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Abstract

The aim of this study was to investigate the toxicity of Syzygium jambos and Solanum guaraniticum leaf extracts, through Artemia salina lethality testing and cytotoxicity evaluations in rat spleen lymphocytes, using methyl tetrazolium (MTT), neutral red uptake (NRU), trypan blue dye exclusion, and lactate dehydrogenase (LDH) leakage assays, besides by their in vitro effects on acetylcholinesterase (AChE) activity. The LC50 calculated in the A. salina bioassay demonstrated that both extracts might well be toxic. Solanum guaraniticum presented cytotoxic effects against lymphocytes, as demonstrated by viable cells count reductions, NR uptake, and increased LDH leakage. Syzygium jambos appears to present immunomodulatory properties, increasing lymphocyte mitochondrial activity and inhibiting AChE activity. The results demonstrated the possible harmful effects of these vegetal preparations and may be helpful in therapeutic decisions and future studies with respect to the toxicology of these extracts, when used as phytotherapeutic medicines.

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Gabriela Bonfanti-Azzolin[a*], Andressa Leal Zambra[b], Bruna Morgan da Silva[b], Karine Santos De Bona[c], Paula Eliete Rodrigues Bitencourt[d], Lariane Cargnelutti[d], Raphaela Maleski Borges[d], Mayara L. De Freitas[d], Ricardo Brandão[e], Josiane Woutheres Bortolotto[b], Aline Augusti Boligon[d], Aline S. Pigatto[f], Maria Beatriz Moretto[d], Thissiane de Lima Gonçalves[d]

[a]*Programa de Pós-Graduação em Atenção Integral à Saúde, Universidade de Cruz Alta, Cruz Alta, RS, Brasil*

[b]*Curso de Farmácia, Universidade de Cruz Alta, Cruz Alta, RS, Brasil;*

[c] *Curso de Farmácia, Universidade Regional Integrada do Alto Uruguai e das Missões (URI), Santo Ângelo, RS, Brazil;*

[d] *Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil*

[e] *Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal de Pernambuco, Recife, PE, Brasil.*

[f] *Universidade Fransciscana, Santa Maria, RS, Brasil*

Abstract

The aim of this study was to investigate the toxicity of *Syzygium jambos* and *Solanum guaraniticum* leaf extracts, through *Artemia salina* lethality testing and cytotoxicity evaluations in rat spleen lymphocytes, using methyl tetrazolium (MTT), neutral red uptake (NRU), trypan blue dye exclusion, and lactate dehydrogenase (LDH) leakage assays, besides by their *in vitro* effects on acetylcholinesterase (AChE) activity. The LC50 calculated in the *A. salina* bioassay demonstrated that both extracts might well be toxic. *Solanum guaraniticum* presented cytotoxic effects against lymphocytes, as demonstrated by viable cells count reductions, NR uptake, and increased LDH leakage. *Syzygium jambos* appears to present immunomodulatory properties, increasing lymphocyte mitochondrial activity and inhibiting AChE activity. The results demonstrated the possible harmful effects of these vegetal preparations and may be helpful in therapeutic decisions and future studies with respect to the toxicology of these extracts, when used as phytotherapeutic medicines.

Keywords: Myrtaceae, Solanaceae, toxicity, vegetal extracts;

1. Introduction

Herbal medicines have received great interest as an alternative to clinical therapy. However, despite the

widespread use of these agents, safety pharmacology studies are relatively restricted and mainly limited to compounds with broad industrial use (Palozi et al., 2019).

In Brazil, different vegetal species are used as as important sources of food and medicine (Palozi et al., 2019). Certain examples such as *Syzygium jambos* (L.) Alston (Myrtaceae), and *Solanum guaraniticum* A. St.-Hil (Solanaceae), are recognized as medicinal species. The first is known by the Brazilian population as "jambolão" and its leaves are mainly used to treat diabetes (Teixeira et al. 2000). Its anti-inflammatory, antibacterial, antifungal, analgesic, diuretic, expectorant and digestive properties are also explored in folk medicines of other countries (Rajkumari et al., 2018). The second specie mentioned above, *Solanum guaraniticum*, is known by the common name "falsa-jurubeba" and is used to treat liver, anemia, and gastric dysfunctions (Penna 1964, Bonfanti et al., 2016).

One common problem regarding the medicinal use of these plants is mistaken identity and eventual use of replacements such as; *Syzygium cumini* and *Solanum paniculatum,* which already have their therapeutic properties elucidated. Safe medicinal use of *Syzygium jambos* and *Solanum guaraniticum* preparations is unsubstantiated by scientific data, and previous studies by our group have already demonstrated enzymatic inhibition caused by these vegetal extracts, and *in vitro* pro-oxidant effects on erythrocytes and tissues (Bonfanti et al. 2013, 2014).

Toxicity screening models provide important preliminary data to help select natural remedies with potentially beneficial health properties (Pour et al. 2011; Mounanga, Mewono, Angone 2015), and *in vitro* cytotoxicity assays are widely used for this purpose. Following exposure to toxic substances, neutral red uptake (NRU), trypan blue dye exclusion, 3-(4,5-dimethylthiazol-2yl)-2,5-biphenyl tetrazolium bromide (MTT), and lactate dehydrogenase (LDH) leakage assays are commonly employed to detect cytotoxicity and other negative effects on cell viability (Fotakis & Timbrell 2006).

Further, the enzyme acetylcholinesterase (E.C. 3.1.1.7, AChE) plays an essential role in the physiological events involving turnover of acetylcholine in the central nervous system. It is an important target of a large number of cholinesterase-inhibiting drugs and toxins (Pohanka 2011). The enzyme also has been acknowledged for having roles in non-neuronal tissue, including regulation of the immune functions (Kawashima & Fujii, 2003, Nizri & Brenner, 2013). Interestingly, several studies have demonstrated that natural substances and dietary components affect AChE activity, suggesting that they can modulate its activities in various tissues (Li et al., 2017; Mendonça de Assis et al., 2019).

Another alternative is to test for toxicity of chemical and natural products based on lethality to *Artemia salina* L. (Artemiidae), the brine shrimp, an invertebrate of saline aquatic and marine ecosystems. The *A. salina* lethality test has been used to determine toxicity by estimating medium lethal concentrations (LC_{50} values), for a series of toxins and plant extracts (Meyer et al. 1982; Choi, 2017).

The current study has been undertaken to investigate the toxicity of *Syzygium jambos* and *Solanum guaraniticum* leaf extracts through *Artemia salina* lethality testing, and to measure AChE activity and cytotoxicity through *in vitro* evaluations of rat spleen lymphocytes.

2. Material and Methods

2.1 Preparation and High-performance liquid chromatography (HPLC) characterization of hydro-

ethanolic leaf extracts

Leaves of *Solanum guaraniticum* and *Syzygium jambos* were collected, dried and submitted to extraction with 80% ethanol in a Soxhlet apparatus until exhaustion. After extraction, the solvent was evaporated by rota-vapor, supplying the dried crude extract. Reverse phase chromatographic analyses were then carried out under gradient conditions using a C18 column (4.6 mm × 250 mm) packed with 5 µm diameter particles; mobile phases being acetonitrile:water (95:5, v/v), and water:phosphoric acid (98:2, v/v). The presence of eleven (11) antioxidant compounds was investigated, namely; gallic, chlorogenic, caffeic and ellagic acids, and catechin, epicatechin, quercetin, quercitrin, isoquercitrin, kaempferol and rutin. Identification of these compounds was performed by comparing their retention times and UV absorption spectrums with those of the commercial standards. The chromatographic peaks were confirmed by comparing their retention times with those of reference standards and by DAD spectra (200 to 500 nm).

2.2 Animals and preparation of spleen lymphocytes

Male adult albino Wistar rats (200-250g, n = 6) were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil (Process number 052/12). The rats were euthanized, and the spleen was aseptically removed, cut into several pieces, and gently crushed in phosphate buffered saline (PBS). Lymphocytes were then collected by centrifugation, erythrocytes were lysed (0.15 M NH_4Cl, 1 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4), and cells were re-suspended in PBS. The cell count was adjusted to contain 3×10^6 cells/mL by trypan blue exclusion.

2.3 Spleen lymphocytes treatments and experimental assays

Solanum guaraniticum and *Syzygium jambos* extracts were added to the cell suspensions at final concentrations of 100, 250, 500, 750, and 1000 µg/mL (Bonfanti et al. 2014). The lymphocytes exposed to the extracts were then incubated at 37°C for 2 h.

After incubation, the treated cells were submitted to MTT assay (Mosmann, 1983), the neutral red uptake test (NRU) (Borenfreund & Puerner 1984), and the trypan blue dye exclusion test (Mischell & Siingi 1980). The measurement of lactate dehydrogenase (LDH) leakage from cells was also performed by determining the LDH activity in the supernatant using a commercial kit (LABTEST, MG, Brazil). AChE activity was determined according to the method described by Ellman et al. (1961) modified by Fritzgerald and Costa (1993). The MTT, NRU, and LDH test results are presented as percentage of control, whereas the results of the trypan blue dye exclusion test are presented as the number of live cells. AChE activity was expressed in µmol AcSch/h/mg protein.

2.4 Artemia salina lethality test

Differing concentrations of the extract dilutions in distilled water were evaluated for lethality with *Artemia salina* (brine shrimp larvae), using the procedure described by Meyer et al. (1982). After 24 h of hatching, the larvae (10 per vial) were transferred to 10 ml vials containing the extract dilutions and saline solution (three triplicates of each concentration). The number of survivors was counted after 24 h and 48 h of incubation, and the number of dead larvae was recorded and used to calculate the 50% lethal

concentration (LC_{50}) by trimmed Spearman-Karber, the conventional Spearman-Karber method being described by Finney (1971).

2.5 Statistical Analysis

In the *Artemia salina* test, the lethal concentration fifty (LC_{50}) and 95% confidence interval were determined from the 24 h and 48 h counts using the Finney (1971) computer program. All other analyses were performed using STATISTICA for Windows, version 6.0 (StatSoft Inc. Tulsa, OK, USA). All data were analyzed using one way ANOVA, followed by Duncan's multiple range test and presented as mean ± standard error of mean (SEM). A value of $p < 0.05$ was considered statistically significant for all analyses.

3. Results and Discussion

In this study, *Solanum guaraniticum* extract gave distinct results in spleen lymphocyte viability tests (Figure 1). After the incubation period, cell suspensions treated with *Solanum guaraniticum* ranging from 250 – 1000 µg/mL presented numbers of live cells significantly lower than untreated cells; this was demonstrated by the trypan blue dye exclusion test. Further, starting from a concentration of 500 µg/mL; the extract also affected lysosomal functionality and cell membrane integrity, as was observed in the NRU and LDH leakage tests. However, no alteration was detected by MTT assay, suggesting that the extract did not affect mitochondrial activity. Considering that the neutral red cytotoxicity assay examines the ability of a cell to incorporate a water-soluble dye (neutral red) into lysosomes, a process requiring cellular energy (Putnam et al. 2002), the extract might interfere with the normal energy-requiring endocytosis processes, as well as causing membrane disruption and releasing LDH into the medium.

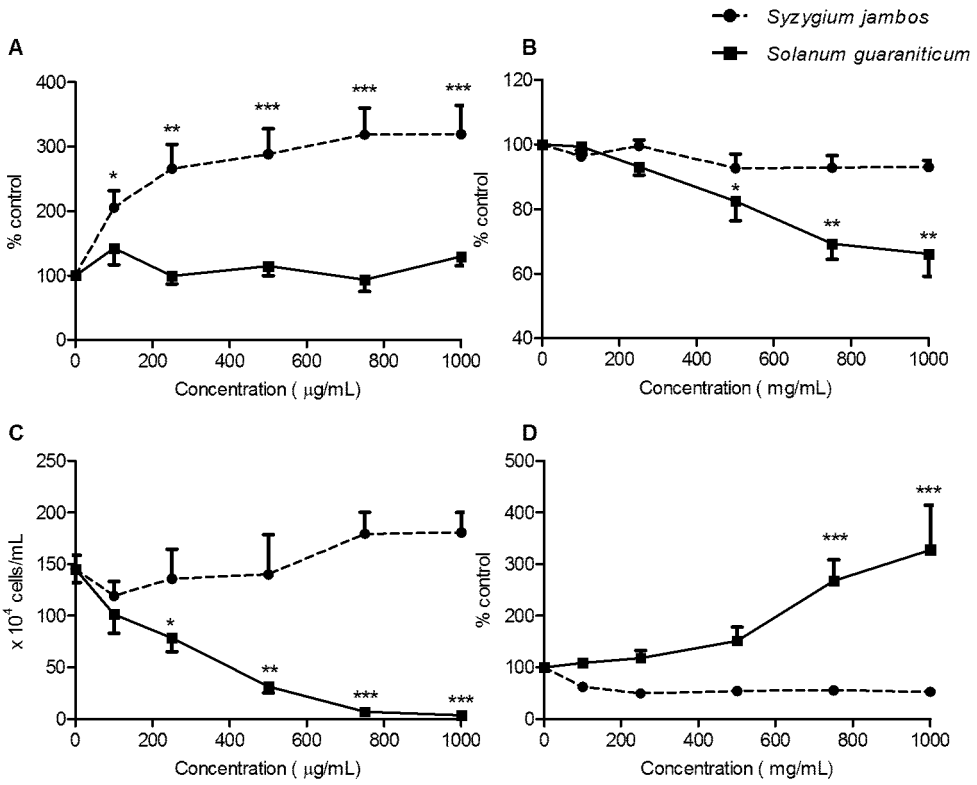


Figure 1. Viability tests of spleen lymphocytes treated with *Syzygium jambos* and *Solanum guaraniticum* extracts: MTT assay (A), neutral red uptake test (B), trypan blue dye exclusion test (C), LDH leakage (D). Results are expressed as mean ± SEM (n = 6) assessed by one way ANOVA followed by Duncan Multiple Comparison post hoc test. (*), (**) and (***) denotes $p<0.05$, $p<0.001$ and $p<0.0001$, respectively, as compared to the respective control samples (without extracts).

On the other hand, none of the viability assays used in this study suggested cell damage after incubation with *Syzygium jambos* extract (Figure 1). These data are in accordance with a previous study showing that *Syzygium jambos* ethanol extract was not toxic to human cells (Sharma et al. 2013). Interestingly, *Syzygium jambos* extract seems to have a stimulatory effect on treated cells, since it increased MTT viability. Taking into account that the MTT assay is used to monitor cell activation and to determine mitochondrial activity (Gerlier & Thomasset 1986), the result could indicate immunostimulant effects since lymphocyte activation is a central event in the inflammatory/immune response (Cavallini et al. 2001).

In line with this, the inhibitory effect of *Syzygium jambos* extract on AChE activity in spleen lymphocytes was also demonstrated (Figure 2). The function of AChE in lymphoid tissue is to regulate the amount of ACh surrounding lymphoid cells according to immunologic demands (Nieto-Cerón et al. 2004, Nizri & Brenner, 2013). In this context, AChE becomes a potential contributor to the pathway which controls inflammatory and immune responses in the blood. It has been demonstrated that AChE inhibitors reduce lymphocyte proliferation and secretion of pro-inflammatory cytokines, and may attenuate inflammation by increasing acetylcholine concentration in the extracellular space (Nizri et al. 2006). Therefore, the results presented in this study provide insight into a possible mechanism of anti-inflammatory action for *Syzygium jambos* extract, which has already been cited in previous studies (Slowing et al. 1994; Sharma 2013).

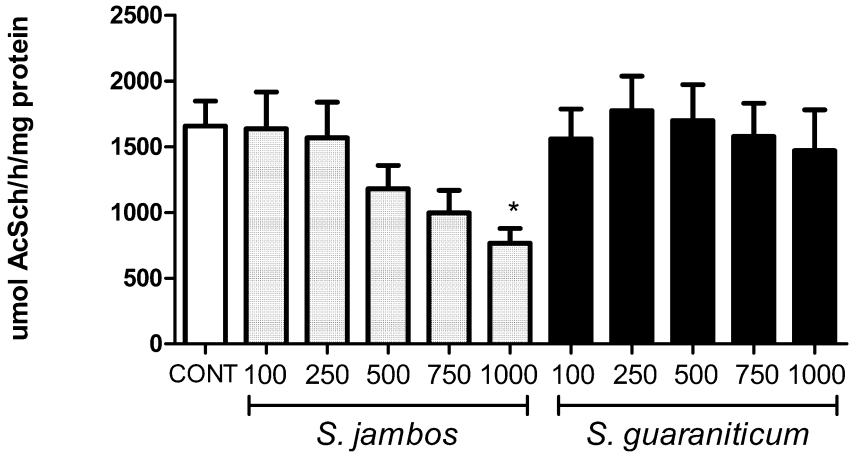


Figure 2. Effect of extracts tested on the acetilcholinesterase activity of spleen lymphocytes. Results are expressed as mean ± SEM (n = 6) assessed by one way ANOVA followed by Duncan Multiple Comparison post hoc test. (*) denotes $p<0.05$ as compared to the control (without extracts).

When assessing the toxicity of plant extracts using the *A. salina* bioassay, an LC_{50} value lower than 1.000 µg/ml is considered bioactive (Meyer et al. 1982). Both of the extracts tested in this work presented

this effect (Table 1). They therefore can be considered biologically active and may be toxic to humans. *A. salina* testing is useful for screening plant extracts in order to predict their lethality. A previous study (Logarto et al. 2011) has already demonstrated a good correlation ($r = 0.85$; $p < 0.05$) between the LC_{50} of the *A. salina* assay, and the LD_{50} of the acute oral toxicity assay in mice. Based on this correlation result, a value of $LC_{50} > 25$ µg/ml in the *A. salina* test corresponds to an *in vivo* LD_{50} value of between 2.500 and 8.000 mg/kg. Therefore, we may deduce that the *in vivo* LD_{50} of oral acute toxicities of *Syzygium jambos* and *Solanum guaraniticum* leaf extracts are likely more than 2.500 mg/kg.

Table 1. *Artemia salina* lethality test of the plant extracts under study

	24 h		48 h	
	LC_{50} (µg/mL)	95% Confidence interval	LC_{50} (µg /mL)	95% Confidence interval
Syzygium jambos	362.70	346.01 – 380.20	391.02	376.28 – 406.34
Solanum guaraniticum	557.04	458.23 – 677.16	113.31	53.37 – 240.55

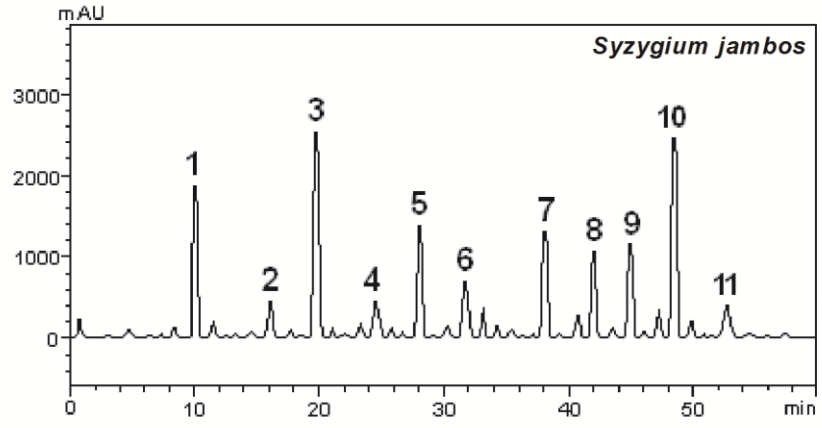
Values are expressed as an average of triplicates.

Syzygium jambos presented similar LC_{50} values when *A. saline* larvae were exposed for 24 or 48 hours to differing concentrations of extract, indicating that its toxicity is not time-dependent. The LC_{50} values (at 48h) of *Syzygium jambos* extract found in this study are also similar to those already reported for its methanolic leaf extract (Mohanty & Cock 2010).

However, the results obtained with *Solanum guaraniticum* extract showed that toxicity increased considerably with exposure time, calling attention to chronic consumption. Another interesting result is that at 24 h, the LC_{50} of *Solanum guaraniticum* was lower than the LC_{50} of *Solanum paniculatum,* already described at 953.9 µg/mL; this suggests that the plant is more toxic than *S. paniculatum* (Silva et al. 2007). This is of particular importance since these plants are frequently used without distinction.

During phytochemical screening of the extracts, HPLC analysis revealed the presence in both extracts of; gallic, chlorogenic and ellagic acids, catechin, epicatechin, rutin, quercitrin, isoquercitrin, quercetin and kaempferol (Figure 3). Further, considering the chromatographic conditions used, caffeic acid was only detected in the *Syzygium jambos* extract. Considering that natural phenolic compounds target multiple metabolic and cell signaling pathways and have several biological effects, we suggest that these phytochemicals are related to the observed effects. However, both vegetal extracts studied deserve to have their compositions explored in future studies, as well as the mechanisms of their demonstrated biological effects.

Compounds	*Syzygium jambos*		*Solanum guaraniticum*	
	mg/g	%	mg/g	%
Gallic acid (1)	27.68 ± 0.02 [a]	2.76	7.41 ± 0.03 [a]	0.74
Catechin (2)	4.47 ± 0.03 [b]	0.44	10.53 ± 0.01 [b]	1.05
Chlorogenic acid (3)	34.96 ± 0.01 [c]	3.49	26.85 ± 0.06 [c]	2.68
Caffeic acid (4)	4.83 ± 0.03 [b]	0.48	ND	-
Ellagic acid (5)	19.35 ± 0.02 [d]	1.93	20.07 ± 0.02 [d]	2.078
Epicatechin (6)	9.76 ± 0.05 [e]	0.97	4.71 ± 0.02 [e]	0.47
Rutin (7)	18.92 ± 0.01 [d]	1.89	39.85 ± 0.01 [f]	3.98
Quercitrin (8)	17.34 ± 0.03 [f]	1.73	11.36 ± 0.05 [b]	1.13
Isoquercitrin (9)	17.55 ± 0.01 [f]	1.75	19.54 ± 0.01 [d]	1.95
Quercetin (10)	36.81 ± 0.02 [c]	3.68	5.17 ± 0.01 [e]	0.51
Kaempferol (11)	4.73 ± 0.05 [b]	0.47	9.73 ± 0.05 [b]	0.97



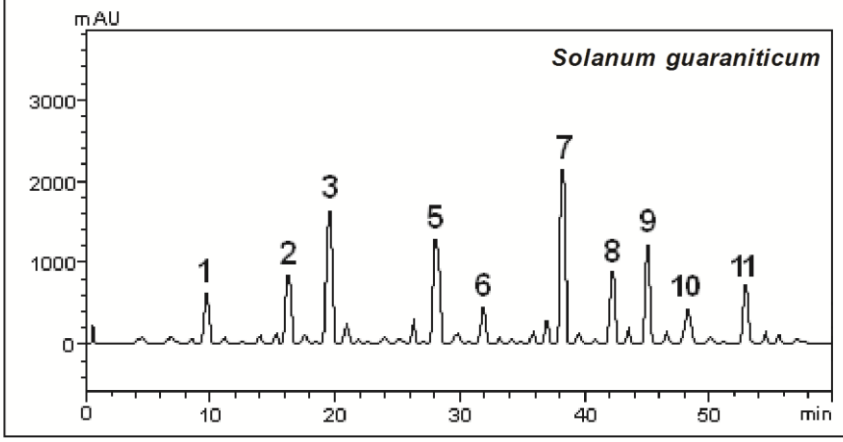


Figure 3. HPLC/DAD profile of extracts tested and quantification of phenolic compounds found at 327nm. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), epicatechin (peak 6), rutin (peak 7), quercitrin (peak 8), isoquercitrin (peak 9), quercetin (peak 10) and kaempferol (peak 11). Quantification results are expressed as mean ± SEM (n = 3) assessed by one way ANOVA followed by Duncan Multiple Comparison *post hoc* test. Means marked with different letters are significantly different ($p < 0.05$). ND = not detected.

5. Conclusion

The present study demonstrated the bioactivity of *Syzygium jambos* and *Solanum guaraniticum* extracts in the *A. salina* lethality test, and calls attention to possible toxic effects regarding its medicinal preparations. Further, *Solanum guaraniticum* presented cytotoxic effects and appears able to affect lysosomal functionality and cell membrane integrity. On the other hand, *Syzygium jambos* extract presents stimulatory effect on lymphocytes, as well as AChE inhibition activity, which suggests immunomodulatory properties. The present results highlight the potential toxicity of these plant extracts, and may be helpful for making them promising pharmacological agents.

6. Acknowledgement

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