

In Vitro* Antioxidant, Anticancer and Anti-cytotoxic Activity of *Lippia alba

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Abstract: *Lippia alba* is a herb widely distributed in Latin America used in the traditional medicine. Considering that medicinal plants present anticancer action, the aim of the work was to verify the *in vitro* antioxidant, cytotoxic, anti-cytotoxic and anticancer effects of *L. alba* extract, correlating the results with phytochemical content. Phytochemicals were evaluated by spectrophotometry; antioxidant activity was evaluated by DPPH, ABTS and Fe²⁺ chelating activity. *In vitro* cytotoxicity and anti-cytotoxicity were evaluated in human lymphocytes and anticancer action was evaluated in sarcoma-180 cells. *L. alba* extract presents good antioxidant, antiproliferative and anti-cytotoxic effects. Total flavonoid content was correlated to the antiproliferative effect; total tannin content was correlated to ABTS antioxidant activity and to health cells maintenance. Tannins were also correlated to the prevention of cisplatin-induced damage. Our finds support the use of *L. alba* as a source of natural antioxidants and as a potent anticancer and anti-cytotoxic agent, reinforcing the use of natural products for health promotion.

Key words: Phenolic content, DPPH, ABTS, iron chelating, sarcoma-180, MTT assay.

1. Introduction

Lippia alba (Mill.) N.E.Br. ex Britton & P. Wilson (Verbenaceae) is an aromatic plant native to South America, widely distributed in Latin America [1]. *L. alba* is a plant with several uses, mainly in the preparation of spices, drinks, infusions and food supplements [2]. There are also uses of this plant in traditional medicine and in ethnopharmacological studies [3], arousing biotechnological interest [4-6].

In vivo and *in vitro* analyzes have demonstrated biological activities of *L. alba*, such as antioxidant, antibacterial and antifungal, evidencing both the presence of phytochemical compounds with diverse properties, as well as their medicinal and therapeutic

potential [1, 7, 8]. However, due to being an extensively used plant with wide potential, investigations that reinforce its safe use in traditional medicine need to be evidenced, since plant metabolites can cause toxic effects and affect molecular and cellular stability [9, 10].

In this context, assays that promote direct contact of phytochemicals with cells are a good method for evaluating the cytotoxicity and anti-cytotoxicity of these substances [11, 12]. Medicinal plants biological activities are extensively described in the literature, such as *L. alba* anticancer action [11, 12], constituting a source of natural products to be explored [13, 14].

Thus, the present work aimed to verify the *in vitro* cytotoxic and anti-cytotoxic effects of *L. alba* extract on human lymphocytes and its anticancer effects on sarcoma-180 cells. In addition, the characterization of the extract was performed by analyzing the flavonoid

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and tannin content and by *in vitro* antioxidant assays.

2. Material and Methods

2.1 Hydroalcoholic Extract

Lippia alba total aerial part was collected in February/2016 from Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural [Incaper] (20°25'23.0"S, 40°28'37.4"W). Herb was dried at room temperature and a voucher specimen was deposited in the Herbarium of Universidade Federal do Espírito Santo (UFES). *L. alba* crude extract was obtained as described by Dutra et al [11].

2.2 Phytochemistry Analysis

Zhishen et al [15] method and Folin-Denis method [16] was used to measure total flavonoid and tannin content, respectively.

2.3 Antioxidant Activity

Antioxidant activity of *L. alba* extract was verified by DPPH(2,2-diphenyl-1-picryl-hydrazyl) [17], ABTS(2,2'-azinobis-(-3-ethylbenzothiazoline-6-sulfonic acid)) [18] and ferrous ions (Fe^{2+}) chelating activity [19].

2.4 In Vitro Cell Assays

Human lymphocytes were obtained, isolated from peripheral blood sample, plated in 96-well plates (2 x 10⁵ cells/well) and used to evaluate *in vitro* cytotoxic and anti-cytotoxic effects of *L. alba* extract [11]. Percentage of cytotoxic damage reduction was calculated as described by Serpeloni et al [20] and Dutra et al [11]. Sarcoma-180 cells were acquired from Banco de Células do Rio de Janeiro. Cells were plated in 96-well plates (2 x 10⁵ cells/well) and *L. alba in vitro* anticancer was evaluated [11]. All protocols were approved by the Research Ethical Committee of UFE (human cells-certificate-2.333.879; murine sarcoma-certificate-89/2015). MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazol

ium bromide) was used to verify cell viability [11].

2.5 Statistical Analysis

Results were expressed as the mean \pm standard error. Normality was evaluated by Shapiro-Wilk test ($p < 0.05$). *L. alba in vitro* cytotoxicity and anti-cytotoxicity was evaluated by ANOVA post hoc Tukey's test ($p < 0.05$) or by multiple t test ($p < 0.05$). Pearson correlation was performed to establish relationships between *L. alba* phytochemicals and biological activities.

3. Results

L. alba extract presents total flavonoid content of 53.35 mg/g \pm 4.72 (mg rutin/g dry extract) and total tannin content of 18.42 mg/g \pm 0.03 (mg tannic acid/g dry extract).

L. alba antioxidant activity is summarized in the Figure 1. In the DPPH assay, *L. alba* extract reached 0.00–80.75% of antioxidant power (Figure 1A), in the ABTS assay reached 38.36–83.80% (Figure 1B) and in Fe^{2+} chelating activity reached 2.99–72.14% (Figure 1C).

L. alba extract reduced human lymphocyte and sarcoma-180 cells viability after 24 h and 48 h of exposure at all tested concentrations (Figure 2). However, *L. alba* extract was more cytotoxic to sarcoma-180 cells than for lymphocytes. *L. alba* extract was more cytotoxic to sarcoma-180 cells after 24 h of treatment (Figure 2A) than after 48 h of treatment (Figure 2B), reinforcing that *L. alba* extract is able to reduce cancer cell proliferation at all tested conditions.

Reduction of damage induced by cisplatin in simultaneous protocol was observed only at the *L. alba* extract concentration of 50.0 and 100.0 $\mu\text{g/mL}$ (Figure 3), reducing cisplatin induced-damage in 18.98% on the cells treated with 50.0 $\mu\text{g/mL}$ of extract and 98.03% on the cells treated with 100.0 $\mu\text{g/mL}$ of extract.

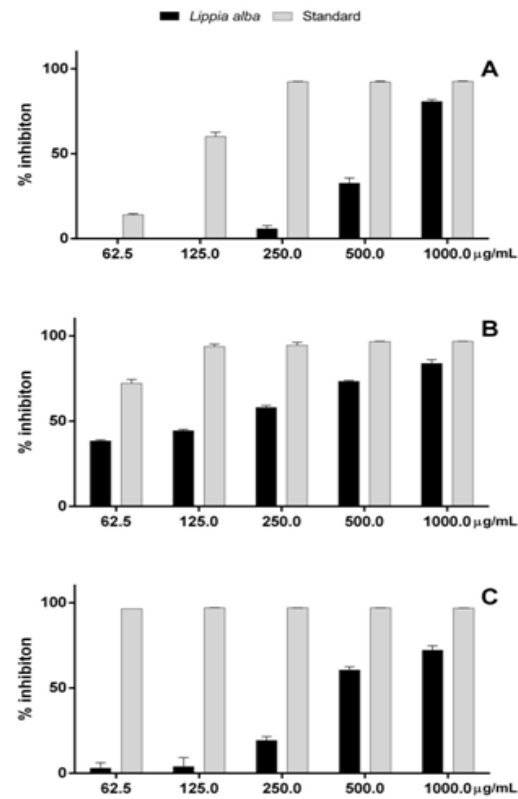


Fig. 1 Antioxidant activity of *L. alba* extract following DPPH, ABTS and Fe²⁺ chelating ions. DPPH (A), ABTS (B) and Fe²⁺ chelating protocols (C).

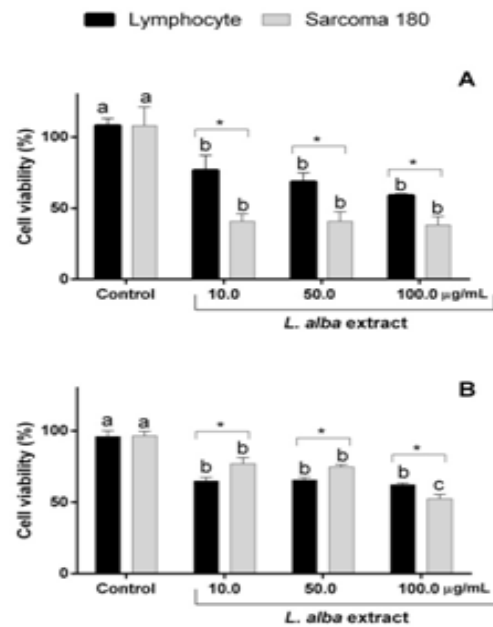


Fig. 2 *In vitro* cytotoxicity of *L. alba* extract (10.0, 50.0 or 100.0 µg/mL) in human lymphocytes and sarcoma-180 cells by MTT assay.

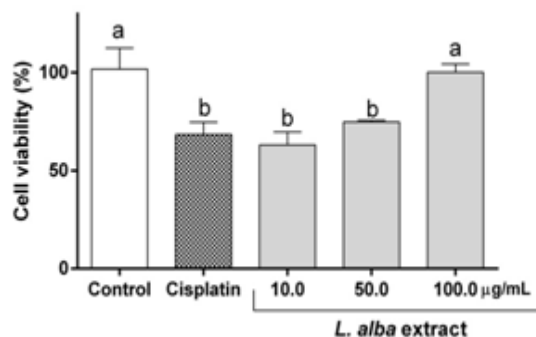


Fig. 3 *In vitro* anti-cytotoxicity of *L. alba* extract (10.0, 50.0 or 100.0 µg/mL) in human lymphocytes following simultaneous protocol by MTT assay.

Explorative analyses obtained on Pearson correlation are presented in Table 1. Negative correlation indicates an inversely proportional relation between the factors. Thus, considering that MTT assay was used to verify the viability of cancer cells after treatment with *L. alba* extract, negative correlations between the MTT assay and the total

flavonoid or tannin content, or antioxidant activities, suggest increasing anticancer effect; as well as, positive correlations between plant metabolites and human lymphocytes cell viability on cytotoxic and anti-cytotoxic protocol suggest the improvement of cellular homeostasis and the prevention of cisplatin-induced damage.

Table 1 Pearson correlation analysis between phytochemicals, antioxidant activities, anticancer and anti-cytotoxic effects of *L. alba* extract.

	Flavonoid	Tannin	DPPH	ABTS	Fe ²⁺ chelating	Hum. Lym. 24 h	Hum. Lym. 48 h	S180 24 h	S180 48 h	% Dam. Red.
Flavonoid	1									
Tannin	-0.979	1								
DPPH	0.477	-0.289	1							
ABTS	-0.498	0.664	0.524	1						
Fe ²⁺ chelating	0.140	0.063	0.937	0.788	1					
Hum.Lym. 24h	-0.961	0.997	-0.217	0.718	0.138	1				
Hum.Lym. 48h	-0.996	0.993	-0.397	0.574	-0.052	0.982	1			
S180 24h	-0.801	0.663	-0.908	-0.119	-0.705	0.605	0.745	1		
S180 48h	-0.982	0.999	-0.302	0.654	0.049	0.996	0.995	0.673	1	
%Dam. Red.	-0.871	0.953	0.015	0.859	0.364	0.973	0.911	0.404	0.948	1

Flavonoid: flavonoid total content; Tannin: tannin total content; DPPH: DPPH assay; ABTS: ABTS assay; Fe²⁺ chelating: iron chelating activity; Hum.Lym. 24 h: human lymphocytes cytotoxicity at 24 hours; Hum.Lym. 48 h: human lymphocytes cytotoxicity at 48 hours; S180 24 h: sarcoma-180 cytotoxicity at 24 hours; S180 48 h: sarcoma-180 cytotoxicity at 48 hours; % Dam. Red.: damage reduction percentage.

Following Pearson correlation, flavonoids were strongly negative correlated with reduced cell viability of sarcoma-180 cells, thus promoting anticancer action *in vitro*. On the other hand, tannins were moderately positive correlated with the antioxidant

action of the ABTS assay and strongly positive correlated with the cell viability of human lymphocytes in the cytotoxicity and anti-cytotoxicity assays, favoring the reduction of cisplatin-induced damage.

4. Discussion

L. alba is a herb extensively used in traditional medicine. Chemical analysis of *L. alba* leaves essential oils suggest a diversity of chemotypes, such as carvone, citral and linalool in the oils [21, 22], as well as, myrcene, tagetone, citral/germacrened, limonene/carvone, β -caryophyllene and eucalyptol/limonene, camphor and eucalyptol [3, 23, 24]. In addition, *L. alba* presents relevant pharmacological role related to its phytochemicals and may be applied in the treatment of liver diseases and intestinal disorders, or be used as an antipyretic, anti-inflammatory, antispasmodic and analgesic [25].

Phytochemistry screening showed the presence alkaloids, tannins and flavonoids in *L. alba* aqueous extract [26]. Oliveira et al [27] investigated fresh and dried *L. alba* ethanol extract fractions. In their study, both extracts with fresh plant and those with dried plant, the highest flavonoid content was observed in the ethyl acetate fraction, followed by the ethanolic fraction. In comparison to the study by Oliveira et al [27], the crude ethanol extract of our study showed a higher total flavonoid content than the ethanol fraction obtained from dried plants (10.01 mg/g). In addition, according to Poyer and Schaefer [28], the total tannin content in the hydroalcoholic extract of the leaves of *L. alba* oscillates between 1.03 and 2.40 mg/g, values lower than those observed in our study (18.42 mg/g).

Many authors attribute the antioxidant activity of plant extracts to the levels of phenolic compounds [11, 12, 29-32]. Regarding the DPPH assay, the ethanol fraction from the study by Oliveira et al [27] showed better antioxidant power than that observed in our study using the crude ethanol extract of *L. alba*, reaching 30.33 to 99.96% of radical inhibition. Reyes-Solano et al [33] investigated the essential oil extracted from *L. alba*. In their study, the total phenolic content in the essential oil samples of *L. alba* ranged between 84.0 and 137.5 $\mu\text{mol/g}$ (galic acid equivalent), and the concentration necessary to inhibit 50% of DPPH radicals ranged from 12.45 to 17.35

mg/ml, and to neutralize ABTS radicals it ranged between 15.6 and 55.2 $\mu\text{mol/g}$ (trolox equivalent). Nonato et al [34], investigated the essential oil of different species of *Lippia*. Nonato and collaborators verified that the compounds present in *L. alba* showed greater antioxidant effectiveness, and even with the potential to chelate iron ions, the essential oil excelled in DPPH scavenging, Fe^{3+} reduction and β -carotene protection.

Our results suggest the use of *L. alba* as an anticancer and in the prevention of cytotoxic damage. Other species of the genus *Lippia*, such as *L. microphylla*, have been described in the literature due to the anticancer action of its essential oil, reaching between 38 and 86% of tumor inhibition *in vivo* [35].

In our study, the ethanol crude extract had a higher total flavonoid content than the ethanol fraction used in the study by Oliveira et al [27]. The best antioxidant activity exerted by flavonoids observed in the study by Oliveira et al [27] may be related to its chemical structure, since flavonoids that loose hydroxyl groups have their antioxidant activity reduced [31, 36].

5. Conclusions

Our findings suggest that *L. alba* hydroalcoholic extract shows antioxidant activity, antiproliferative and anti-cytotoxic effects. Total flavonoid content has been correlate to the antiproliferative effect, as well as, total tannin content has been correlate to ABTS antioxidant activity and to health cells maintenance. Total tannin was also correlated to the prevention of cisplatin-induced damage. This finds also suggests the potential use of *L. alba* as a source of natural antioxidants and potent anticancer and anti-cytotoxic agent, reinforcing the effectiveness of the biological activities of natural products.

Conflicts of interest

The authors have no conflicts of interest.

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