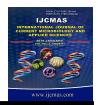


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Antimicrobial and Antioxidant Activity of the Leaves, Bark and Stems of *Liquidambar styraciflua* L. (Altingiaceae)

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ABSTRACT

Keywords

Liquidambar styraciflua, antioxidant activity, antimicrobial activity, microdilution method, DPPH assay

Article Info

Accepted: 16 December 2015 Available Online: 10 January 2016 The genus Liquidambar L. is the best-known genus of the Altingiaceae Horan family, and species of this genus have long been used for the treatment of various diseases. Liquidambar styraciflua L., which is popularly known as sweet gum or alligator tree, is an aromatic deciduous tree with leaves with 5-7 acute lobes and branched stems. In the present study, we investigated the antimicrobial and antioxidant activity of aerial parts of L.styraciflua. Antimicrobial activity was evaluated using the microdilution methodology. The DPPH and phosphomolybdenum methods were used to assess the antioxidant capacity of the samples. The extracts showed moderate or weak antimicrobial activity. The essential oil had the lowest MIC values and exhibited bactericidal action against Escherichia coli, Enterobacter aerogenes and Staphylococcus aureus. The ethyl acetate fraction and the butanol fraction from the bark and stem showed the best antioxidant activity. This study revealed that the essential oil of L. styraciflua leaves displays antimicrobial activity and can be used as an alternative against pathogenic microorganisms. The extracts and fractions of this species have a high antioxidant capacity, revealing new sources of antioxidants for the food and pharmaceutical industries.

Introduction

Infectious diseases are a prominent cause of morbidity and mortality worldwide, despite technical and scientific advances. One of the main factors responsible for this status is the ability of microorganisms to acquire mechanisms of resistance to antimicrobial

agents (Silver and Bostian, 1993). Thisability has driven the search for substances with antimicrobial activity in natural products, as many secondary metabolites of plants have been proven to have antimicrobial properties in vitro (Chauhan *et al.*, 2015).

Various degenerative diseases, such as cancer and Parkinson's disease, can be caused by the uncontrolled production of reactive oxygen species. The use of antioxidants to aid in the prevention and treatment of these diseases has been studied due to their potential to reduce oxidative stress (Sadi *et al.*, 2015). Many medicinal plants contain a large number of phenolic compounds, such as flavonoids and tannins, which exhibit antioxidant activity (Araujo *et al.*, 2008), encouraging research in this area.

A little explored family is Altingiaceae Horan, which consists of approximately fifteen species that are divided into three genera: *Liquidambar* L., *Altingia* Noronha and *Semiliquidambar* HT. Chang (Ickert-Bond and Wen, 2006). The extracts and resins of plants from this family are used in traditional Chinese and Ayurvedic medicine for the treatment of stomach pain, bronchitis, inflammation, amenorrhea and skin diseases, such as eczema and scabies (El-Readi *et al.*, 2013).

The genus *Liquidambar* is the best-known genus of the family and consists of four intercontinental species in the temperate the northern hemisphere: zone of Liquidambar formosana, Liquidambar acalycina, Liquidambar orientalis and Liquidambar styraciflua (Ickert-Bond et al., 2005). One of the species of this genus, L.styraciflua L., which is commonly known as sweet gum, red gum, alligator tree and Liquidambar, has a wide natural distribution in the southern and southeastern United States, extending to Mexico and Central America. This organism is a deciduous tree that is 20-25m high and has leaves with 5-7 acute lobes that are green in the spring and summer but turn purple, red, or yellow in the fall (Lorenzi et al., 2003). More than 10,000 years ago, Native Americans used the storax of this species for the treatment of various

diseases, such as stomach disorders, wounds and cough; however, the essential oil extracted from the leaves is also known to exhibit medicinal properties (Lingbeck *et al.*, 2015).

Some studies of species of the genus Liquidambar demonstrated antimicrobial potential (Sağdic et al., 2005; Kim et al., 2008; Lee Y-S et al., 2009); however, no studies have been conducted with the essential oil and extracts of the species L. styraciflua. Some studies demonstrated antioxidant, antitumor, anticoagulant and hepatoprotective activity for the species L.orientalis and L.formosona (Wang et al., 2010; Konno C et al., 1988; Yang et al., 2011). The essential oil of the leaves and stem of L. styraciflua has anti-inflammatory activity and antioxidant activity with low cytotoxicity (El-Readi et al., 2013), but there are no reports in the literature concerning the antioxidant capacity of extracts and fractions of the aerial parts of this species.

The lack of studies related to this species encourages this work, which aims to evaluate the antimicrobial and antioxidant activity of the extracts, fractions and essential oil of *L.styraciflua*.

Materials and Methods

Plant Material

The botanical material was collected from L.styraciflua specimens of L., ALTINGIACEAE, which were grown in the of experimental field the Brazilian Agricultural Corporation, Research EMBRAPA Forests (Colombo - PR). The leaves, bark and stems of L. styraciflua were collected during the fall and spring of 2011/2012.

The hydroalcoholic extract of the leaves and the hydroalcoholic extract of the bark and stems of *L. styraciflua* were prepared using a maceration technique at a moderate temperature. From these extracts, we prepared fractions into a separatory funnel using the liquid-liquid partition method and solvents with increasing polarity (n-hexane, chloroform, ethyl acetate, butanol and ethanol 70%).

The essential oil was extracted from dried leaves of *L. styraciflua* via the hydrodistillation method, using the Clevenger apparatus.

Reagents and Chemicals

All chemicals and solvents were purchased from Sigma Aldrich Co. (St. Louis, MO, USA) and Merck Millipore (Darmstadt, Germany).

Antimicrobial Activity

For the antibacterial tests, we selected eight standard strains of bacteria from the company NEWPROV, including four Grampositive bacteria and four Gram-negative bacteria. In the antifungal tests, we used five standard yeast strains from the Mycology Laboratory of Clinical Hospital (Curitiba-PR), which are often isolated from patients. The culture media used in this study included Mueller-Hinton broth and Sabouraud broth, which were both obtained from HIMEDIA. After growth on Petri plates containing a suitable culture medium, the bacteria and yeasts were inoculated into sterile saline (0.9%) and the turbidity was adjusted to McFarland standard n°0.5 to concentration obtain a standard microorganisms (1 x 10⁸ colony-forming unit/mL).

The minimum inhibitory concentration (MIC) values of the extracts and essential oil

against the selected microorganisms were determined in sterile 96-wells or holes (TPP) according to previously described methodologies (Ayres *et al.*, 2008; Hanamanthagouda *et al.*, 2010; Silva *et al.*, 2011), with some modifications. The recommendations of Clinical and Laboratory Standards Institute (2005) were applied.

The hydroalcoholic extracts were added to an initial concentration of 2,000 $\mu g/mL$, and the serial dilution procedure was then performed with the first culture medium to obtain decreasing concentrations (2,000 $\mu g/mL$ to 15.625 $\mu g/mL$). For the essential oil, an initial concentration of 1,000 $\mu g/mL$ with the solvent 10%Tween 80 was used, and concentrations of 1,000 $\mu g/mL$ to 3.9 $\mu g/mL$ were obtained by serial dilution with medium.

In the last columns of the plate,we added the positive controls (30 μ g/mL of erythromycin for bacteria; 50 μ g/mL of ketoconazole and 50 μ g/mL of terbinafine for yeast), the negative controls (solvent) and the sterility control for the culture medium.

To verify the ability of the sample to inhibit the growth of microorganisms or cause the death of microorganisms, a methodology described by Veljic *et al.* (2010) was used, with some modifications. From wells that exhibited an MIC, we collected a10 μ L aliquot and transferred the aliquot to Petri dishes containing the appropriate culture medium for each microorganism.

After the incubation period, samples that showed no visible microbial growth, indicating the death of 99.5% of the microorganisms in the inoculum, were considered to have bactericidal/fungicidal activity. The samples with visible microbial growth were considered to have bacteriostatic/fungistatic activity.

Antioxidant Activity

The DPPH method that was used to determine the antioxidant capacity followed the method described by Sousa *et al.* (2007), with some modifications. All samples (the ethyl acetate fraction, the butanol fraction, and the remaining aqueous-alcoholic raw extract of both organs of the species) were subjected to this test at concentrations of 25 to 250µg/mL. We used solutions of ascorbic acid, rutin and gallic acid as positive controls, all at the same concentrations as the test samples.

The analytical curve was generated with a DPPH solution, yielding a straight-line equation (R = 0.9996), which was used for further calculations. The samples and positive controls were tested in a reaction mixture (0.3 ml of the sample or positive control + 2.7 mL of the 40µg/ml DPPH solution) in triplicate, and after 30 minutes, the absorbance of the samples was measured using **UV-Vis** 515 nm a spectrophotometer (Shimadzu 1601, Kyoto, Japan).

From the equation obtained using the calibration curve and the average DPPH absorbance of each sample and positive control, it was possible to determine the remaining concentration of DPPH as a percentage, as well as the percentage of antioxidant activity at all tested concentrations.

For the method in which phosphomolybdenum complexes are reduced to determine the total antioxidant capacity (Prieto *et al.*, 1999), the same samples and positive controls were used, at the same dilutions. The reaction mixture was formed by mixing 100µL of the positive control or sample with 1 ml of the reagent solution. All reactions were performed in

triplicate. These samples were incubated for 90 minutes in a 95°C water bath, and after cooling, the absorbance of the samples at 695nm was measured (UV-Vis spectrophotometer Shimadzu 1601, Kyoto, Japan).

The analytical curve was generated witha $250\mu g/mL$ ascorbic acid solution using six different concentrations, following the same procedure that was used for the other samples. The total antioxidant capacity was determined by interpolating the average absorbance of the samples and positive controls against the calibration curve and expressed as μg of ascorbic acid/g plant material (μg AA/g PM).

The biological activity results were analyzed using the statistical program SISVAR (Ferreira, 2011)and results expressed as mean ± standard deviation (SD) from three experiments. Analysis of variance was performed to detect the existence of differences among the factors (One way ANOVA), and the identified differences were subjected to significance comparisons using the Tukey test.

Results and Discussion

Based on the obtained results (Table 1), we can confirm that the extract from the leaves moderate antibacterial activity has (Machado et al., 2005) against Salmonella typhimurium, Escherichia coli, Enterobacter aerogenes, Proteus mirabilis. Staphylococcus Streptococcus aureus, pyogenes and Bacillus subtilis. However, the antibacterial activity of this extract against the microorganism Enteroccus faecalis was low, with an MIC value of 2,000 µg/mL. The effect of this extract against the eight in bacteria used this study bacteriostatic.

Regarding the investigated yeasts (Table 2), this extract showed activity against only two species: *Candida glabrata*, for which the extract exhibited weak activity, and *Trichosporon beigelli*, for which the extract exhibited moderate activity. The effect of this extract against both strains was fungistatic.

The extract from the bark and stem exhibited moderate antibacterial activity against *S. typhimurium*, *E. coli*, *E. aerogenes*, *S. aureus*, *S. pyogenes* and *B. subtilis*, with MIC values of 500 µg/mL. However, against the strains *P. mirabilis* and *E. faecalis*, this extract showed weak activity. Bactericidal activity of this extract was observed only against *E. coli*.

Among the investigated yeasts, the only species that was not sensitive to the action of this extract was *Candida krusei*. The MIC values of this extract against *C. glabrata*, *C. albicans*, *C. tropicalis* and *T. beigelli* revealed a low activity of the extract against these microorganisms. In all of these cases, the extract exhibited fungistatic activity.

Analyzing the results for the essential oil (Table 1), it can be seen that the MIC values were low against all bacteria, except for S. pyogenes. Against S. typhimurium, E. coli, E. aerogenes, S. aureus and B. subtilis, the essential oil showed MIC values of 31.25 representing good antibacterial μg/mL, activity. Attention should be paid to the action of the essential oil against the bacteria E. coli, S. aureus and E. aerogenes, as the essential oil was capable of causing the death of these organisms, thus confirming bactericidal activity. Concerning the other bacterial strains. the oil exhibited bacteriostatic activity.

Regarding the investigated yeasts (Table 2), only *C. glabrata* and *C. krusei* were not

sensitive to the action of the essential oil. Against *C. albicans*, *T. beigelli* and *C. tropicalis*, the essential oil exhibited moderate activity. The action of the essential oil against these yeasts was fungistatic in all cases.

In relation to the antioxidant capacity, the results obtained using the DPPH method (Table 3) revealed that all of the samples have antioxidant activity, with a dose-dependent profile. In addition, a significant difference was observed between the samples (p <0.01) and reference patterns, as well as between the samples tested in this assay.

The butanol fraction of the bark and stem showed the strongest antioxidant activity, which was stronger than those of the reference standards that were used in this study. The value displayed by the ethyl acetate fraction of this same plant organ represented the second strongest antioxidant effect; however, the average did not differ from those of the ascorbic acid standard and the ethyl acetate fraction of the leaves. The antioxidant activity found for the crude extract of the leaves was statistically similar to that exhibited by the gallic acid and rutin standards.

From the results obtained using phosphomolybdenum methodology, it can also be seen that for both the samples and the reference patterns, the total antioxidant capacity is dose-dependent, with the highest concentration tested here (250 µg/mL) corresponding to the highest antioxidant capacity of all of the analyzed solutions. According to the statistical analysis (Table 4), the ethyl acetate fraction of the bark and stems exhibited the strongest total antioxidant capacity and was superior to the reference standards used (gallic acid and rutin).

Table.1 Minimum Inhibitory Concentration (MIC in μg/ml) and Antimicrobial Activity of Extracts and Essential oil of *Liquidambar styraciflua* in Bacteria Control

Micrographicm	Samples			
Microorganism	Gram	Oil Leaves	Barkandstems	
Salmonella typhimurium		31,25	500	500
ATCC 14028	-	Bacteriostatic	Bacteriostatic	Bacteriostatic
Escherichia coli		31,25	250	500
ATCC 25922	•	Bactericidal	Bacteriostatic	Bactericidal
Enterobacter aerogenes		31,25	250	500
ATCC 13048	-	Bactericidal	Bacteriostatic	Bacteriostatic
Proteus mirabilis		62,5	500	1000
ATCC 25933	-	Bacteriostatic	Bacteriostatic	Bacteriostatic
Staphylococcus aureus		31,25	250	500
ATCC 25923	+	Bactericidal	Bacteriostatic	Bacteriostatic
Enterococcusfaecalis	+	500	2000	2000
ATCC 29212		Bacteriostatic	Bacteriostatic	Bacteriostatic
Streptococcus pyogenes		NA	250	500
ATCC 19615	+		Bacteriostatic	Bacteriostatic
Bacillus subtilis		31,25	500	500
ATCC 6633	+	Bacteriostatic	Bacteriostatic	Bacteriostatic

NA = No Activity

Table.2 Minimum Inhibitory Concentration (MIC in μ g/ml) and Antimicrobial Activity of Extracts and Essential Oil of Liquid Ambarstyraciflua in Yeasts Control

Microorganism		Samples	
	Oil	Leaves	Barkandstems
Candida glabrata	NA	2000	2000
		Fungistatic	Fungistatic
Candida albicans	250	NA	2000
	Fungistatic		Fungistatic
Candida tropicalis	125	NA	2000
	Fungistatic		Fungistatic
Candida krusei	NA	NA	NA
Trichosporon beigelli	250	250	1000
1	Fungistatic	Fungistatic	Fungistatic

NA = No Activity

Table.3 Percentages of Antioxidant Activity of Samples and Positive Controls (concentration 250 μg/mL) by the DPPH method. Averages (n=3) Followed by the Same Letter do not differ by Tukey Test (p<0,01)

Samples	Averages ± SD	Tukey test
Butanol fraction of barks and stems	$104.386 \pm 0,001$	a
Ethylacetate fraction of bark and stems	$97.699 \pm 0,001$	b
Ascorbic acid	$96.596 \pm 0,002$	b, c
Ethylacetate fraction of leaves	94.575±0,001	b, c
Hydroalcoholic extract of leaves	93.955±0,001	c
Gallicacid	93.544±0,001	c
Rutin	93.263±0,001	c
Hydroalcoholic extract of bark ands tems	82.807 ± 0.017	d
Butanolfraction of leaves	77.758 ± 0.012	e
Hydroalcoholic fraction of bark and stems	52.877±0,001	f
Hydroalcoholic fraction of leaves	36.313 ± 0.037	g

Graph.1 Percentages of relative antioxidant activity of samples (EL = extract of leaves, EAL ethylacetate fraction of leaves, BL = butanol fraction of leaves, HL = hydroalcoholic fraction of leaves, EBS = extract of bark and stems, EABS = ethylacetate fraction of bark and stems, BBS = butanol fraction of bark and stems, HBS = hydroalcoholic fraction of bark and stems) and positive controls (AA = ascorbic acid, GA = gallic acid, RT = rutin) in relation to ascorbic acid by phosphomolybdenum methodology. Averages (n=3) followed by the same letter do not differ by Tukeytest(p<0,01)

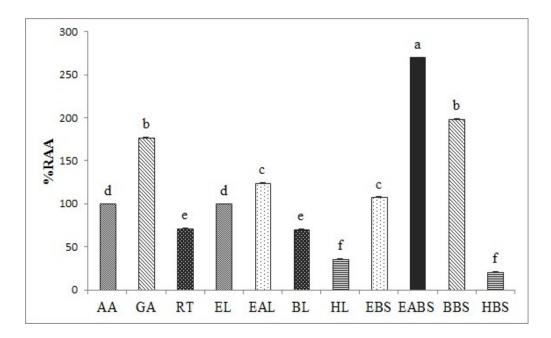


Table.4 Total Antioxidant Capacity of Samples and Positive controls (concentration 250 μg/mL) by the Phosphomolydenum Method. Averages (n=3) followed by the Same Letter do not Differ by Tukey Test (p<0,01)

Samples	Averages (μ gAA/gPM) \pm SD	Tukey test
Butanol fraction of barks and stems	$700.797 \pm 0,035$	a
Ethylacetate fraction of bark and stems	514.710 ± 0.021	b
Ascorbic acid	$458.043 \pm 0,013$	b
Ethylacetate fraction of leaves	322.101 ± 0.015	c
Hydroalcoholic extract of leaves	283.116 ± 0.033	c
Gallicacid	$256.739 \pm 0,010$	c
Rutin	$187.609 \pm 0,030$	d
Hydroalcoholic extract of bark ands tems	181.956 ± 0.015	d
Butanolfraction of leaves	$89.638 \pm 0,027$	e
Hydroalcoholic fraction of bark and stems	$71.522 \pm 0,022$	e

The butanol fraction of the bark and stem had a total antioxidant capacity similar to that of the gallic acid standard, while the butanol fraction of the leaves exhibited a total antioxidant capacity similar to that of the rutinstandard but lower than that of the same fraction of the bark and stem. The hydroalcoholic extracts and the ethyl acetate fraction of the leavesyielded statistically equivalent results, and all of the extracts were superior to rutin but inferior to gallic acid. Through a comparison with ascorbic acid, which was considered to have 100% antioxidant activity for the purposes of the calculations, another comparison profile was established between the samples and the reference standard by calculating the percentages of relative antioxidant activity at a concentration of 250 µg/mL (Graph 1).

Analyzing these results, we found that the ethyl acetate fraction of the bark and stem was again superior to all of the samples and reference standards, displaying antioxidant activity three times higher than the ascorbic acid. The reference standard gallic acid also showed a higher value than ascorbic acid, along with the butanol fraction of the bark and stem, and these results were statistically similar.

The hydroalcoholic extract of the leaves showed a relative percentage of antioxidant activity similar to that of ascorbic acid, while the butanol fraction of this plant organ was lower than but similar to that of the reference standard rutin.

The antimicrobial activity observed for extracts of this species may be related to the presence of polyphenols, such as flavonoids and tannins, and also to the presence of steroids and/or triterpenes. These classes of secondary metabolites are present in the hydroalcoholic extracts of both of the analyzed organs (Baril, 2013), and the literature contains reports of the antimicrobial properties of these chemical compounds (Lima, 2002).

The essential oil from the leaves of *L. styraciflua* showed the best antimicrobial activity and also exhibited bactericidal activity against some species, such as *S. aureus*. These data are extremely valuable because this microorganism is an important etiologic agent and has become a paradigm of bacterial infections. *S. aureus* stands out due to its high frequency and pathogenicity, as well as its high versatility in acquiring antimicrobial resistance, making it a

universal concern (Ferronato *et al.*, 2007; Ferreira *et al.*, 2010).

Approximately volatile secondary 64 metabolites have been identified in the essential oil of L. styraciflua leaves, including major monoterpene the hydrocarbon components d-limonene (20.71% to 22.34%), α -pinene (26.17% to27.95%) and β -pinene(10.06% to 11.25%) (El- Readi et al., 2013). The antimicrobial activity of a wide variety of essential oils is recognized, and in most cases, this activity is primarily attributed to isoprenes, such as monoterpenes, sesquiterpenes and related alcohols, phenols and other hydrocarbons (Koroch et al., 2007), which are all compounds that are present in the essential oil of this species.

However, as the essential oil has a complex chemical composition, its antimicrobial activity can likely be assigned not to a single mechanism but to several mechanisms, which may result in synergistic effects or even antagonistic effects (Silva *et al.*, 2011).

The antioxidant activity results show that the fractions and the extract obtained from the bark and stem of L.styraciflua exhibited stronger activity than the substances used as references of antioxidant activity. Those fractions and extracts also exhibited higher values than those found for the fractions and extracts of the leaves of this species. We obtained the best results for antioxidant activity using the butanol and ethyl acetate fractions of both organs from L.styraciflua. The polar nature of these compounds allows polyphenol for better extraction of compounds, which have a multifunctional profile in the plant and may act as reducing agents, hydrogen donors and suppressors of singlet oxygen (Koroch et al., 2007).

Therefore, the antioxidant activity of these compounds can occur through several

mechanisms, such as blocking, interfering with or suppressing the activities of the enzymes involved in the generation of reactive oxygen and nitrogen species, acting as chelating agents for transition metals and making them inactive, or neutralizing free radicals into stable compounds (Mosquera *et al.*, 2009). These characteristics of this group of secondary metabolites that are prominent in fractions and extracts of *L.styraciflua* (Baril, 2013) may underlie the high antioxidant capacity observed in this work.

The antioxidant activity of the essential oils from the leaves and stem of *L. styraciflua* was assessed by El-Readi *et al.* (2013) using three methods, including the DPPH test. Both essential oils had antioxidant activity, but the essential oil from the stem was more active than the essential oil from the leaf: however, both oils exhibited weaker activity than vitamin C.

In the present study observed differences in the results obtained for the *L.styraciflua* samples and the reference standards using the two methodologies employed in this study. This finding proves that many complex mechanisms are used to both inhibit oxidation and neutralize free radicals; therefore, the results obtained using the two methods should not be compared, as the principle of the reaction is not the same (Malinowski, 2010).

Reactive oxygen species may be the principal factor involved many in degenerative diseases. For this reason, antioxidants may have preventive and therapeutic effects on these diseases (Koroch et al., 2007) and may act to reduce the development of diseases such as atherosclerosis, diabetes, hypertension, and degenerative coronary and neurological diseases and to reduce mutagenicity and cancer (Barbosa, 2010).

In conclusion, based on the results of this research, that the essential oil extracted from the leaves of *L. styraciflua* exhibits antimicrobial activity. Emphasis should be given to the action of the essential oil against the bacteria *E. coli*, *E. aerogenes* and *S. aureus*, as the essential oil exhibited bactericidal activity against these strains. Therefore, natural products like the essential oil extracted from the leaves of *L. styraciflua*, are emerging as a viable way to identify alternative treatments for these pathogenic microorganisms.

The antioxidant capacity of the aerial parts of *L. styraciflua* was confirmed by this study, based on the stronger antioxidant activity of the extracts and fractions obtained from the bark and stem of this species. Due to the great interest in the use of natural products, the results of this study have importance in determining the beneficial effects of such products and may be used as new sources of antioxidants for the food industry and the pharmaceutical industry.

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