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Quimica

Flavonoids isolated from *Sida santaremnensis* H. Monteiro ("Guanxuma") and evaluation of biological activities

Flavonoides isolados de Sida santaremnensis H. Monteiro ("Guanxuma") e avaliação das atividades biológicas

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ABSTRACT

Introduction: *Sida santaremnensis* H. Monteiro (Malvaceae) is a plant popularly known as "vassourinha" or "guanxuma" that has been described as vasorelaxant, antiulcerogenic, antinociceptive and antiedematogenic. **Objective:** To contribute with the phytochemical and pharmacological profile of *Sida santaremnensis* through the isolation, purification and determination of chemical constituents of this plant, as well as through the evaluation of the anti-inflammatory and antitumor activity and leishmanicidal effect of the isolated constituent in a greater amount. **Methods:** The isolation of substances from the plant herein studied was performed with column chromatographic and analytical thin-layer methods and structural determination made by spectroscopic methods, such as Nuclear Magnetic Resonance, Hydrogen and Carbon 13, and comparisons with data from the literature. To assess pharmacological activities, cell viability tests, determination of nitric oxide levels, leishmanicidal activity, among others, were performed. **Results:** Two flavonoids from *S. santaremnensis* were obtained, kaempferol (S-1) and kaepferol 3-O-β-D-glycosyl-6"-α-L-rhamnoside (S-2), the latter in a greater amount. The evaluation of the antitumor activity of the glycosylated flavonoid demonstrated that it does not present hemolytic activity against the human promyelocytic leukemia cell line (HL-60), and that it presented weak leishmanicidal activity. The immunopharmacological evaluation of kaempferol 3-O-β-D-glucosyl-6"-α-L-rhamnoside revealed that it presents a possible anti-inflammatory action related to the inhibition of the production of nitrite by LPS-stimulated macrophages. **Conclusion:** These data demonstrate that kaempferol 3-O-β-D-glucosyl-6"-α-L-rhamnoside has low toxicity, in addition to antileishmania, anti-inflammatory and immunomodulatory properties, which makes it a therapeutic potential for infectious and inflammatory diseases mediated by macrophages.

Keywords: Immunological cytotoxicity, Immunomodulation, Inflammation, Malvaceae, Natural products

RESUMO

Sida santaremnensis H. Monteiro (Malvaceae) é uma planta conhecida popularmente como "vassourinha" ou "guanxuma", que vem sendo descrita como vasorelaxante, antiulcerogênica, antinociceptiva e antiedematogênica. Objetivou-se contribuir com o estudo fitoquímico e farmacológico de *Sida santaremnensis* a partir do isolamento e determinação estrutural de constituintes químicos dessa planta, bem como pela avaliação da atividade anti-inflamatória e antitumoral do constituinte isolado em maior quantidade. O isolamento de substâncias da planta estudada foi realizado por métodos cromatográficos em coluna e em camada delgada analítica e a determinação estrutural feita por métodos espectroscópicos, como a Ressonância Magnética Nuclear de Hidrogênio e Carbono 13, e comparações com a literatura. Para avaliação das atividades farmacológicas foram realizados ensaio de viabilidade celular, determinação dos níveis de óxido nítrico, atividade leishmanicida, entre outros. Foram obtidos como resultados dois flavonoides de *S. santaremnensis*, o canferol (S-1) e canferol 3-*O*-β-D-glycosyl-6”-α-L-ramnosídeo (S-2), este último em maior quantidade. A avaliação da atividade antitumoral do flavonoide glicosilado demonstrou que ele não apresenta atividade hemolítica nem citotóxica contra linhagem de células de leucemia promielocítica humana (HL-60), porém apresentou fraca atividade leishmanicida. A avaliação imunofarmacológica dessa mesma substância revelou uma possível ação anti-inflamatória relacionada à inibição da produção de nitrito por macrófagos estimulados com LPS. Conclui-se que estes dados demonstram que o canferol 3-*O*-β-D-glicosil-6”-α-L-ramnosídeo apresenta baixa toxicidade, além de propriedades antileishmania, anti-inflamatória e imunomoduladora, o que o torna um potencial terapêutico para doenças infecciosas e inflamatórias mediadas por macrófagos.
Palavras-chave: Citotoxicidade imunológica, Imunomodulação, Inflamação, Malvaceae, Produtos Naturais

1 INTRODUCTION

The species *Sida santaremnensis* H. Monteiro belongs to the family Malvaceae and is known as “vassourinha” or “guanxuma”. Found in Brazil especially in the Northeast and Southeast, they are herbaceous, semi-shrub plants considered as weed with little commercial value (ARMIÉN *et al*., 2007).

The chemical constituents described for *S. santaremnensis* correspond to a mixture of sitosterol 3-O-b-D-glucopyranoside and stigmasterol 3-O-b-D-glucopyranoside, isolated from the hexanic phase (ASSIS, 2016), as well as kaenperol 3-O-β-D-glucose-6”-α-L-rhamnoside isolated from the ethyl acetate (MELO, 2013). Despite the fact the referred species does not have traditional use, preliminary studies conducted with the crude ethanolic extract pointed to vasorelaxant (ARCANJO *et al*., 2011), anti-ulcerogenic (OLIVEIRA *et al*., 2008), antinociceptive (MENDES *et al*., 2008) and antiedematogenic (MOURA, 2010) activities.

Amidst the biological activities mentioned, the capacity to inhibit the formation of edema is highlighted, taking into account the worldwide prevalence of inflammatory diseases and the need to develop new therapies for the control of such pathologies. In light of that, nitric oxide (NO), which is gas produced by different cells in vertebrates, particularly humans,

and with a number of biological functions, stands out as a marker for inflammatory reactions (MOURA, 2010). Besides, other parameters for the evaluation of the biological activity must be considered as the measure of cytotoxicity in different experimental models, such as those for the antitumoral and leishmanicidal activities (MAO *et al*., 2013).

Although preliminary researches have shown pharmacological potential for the crude ethanolic extract of *S. santaremnensis*, the chemical and pharmacological knowledge of its isolated compounds is incipient. Therefore, the present work aimed at isolating chemical constituents of the referred species and evaluating the anti-inflammatory and antitumoral activities of the substance isolated in a greater amount during the development of the work.

2 MATERIALS AND METHODS

2.1 Phytochemical study

2.1.1 Collection of the plant material, obtaining and partitioning of the Crude Ethanolic Extract *(CEE) of S. santaremnensis*

The aerial parts of *S. santaremnensis* were collected at the Parque Piauí (Teresina-PI) in April 2007 and identified by botanist Prof. Dr. Gardene Maria de Sousa. An exsiccate was stored at Graziella Barroso Herbarium, under the number 21867. The registration on the SISGEN platform (National System for the Management of the Genetic Patrimony and the Associated Traditional Knowledge) is under the number AD36537.

The plant material was dehydrated at room temperature for 30 days and ground on a SP 30 – SPLabor mechanical mill, yielding 2,500 g of powder, which underwent maceration for a period of 48h in 96% ethanol; this process was repeated eight times. After filtration and concentration of the extractive solution on a Quimis rotary evaporator, the material was placed on a TELSTAR table-top lyophilizer yielding 105.1 g of the CEE.

The CEE (100.0 g) was dissolved in a hydro-ethanolic solution (MeOH:H_2O, 7:3) and underwent partitioning with solvents in increasing gradient of polarity, resulting in the following phases: hexane (39.4g), dichloromethane (11.1g), ethyl acetate (8.9g) and hydro-

ethanolic (39.3g). The solvents used in the research were analytically pure from either the Vetec or Dinâmica brands.

2.1.2 Chromatographic fractionation of the ethyl acetate phase of *S. santaremnensis*

An aliquot of the ethyl acetate phase (3.0g) was subjected to chromatography on a LH-20 sephadex column and eluted with methanol yielding 48 fractions of 5 mL each (Column I). The fractions were analyzed by means of thin-layer chromatography (TLC) in silica gel, revealed in iodine vapor and grouped according to the retention factors (Rf's), yielding six fraction groups: 01/05, 06/11, 12/26, 27/30, 31/41 and 42/48.

The fraction 12/26 was rechromatographed (Column II) under the same previous conditions, yielding 20 fractions, which after grouped resulted in six groups (01/04, 05/07, 08/10, 11/13, 14/15 and 16/20). The subtraction 05/07 resulting from the column II was classified as pure, codified as S-1 (0.011 g) and directed for the obtaining of the ^{1}H and ^{13}C NMR spectra.

The fraction 31/41 resulting from the column I was rechromatographed yielding 39 fractions of 5mL each (Column III). After TLC and Rf´s analysis, they were divided into five groups (01/03, 04/10, 11/22, 23/27 and 28/39). The subfraction 28/39 was classified as pure, codified as S-2 (0.050 g) and directed for the obtaining of the ^{1}H and ^{13}C NMR spectra.

The ^{1}H and RMN ^{13}C nuclear magnetic resonance spectra of the isolated constituents were obtained on a MERCURY-VARIAN spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C). The deuterated solvent used was methanol (CD_3OD).

2.2 Pharmacological study

The pharmacological studies were performed with the substance codified as S-2 once it was isolated in a higher amount, what enabled the conduction of the experiments.

2.3 *Peritoneal macrophage culture*

Swiss mice were stimulated with 2 mL of thioglycolate (4%) in the peritoneal cavity. After four days, the animals were euthanized for the obtaining of the macrophages. For that, 10 mL of the sterile-filtered phosphate buffered saline (PBS) supplemented with 3%

fetal bovine serum (FBS) was injected in the peritoneal cavity and the peritoneal washing was collected. The cellular suspensions were centrifuged at 1,200 rpm at 4 ºC for 10 minutes and resuspended in 1 mL of complete RPMI medium (RPMI + 10% FBS, 100 U.I. of penicillin and 100 µg/mL of streptomycin - Sigma); the cellular viability was determined using Trypan Blue stain. The cells were resuspended in the concentration of 4 x 10^5 cells/mL of complete RPMI medium and distributed in 96-well culture plates (200 µL/well) (CHOUDHARI *et al*., 2013; VASCONCELOS *et al*., 2014; VASCONCELOS; DAVIES and GORDON, 2005). Next, the cells were cultured for 24 hours, either in the presence or in the absence of 1µg/mL of lipopolysaccharide (LPS), followed by the addition or not of different concentrations of the S-2 substance. After 24 hours of incubation, the analysis of cellular viability was made (CHOUDHARI *et al*., 2013).

2.2.1 Determination of the viability

Cellular viability was determined with the use of 3-methyl-[4-5-dimetiltiazol-2-il]-2,5-diphenyltetrazolium bromide (MTT - Sigma). The cells were added with 90 µL of complete RPMI medium and 10 µL of a suspension of 3-methyl-[4-5-dimetiltiazol-2-il]-2,5-diphenyltetrazolium bromide (MTT - Sigma) in the concentration of 5 mg/mL. The cell suspensions were incubated for 4 hours at 37°C and 5% CO_2. After this period, the supernatant was removed and 100 µL of dimethyl sulfoxide (DMSO) was added in each well to dissolve the formazan crystals formed. The cellular viability was quantified by the measure of the optical density at the wavelength of 570 nm, determined by means of a microplate reader (Spectramax 190 – Molecular Device) (MOSMANN, 1983; LÖFGREN *et al*., 2008).

2.2.2 Quantification of the nitric oxide (NO) levels

The production of NO was evaluated *in vitro* with the colorimetric method named Griess reaction (GREEN *et al*., 1982). 100 µL of Griess reagent was added to 100 µL of the supernatants obtained from the macrophage culture. After 10 minutes at room temperature, reading at 540 nm was made (Spectramax 190 – Molecular Device). The results in µM obtained were determined by the comparison with the standard curve for the concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.5 µM.

2.2.3 Cytotoxicity test in sheep erythrocyte

The hemolytic activity was evaluated in sheep erythrocyte incubated with the S-2 substance (6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/mL) for 1 hour, followed by reading at 540 nm. The maximum and blank lysis controls were PBS and distilled water (GREEN *et al*., 1982).

2.2.4 Evaluation of the antitumoral activity

Cells of human promyelocytic leukemia (HL-60) were incubated (5×10^4 cells/well) with the S-2 substance (5, 50 and 500 µg/mL) for 24 h, at 37ºC and CO_2 5.0%. Next, MTT (5 mg/mL in PBS) (m/v) was added to the supernatant, and after 4 h, DMSO was added and shaken for 30 min. The plates were read at 570 nm (GONÇALVES *et al*., 2016).

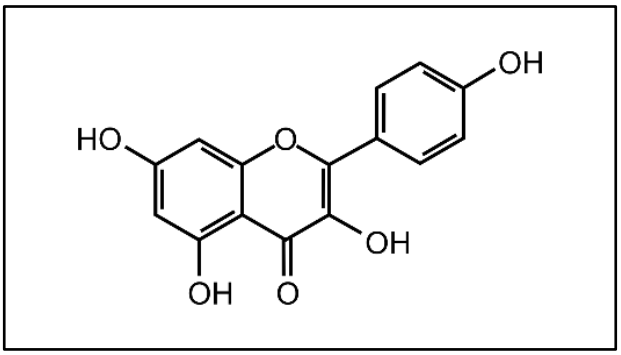
2.2.5 Evaluation of the leishmanicidal activity *in vitro*

For this assay, either the S-2 substance (6.25; 12.5; 25; 50; 100; 200; 400 and 800 µg/mL) or amphotericin B (2 µg/mL) was incubated with promastigote forms of *Leishmania major* (MHOM/IL/80/Friedlin) previously seeded in supplemented Schneider’s medium (1×10^6 leishmanias/100 µL of medium). After incubation in a B.O.D. oven at 26 ºC for 42 h, resazurin 1×10^{-3} mol/L was incubated for 6 h and the reading was made at 550 nm (VALADARES *et al*., 2011).

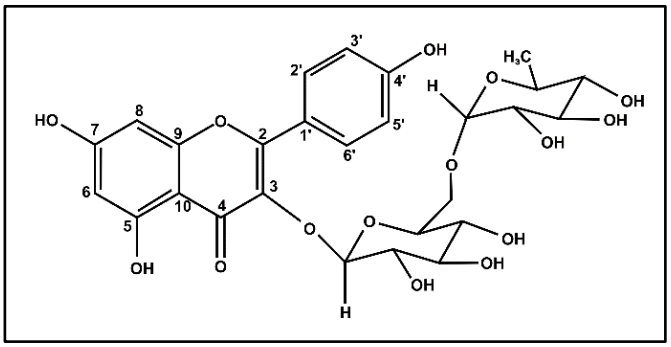
3 RESULTS AND DISCUSSIONS

The chromatographic process of the ethyl acetate phase of *S. santaremnensis* resulted in the isolation of two flavonoids, after the analysis of the RMN ^1H and ^{13}C spectra and comparisons with literature data (VALADARES *et al*., 2011; AGRAWAL, 1989). These substances were identified as: 3,5,7,4’ tetrahydroxyflavone (kaempferol), initially coded as S-1, and kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside, coded as S-2 (Table 1). Both substances were presented as a yellow powder. Their ^1H and ^{13}C RMN data are shown in Table 1.

Table 1 – RMN ^{1}H and ^{13}C spectral data (d, CD_3OD, 500 and 125 MHz) of the flavonoids isolated from *Sida santaremnensis*



kaempferol **(S-1)**



kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside **(S-2)**

	H	**C**	**H**	**C**
2	-	148.0	-	159.23
3	-	137.1	-	135.48
4	-	-	-	179.06
5	-	164.3	-	162.51
6	6.17 (*d*, 1H, *J*=2 Hz)	-	6.11 (*d*, 1H, 5.0 Hz)	99.93
7	-	165.7	-	165.68
8	6.39 (*d,* 1H, *J*=2 Hz)	94.5	6.27 (*d,* 1H, 5.0 Hz)	94.96
9	-	158.3	-	158.16
10	-	104.5	-	105.45
1’	-	123.7	-	122.55
2’,6’	8.08 (*d,* 2H, *J*=9.0 Hz)	130.7	7.99 (*d,* 2H, *J*=10 Hz)	132.29
3’,5’	6.89 (*d,* 2H, *J*=9.0 Hz)	116.3	6.83 (*d,* 2H, *J*=10 Hz)	116.02
4’	-	160.6	-	161.19
1”			5.04 (*d*, 1H, *J*=5.0 Hz)	104.76
2”			3.44 *m*	75.63
3”			3.44 *m*	77.97
4”			3.26 *m*	71.27
5”			3.35 *m*	76.91
6”			3.77 *m* 3.35 *m*	68.50
1”’			4.50	102.24
2”’			3.65 *m*	71.90
3”’			3.53 *m*	72.22
4”’			3.27 *m*	73.83
5”’			3.42 *m*	69.59
6”’			1.11 (*d,* 1H, *J*=5.0 Hz)	17.86

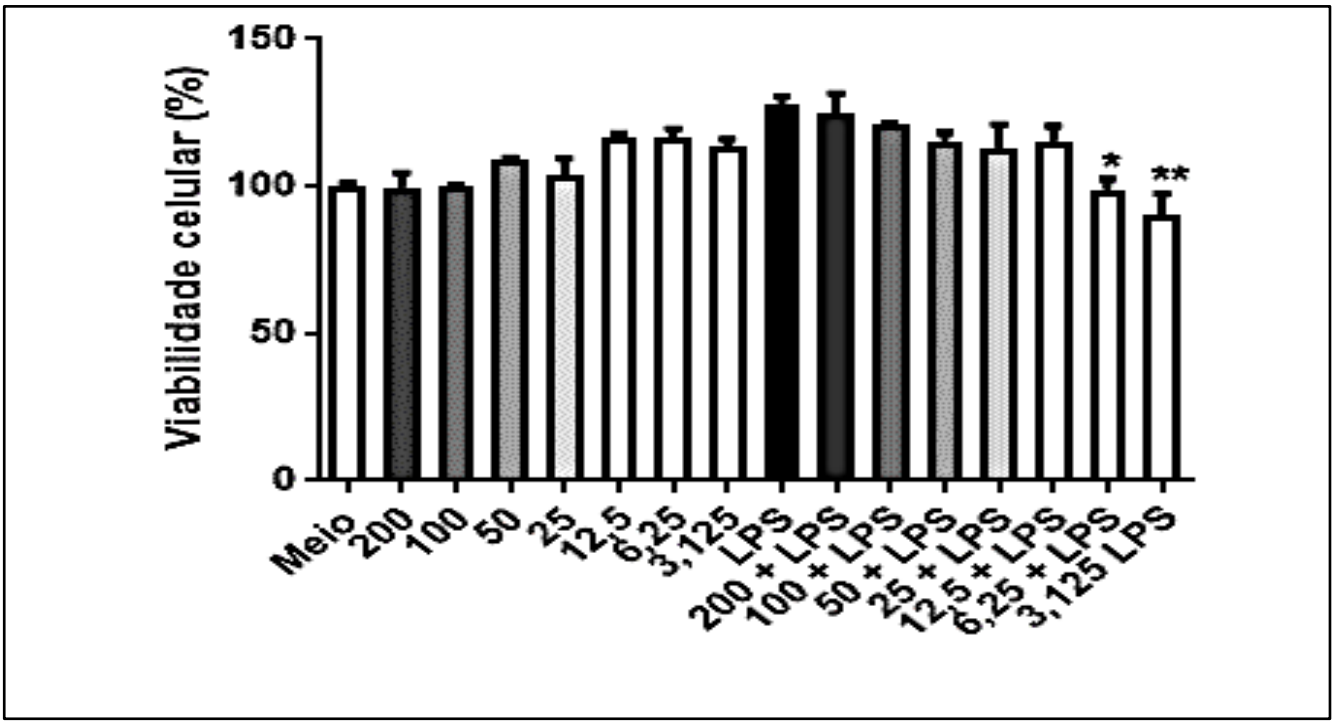
Source: Research data

Kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside **(S-2)** was tested for its capacity to alter cellular viability. The murine peritoneal macrophage cultures stimulated with LPS and seeded in the presence of the glycosylated kaempferol showed that it inhibited cellular proliferation. The compound studied did not alter the viability of the macrophages in the

concentrations from 3.125 to 200 μM, indicating low toxicity. Nonetheless, the cultures stimulated with LPS presented an increased percentage of viability when compared to the non-stimulated cultures, suggesting LPS-induced cellular proliferation. On the other hand, the cultures stimulated with LPS and treated with this substance in the concentrations of 3.125 μM and 6.25 μM presented a decrease in cellular viability ($p<0.05$), indicating that it can exert an inhibitory effect in the activation and proliferation of LPS-stimulated macrophages, thus suggesting anti-inflammatory activity (Figure 1).

These data demonstrate that Kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside has cellular antiproliferative property and potential anti-inflammatory activity.

Figure 1 – Effect of S-2 in cellular viability. The data were analyzed using ANOVA test followed by Tukey´s test and are represented as mean ± SEM from three experiments isolated in triplicate (n= 3x3).



Fonte: Autores, 2021

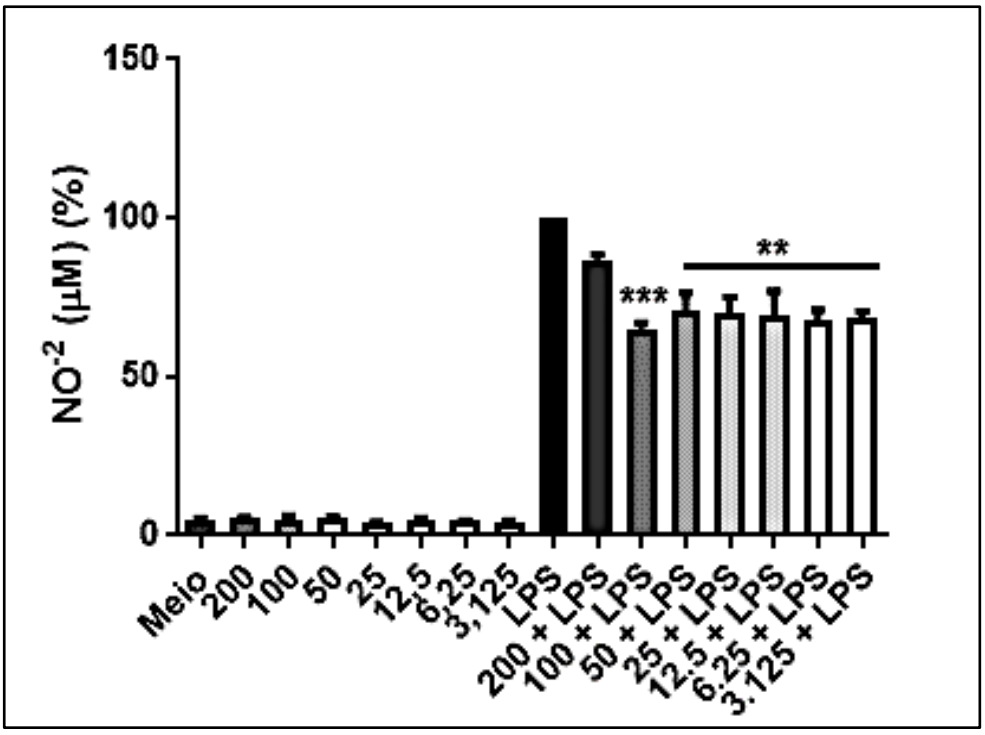
The production of NO was also analyzed. Kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside inhibited the formation of nitrite in LPS-stimulated macrophage cultures, indicating the potential for anti-inflammatory activity. The macrophage cultures used in this study were stimulated with bacterial LPS, which is known as a potent activator of

macrophages, and secreted high concentrations of NO quantified in the supernatant of the cellular cultures.

The stimulation of the peritoneal macrophages with LPS resulted in an increase in the NO synthesis when compared with the cultures of cells seeded only with culture medium known as negative control (Figure 2).

Kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside decreased the production of NO in the LPS-stimulated macrophages, in the concentrations ranging between 3.125 µM ($p<0.01$) and 100 µM ($p<0.001$) that were tested.

Figure 2 – Effect of the S-2 in the levels of nitrite in LPS-stimulated macrophages. The results were expressed as ± SEM (n=3x2) and analyzed using ANOVA followed by Tukey´s post-test. ** $p < 0.01$ and ***$p<0.001$ were considered when compared with the control group.

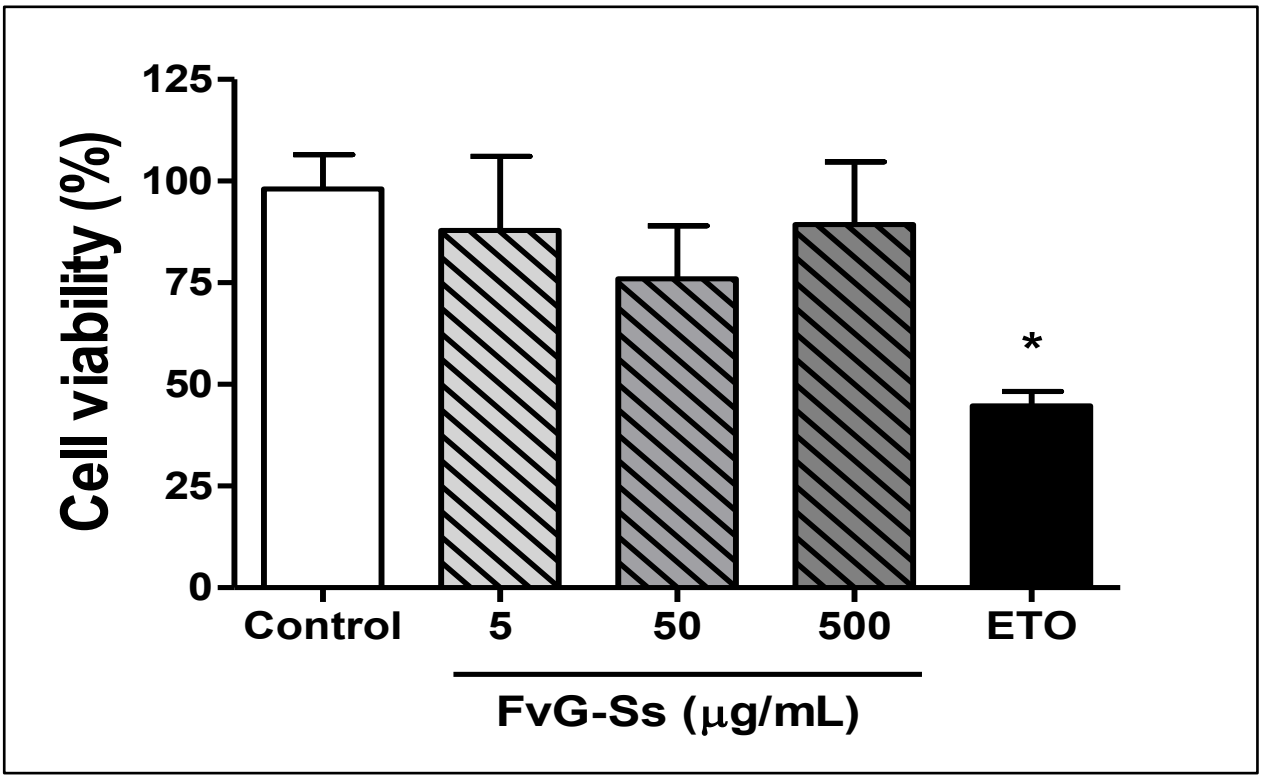


Fonte: Autores, 2021

Kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside presented hemolytic activity of only 5% in the highest concentration tested (800 µg/mL), indicating lack of cytotoxicity. Upon running the MTT test in HL-60 cells and comparing the cellular cultures either in the absence or in the

presence of S2 in the concentrations of 5, 50 and 500 µg/mL, no visible alteration in the cellular viability was observed (Figure 3), indicating that the referred flavonoid does not show cytotoxicity against the lineage tested. As expected, the etoposide-pattern drug (50 µg/mL) reduced about 50% the viability of HL-60 cells from the control ($p<0.05$).

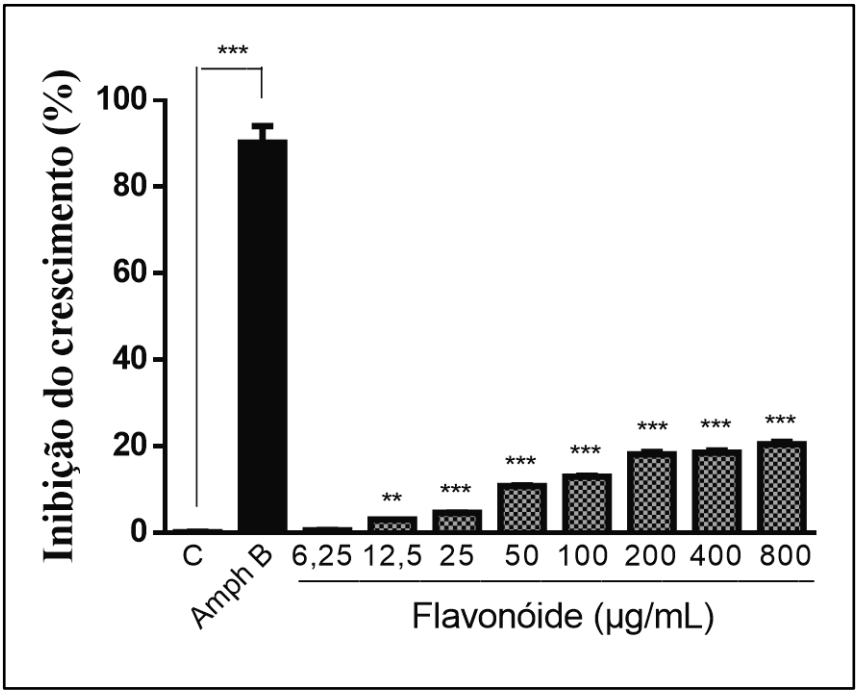
Figure 3 – Evaluation of the cytotoxicity of S-2 over HL-60 cells. 50 µg/mL etopside (ETO) was used as the positive control. The values are represented as means ± SEM (n= 3x3). ANOVA followed by Dunnet´s test.



Fonte: Autores, 2021

Kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside inhibited in 20% the growth of promastigote forms of *L. major*, with theoretical IC_{50} value of 8561.865 µg/mL (Figure 4). In spite of the low leishmanicidal activity, higher cytotoxicity was observed for the promastigotes in relation to the erythrocytes and macrophages through the hemolysis test and MTT, respectively, indicating higher selectivity for the parasite in relation to the mammalian cells.

Figure 4 – Effect of the S-2 (6.25 – 800 µg. mL^{-1}) or amphotericin B (Amph B) (2 µg/mL) in the growth of promastigote forms of Leishmania major. Data represent the average percentage of dead promastigotes ± standard error (n=3x3). **p<0.01 and ***p<0.001 in relation to the control (C).



Fonte: Autores, 2021

Kaempferol has already been described in literature in other species of the Malvaceae family, as for instance *Herissantia crispa* (VALADARES *et al*., 2011; AGRAWAL, 1989) and *Pavonia malacophylla* (CHAVES, 2016). However, it is being described for the very first time in *S. santaremnensis.* In the *Sida* genus, this chemical constituent has already been isolated in the species *S. ellioti, S. rhombifolia, S. hirsuta* and *S. spinosa.* Also, there are several pharmacological activities reported in the literature for the *Sida* genus, where one can highlight the antibacterial (BISIGNANO *et al*., 2000), anticoagulant (CHANG *et al*., 1998), anti-inflammatory (DELLA *et al*., 1988), anticarcinogen (DIMAS *et al*., 2000) and anti-HIV (C8166 cell culture) (CARLO; MASCOLO; IZZO, 1999), , antidiarrheal (KATAOKA *et al*., 2001), antispasmodic (LEE *et al*., 2005) and antiulcerogenic (MAHMOOD *et al*., 1996), as well as the inhibition of the complement system (JUNG *et al*., 1998).

Kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside is being isolated in *S. santaremnensisi* for the second time. However, in the previous study, it could not be obtained in a sufficient

amount for the pharmacological tests (MELO, 2013). This substance has been previously related in the species *S. rhombifolia* and *S.* acuta (COSTA, 2007; CHAVES, 2016).

Several publications have reported the activity of flavonoids inhibiting the cellular proliferation/viability. Flavonoids such as flavonol, galangin, kaempferol, quercetin and myricetin induce neither toxicicity nor cellular death, what enables their application as plant-derived drugs in inflammatory diseases (AHMED *et al*., 2010; MORORÓ *et al*., 2018).

Although some flavonoids are described as antitumoral species, such as those isolated from the extract of the plant of the Amazon region *Bauhinia purpurea,* like kaempferitrin, quercetin and rutin, the glycosylated kaempferol herein described was not able to reduce the cell viability in the HL-60 lineage (LIMA *et al*., 2016).

The reduction in the production of NO indicates the capacity of the glycosylated kaempferol to modulate the activity of macrophages and, consequently, the inflammatory response. As a rule, preliminary studies associated the presence of sequestrating agents of NO as immunomodulatory agents, once they can both decrease the harmful effect of inflammation and aid in homeostasis (LIMA *et al*., 2016). Also, the increased production of NO reflects the level of inflammation and works as a parameter to evaluate the effect of drugs on the inflammatory process (CHOUDHARI *et al*., 2013).

The inhibitory effect of the hydroethanolic extract of *Herissantia tiubae (another species of Malvaceae) on the production of NO in a murine macrophage culture was also identified in a previous study (SANTOS et al., 2014).*

Additionally, the glycosylated kaempferol presented lower leishmanicidal activity against promastigote forms of *L. major*. This result is in agreement with a previous report on the leishmanicidal activity for another kaempferol derivative, kaempferol 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside, which presented a stronger activity against amastigote forms of *L. amazonensis* internalized in macrophages, a model that simulates the clinical condition of the parasite in the host body in leishmaniasis (MUZITANO *et al*., 2006).

Kaempferol and Kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside, isolated from *S. santaremnensis*, are in agreement with those reported in literature as predominant in the genus *Sida,* being the first novel in the species.

4 CONCLUSION

The results herein obtained highlight that Kaempferol 3-O-β-D-glycosyl-6”-α-L-rhamnoside presents anti-inflammatory potential that involved the inhibition of the production of NO. Additionally, it did not present cytotoxicity against the cells of the HL-60 tumoral lineage and also presented weak leishmanicidal activity. These results show the importance of future research with *S. santaremnesis*, in a way to deepen both the chemical and the immunological knowledge, seeking to develop future novel drugs derived from plant-based products for the treatment of inflammatory diseases.

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