha-grande", as it is called popularly, is indicated for menstrual disorders, bronchitis and catarrh (Liberherbarum, 2015b); and *Lantana undulata* Schrank, such as *L. camara*: "cambará "and "cambará-chumbo", is indicated in the treatment of ashma, bronchitis, fatigue and bronchial catarrh (Liberherbarum,

2015c). The antiviral activity of *Lantana* species have been described in the literature. Balasubramanian et al. (2007) described antiviral activity of *L. camara* methanolic extract against *white spot syndrome virus* in shrimp. Garcia et al. (2010) showed the anti-*humanalphaherpesvirus* activity and direct inactivation of *dengue virus* by *L.grisebachii* essential oil. However, until now, no report of antiherpesviral activity by the species chosen here was seen. Taking into consideration the environmental conditions of plants growing in Bahia region which can may interfere in phytochemical and biological characteristics, the widely distributed *Lantanacamara* was chosen to compare with two local species. Thus, the objective of the present study was to compare the phytochemical profile, the toxic effect on Vero cell and the antiviral activity against *suidalphaherpesvirus* of *L. camara* L., *L. macrophylla* S. e *L. undulata* Sethanolic extracts.

MATERIAL AND METHODS

Botanic material: Aerial parts of the three species were collected in public domains of Uruçuca, Bahia, Brazil. The geographic coordinates were long: -39.039722; lat: -14.469722) for *L. camara* Linn; long: -39.0275; lat: -14.426667 for *L. macrophylla* Schauerand long: -39.054722; lat: 14.440833 for *L. undulata* Schrank. Specifically, botanic materials were collected in April 2015 for *L. camara* and *L. undulata* and August 2015 for *L. macrophylla*. These two periods were characterized by the severe drought that occurred in the region, being in the month of April the average rainfall of approximately 5.9 mm and in August of 11.8 mm (Inmet, 2016). The plant identification was confirmed by Luiz Alberto de Mattos at Universidade Estadual de Santa Cruz herbarium, Ilhéus, Bahia, Brazil and voucher numbers were HUESC21,064 (*L. camara*), HUESC 21,065 (*L. macrophylla*) e HUESC 21,066 (*L. undulata*).

Extract preparation: Leaves were selected and dried under forced ventilation at 50ºC. Thereafter,241.63 g of *L. macrophylla* powdered leaves were mixtured in 10 L ethanol; 50.13 g of *L. camara* powdered leaves was mixturedin 430.0 mL of ethanol; and 50.00 g of *L. undulata* powdered leaves was mixture in 100 mL of ethanol. After 24 h through direct contact with solvent and with mechanical agitation, marcs were filtered through What mannnumber 1 filter paper and evaporated under reduced pressure (Fisatom® Mod 550) at 50-60ºC. The extraction procedure was repeated three times for each plant yielding80.60 g of crude extract for*L. macrophylla*; 10.60 g for *L. camara*; and 3.11 g for *L. undulata.*All extracts were stored at 4-8ºC.

Phytochemical bioprospection: Phenol, condensed tannin, antocyanins, anthocyanidins, flavones, flavanons, flavonols, xanthonesleucoanthocyanidins, catechins, catechinases flavones, chalcones, aurones, steroids, triterpenoids, saponin, alkaloids and cumarins were qualitatively analysed in ethanolic extracts from leaves using methodology described by Matos (1997). Terpens analysis was performed by thin layer chromatography using lantanolic acid, ursolic acid and a mixture of oleanolic and ursolic acids as standard compounds. For that, ethanolic extracts were solubilized in chloroform and eluted chromatoplates covered with 60 g of silica gel (Vetec®) as stationary phase (0.25 mm)and ethyl acetate (Vetec®) 15 mL: hexane (Fmaia®) 20 mL, as eluent. Revelation was made with iodineand vanillin spray (Mattos, 1997). Retention factors(R_f) were measured.

Condensed tannins content: The tannins content was performed following the methodology described by Perez et al. (1999), with modifications. Aqueous acetone (80%) solutions at 2 mg. $mL^{-1}$of *L. camara* and at 4 mg. $mL^{-1}$of *L. macrophylla* and *L. undulata* were obtained from the ethanolic extracts. Thus, to a 500 µL of each solution, 3 mL of methanolicvanilline 4% solution were added. The mixture was homogenized and 1.5 mL of hydrochloric acid was added. After 20 minutes, samples were analyzed using spectrophotometer Nova 1600 UV at 500 nm. Dilutions at 5.0; 35.0; 55.0; 75.0 and 95.0 $\mu g.mL^{-1}$of catech in were used as standard curve. The equation was y = 0.003x - 0.0026 with R^2 de 0.981, being detection limit of 0.04 $\mu g.mL^{-1}$and quantification limit of 0.13 $\mu g.mL^{-1}$.

Cell culture: The Vero cell line (ATCC® CCL-81™) was maintained in Eagle essential medium (MEM) (Vitrocell, Atená®), supplemented with 8% bovine fetal serum (BFS) (Vitrocell, Atená®) at 37ºC with 5% CO_2. For cytotoxic, antiviral, and virucidalassay, Vero cells were seeded in 96 wells microplate at $3x10^4$cells density 24 h before tests.

Virus: The EMBRAPA: BRMSA 3, 00588 do *suidalphaherpesvirus* type 1 (SuHV- 1) was kindly provided by EMBRAPA/Concordia. Virus was routinely grown on Vero cell to obtain the viral stock. Initially, Vero cells were infected with SuHV-1 to obtain virus stock. After 72 h of incubation, cytopatic effect (ECP) was observed and the material was submitted to the clarification process for viral stock storage. Then, 50% Tissue Culture Infective Dose (TCID50) was established.

Cytotoxic evaluation: The cell viability was evaluated by morphological aspects to establish maximum non-cytotoxic concentration (MNCC)(Barros et al.,2011)and by the colorimetric method MTT (3- (4,5-dimethylthiazol-2-yl) -2,5 -diphenyltertrazolium bromide), where the concentration causing cytotoxic effect to 50% of the cell culture (CE_{50}) was determined (Mosmann,1983).

Antiviral assay: For antiviral and virucidal activity, Vero cells were seeded in 96-well microplates. After 24 h of culture, medium was discarded and plant extracts were added to the cell cultures in their respective MNCC.Cell monolayers were then treated with the extracts at their respective MNCC for 1 h and were inoculated with the logarithmic dilutions (10^{-1} to 10^{-7}) of each correspondent virus. Controls consisted of untreated infected (virus titer), treated non-infected (cytotoxicity control) and untreated non-infected (cell control) cells. The tests were done in triplicate and repeated twice.The antiviral activity of the extracts was determined by the reduction of virus titers through Reed and Muench (1938) to establish 50% tissue culture infective dose (TCID50). The difference of viral titer between treated and untreated control cultures was expressed as viral inhibition index (VII) (KOSEKI et al., 1990). Also the inhibition percentage (IP) was calculated using the anti-logarithmic $TCID_{50}$: PI = (1 - antilogT / antilogC) x 100where T corresponds to the virus titer of the extract treated cells and C is the viral titer of the untreated cells (Nishimura et al., 1977).

Virucidal assay: The virucidal effect was assessed using the technique described in Schuhmacher et al. (2003) and Barros et al. (2010) with modifications. Equal parts of the extract at MNCC and the virus dilutions (10^{-1} to 10^{-7})were mixed and left to rest at room temperature (25 - 28 °C) for 1 h. Controls consisted of equal parts of virus and MEM without serum, medium without serum, and the extracts in their respective MNCC only. Then, 100 µL final volume of the treatments were added to 96 well microplates containing 24 h seeded Vero cells, in triplicate. After 72 h of incubation the cytopathic effect was evaluated to established IIV and IP values. These tests were repeated three times.

RESULTS AND DISCUSSION

Through phytochemical prospection, a variety of compounds was seen in the three *Lantana* species investigated in this study. Condensed tannins were found in all three species and quantification revealed significant difference between species highlighting the higher amount produced by *L. macrophylla* (Table 1). Besides condensed tannins, *L. undulata* leaves presented flavonones, flavonols and xantones, flavones and cathechins, and alkaloids being the most variables in composition. *L. camara* leaves presented also saponins and flavonol and *L. macrophylla* leaves presented only alkaloids besides condensed tannins. Alkaloides, phenolic compounds, flavonoid, tannin, saponin, glycosides and triterpenoids were already reported in *Lantana* species (Venkataswamy et al., 2010;Naz and Bano, 2013) showing that a kind of variability is expected in this genus. However, it is worth to note that although tannins and flavonoids commonly occur in *Lantana* species, the *L. macrophylla* studied here did not show flavonoids. Also, terpenoids were not detected by the methodology used. The fact that some metabolites are not detected may be due to small amounts found in the plant organ or limitations in the techniques used. The metabolomics analysis of natural variation is important to understand how a plant species functions in the wild or the field (Soltis and Kliebenstein, 2015). Here, the differences on metabolites classes identified for *L. macrophylla* raises doubts in its placement in *Lantana* genus (Lu-Irving and Olmstead, 2013) indicating genetic variation of this specie. *L. macrophylla* presents remarkable morphological characteristic and it is generally found as isolated individuals and not as abundant as the other two.

The TLC and $R_f$analysis showed the presence of terpenoids in the leaves of two of the *Lantana* species analyzed. Lantanolic acid was detected in *L. camara*, oleanolic and ursolic acids in *L. macrophylla* (Figure 1).The presence of such acids in *Lantana* species is known. Lantanolic acid (Barua et al., 1971; Begum et al., 1995) and oleanolic acid (Begum et al., 1995) were described in aerial parts of *L. camara*; oleanolic and ursolic acids were found in *L. indica* roots (Singh et al., 1990); and, oleanolic, ursolic, and lantanolic acids were detected in *L. macrophylla* leaves (da Conceição et al., 2012) indicating *Lantana* genus as a common source of triterpenic acids. The biological activity of these triterpenic acids has been reported in the literature. Liu (1995) reported antimicrobial, hepatoprotection, antitumor andanti-inflammatory as biological activity related to oleanolicand ursolicacids. Begum et al. (2008) identified antihelminticacitivity of lantanolic acid and, more recently, anti-humanherpesvirus activity of ursolic

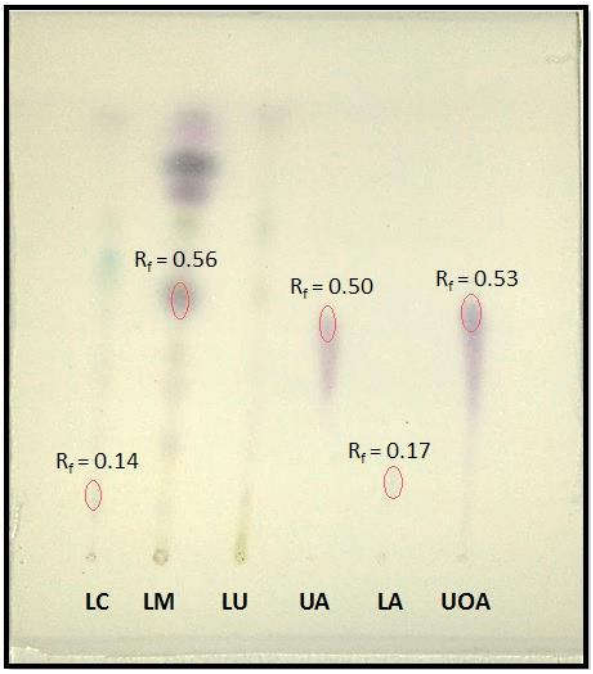

Figure 1. Thin layer chromatography of *L. camara* (LC); *L. macrophylla* (LM); *L.undulata* (LU) ethanolic extracts. Ursolic acid (UA), lantanolic acid (LA) and a mixture of ursolic and oleanolic acid (UOA) were used as standard. Red circles indicate main spot for $R_f$determination

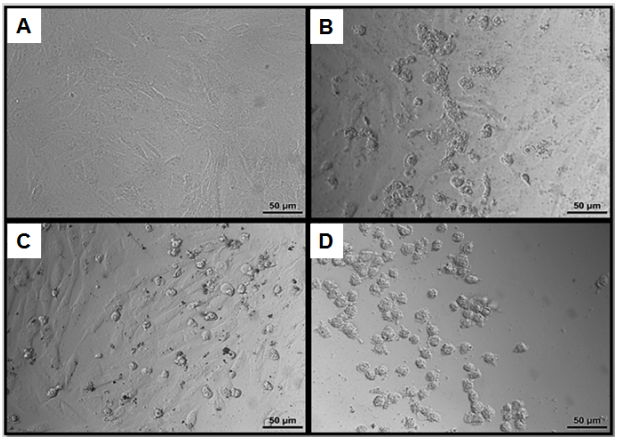

Figure 2. Morphological changes of Vero cells treated with *Lantana* ethanolic extracts. A- Control cells (MEM without serum); B- Cells treated with *L. camara* ethanolic extract at 1000 µg.mL^{-1}; C- Cells treated with *L. macrophylla* ethanolic extract at 1000 µg.mL^{-1} (1000 µg/mL); D- Cells treated with *L. undulata* ethanolic extract at 20 µg/mL.200 x (DMI3000 B Leica®)

Table 1.Condensed tannins content in Southern Bahia *Lantana* species leaves

Species	Tannincontent (g.100g^{-1})
L. câmara	1.018a ± 0.08*
L. macrophylla	1.964b ± 0.21
L. undulata	1.643b ± 0.08

*p<0.05 by ANOVA test followed by Tukey (BioEstat 5.0).

Table 2.$CC_{50}$and MNCC values (µg.mL^{-1}) of leaves ethanolic extracts from three *Lantana* species on Vero cells

Plant species	CC_{50}	MNCC
L. camara	208.4	250
L. macrophylla	285.1	125
L. undulata	5.7	2.5

Table 4. Antiviral and virucidal activity of *Lantana* species leaves ethanolic extract against *suidalphaherpesvirus* type 1 (SuHV-1)

Species	Antiviralactivity		Virucidalactivity	
	VII	IP (%)	VII	IP (%)
L. camara	0.63	77	0.33	54
L. macrophylla	0.75	83	2.49	99.7
L. undulata	0	-	0.67	79

VII: Viral inhibition index; IP: Inhibition percentage.

(Bag et al., 2012) and oleanolic acid (Mukherjee et al., 2013) were also reported. Thus, the presence of these triterpenic acids indicates a great biological potential of *L. camara* and *L. macrophylla* in Southern Bahia environment. The cytotoxic effects of all extracts were evident on Vero cells. Toxic signs were characterized by cell wrinkling and rounding, and cell destruction (Figure 2). *L camara* and *L. macrophylla* were less toxic while *L. undulata* showed a high toxicity (20 $\mu g.mL^{-1}$) on Vero cell and $CC_{50}$and MNCC of each species is presented in Table 2. In this study, following the criteria stated by Okonogi et al. (2007) and Oonsivilai et al. (2008) where CC_{50}values of 100 $\mu g.mL^{-1}$are considered non-cytotoxic confirm that *L. camara* e *L. macrophylla*are promising for biological tests in Vero cells.

From this study, we also could see that attention must be done when using cytotoxic assay based on cell metabolism. The usage of two methods (cell function and morphology observation) was relevant in this study. For example, *L. macrophylla* extract showed cut-off value for MTT technique of 285.1 $\mu g.mL^{-1}$compared to125 $\mu g.mL^{-1}$obtained by morphological observation. This fact is justified by variations between both techniques, the interference of chemical substances, the culture conditions as well as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide intrinsic toxicity (Riss, 2015). Regarding antiviral activity, although an optimum antiviral index is still controversial in the literature (Felipe et al., 2006; Lupini et al., 2009; Kaziyama et al., 2012), *L. macrophylla* was the most promising species, with VII of 0.75 andIP of 83%. Different from antiviral test, by virucidalapproach, all plants were active against SuHV-1, being *L. macrophylla* (IP= 99.7%) and *L. undulata* (IP= 79%)the most promising ones. Considering the tannins presence and content, it seems that these molecules in *Lantana* species play a role in virucidal activity. *L. macrophylla* ($1.96g.100g^{-1}$) and *L. undulata* ($1.64\ g.100g^{-1}$) which had significant ($p<0.5$) higher content of tannins had the more evident virucidal action. Also, the effects of tannins on health (Okuda, 2005) and on herpesvirusparticle (Lohezic et al., 1999; Serrano et al., 2008) are reported in the literature, being this, related to their toxicity. The more toxic they are, the more active as antiviral they are (Takechi, 1985). In this study, the two plants with the highest tannin content showed a more toxic effect to Vero cells and significant virucidal effect. Thus, here we highlight the effect of *L. macrophylla*leaves extract against herpesvirus. Taking into consideration that virucidal compounds are considered on antiseptic (Jassim and Naji, 2003)or disinfectant production interrupting the chain of environmental transmission (Sauerbrei et al., 2012) this species extract can be considered promising. In folk medicine, *L. macrophylla*is indicated for catarrhal cough and bronchitis which can be caused by virus. Therefore, it would be of interest to test this plant extract against other enveloped virus such as influenza or rhinovirus as well as how its molecules interfere on viral particle.

In conclusion, the present work showed the comparative phytochemical, citotoxic and antiherpesviral action study of three different *Lantana*species occurring on Mata Atlantica biome.

Authors' contributions

LDS (master student) carried out all experiments and drafted the manuscript. FFO contributed to thin layer cromatograph experiments. RAO contributed to phytochemical bios prospection experiments. MJBF contributed to virological experiments.AOC guided all study and participated in the design and draft of the manuscript. All the authors have read the final manuscript and approved the submission.

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