Chemical and Biological Evaluation of Ethanol Extract from *Plectranthus barbatus* Andrews Leaves (Lamiaceae)

ABSTRACT

Polyphenols can act as antioxidants, antibacterial, antifungal, or insecticidal agents. Given that microbial infections and arboviruses transmitted by *Aedes aegypti* significantly contribute to global morbidity and mortality, there is an urgent need to discover new antimicrobials and insecticides. This study investigated the ethanol extract (PbLE) of *Plectranthus barbatus* Andrews (Lamiaceae) leaves for its chemical constituents, antioxidant, antimicrobial, anti-hemolytic, and larvicidal activities. Qualitative analysis by TLC revealed cinnamic derivatives, flavonoids, terpenes, and steroids in PbLE. Quantitative analysis showed that PbLE contained 98.64 ± 0.87 mg EAG/g of total phenols, with flavonoids comprising 21.84 ± 0.34 mg EQ/g (c.a. 22%). PbLE demonstrated scavenging activity against $ABTS^{+}$ (IC50 = 3246.00 ± 0.97 µg/mL) and DPPH (IC50 = 1371.75 ± 0.94 µg/mL) free radicals and exhibited reducing ability in the phosphomolybdenum method (IC50 = 899.33 ± 0.73 µg/mL). Although PbLE did not affect the growth or survival of pathogenic microorganisms, it significantly reduced (c.a. 80% for the extract at 256 mg/mL) the hemolysis caused by *Staphylococcus aureus* in human erythrocytes. Furthermore, PbLE (7 mg/mL) caused 40% mortality of third-instar larvae of *A. aegypti* after 48-h exposure. In conclusion, PbLE, rich in phenolic compounds, acts as an antioxidant that mitigates the virulence of *S. aureus* to human erythrocytes and reduces the viability of *A. aegypti* larvae.

Keywords: Brazilian boldo, antioxidant, *Staphylococcus aureus*, antivirulence, larvicide, *Aedes aegypti*

1. INTRODUCTION

Polyphenols are a diverse group of compounds synthesized through the plant secondary metabolism. They are characterized by the presence of at least one aromatic ring with attached hydroxyl groups. These compounds are renowned for their antioxidant properties, including scavenging free radicals and reactive oxygen/nitrogen species, reducing oxidized intermediates, inhibiting oxidases and peroxidases, and activating antioxidant enzymes such as catalase and superoxide dismutase. Additionally, polyphenols can act as antibacterial, antifungal, or insecticidal agents [1, 2].

Microbial infections pose a significant global health challenge, exacerbated by the rise of antimicrobial resistance, a critical public health issue that complicates the treatment of

infections [3, 4]. This situation underscores the urgent need for the discovery of new antimicrobial agents with improved efficacy against resistant microorganisms and reduced toxicity to humans [5].

Aedes aegypti L. (Diptera; Culicidae) is one of the most widespread mosquito species globally, except in Antarctica. Its life cycle includes egg, larval (four instars), pupal, and adult stages [6]. Female mosquitoes are hematophagous, requiring blood meals for ovarian maturation and egg production [7]. Through their bites, *A. aegypti* females transmit several significant viruses, including yellow fever (YFV), dengue (DENV), chikungunya (CHIKV), Zika (ZIKV), and Mayaro (MAYV). In 2023, yellow fever was endemic in 34 African countries and 13 countries in Central and South America [8]. Dengue alone accounted for over 6.5 million cases and 7,300 deaths, with an estimated 100 to 400 million infections annually [9]. Chikungunya reported 410,754 cases across 17 countries in the Americas in 2023, with 419 deaths, marking the highest number of cases in recent years [10, 11]. In the same year, Zika cases in the Americas totaled 8,756, while Mayaro emerged as a notable public health concern, spreading across the region [12, 13]. Controlling vector populations is crucial for managing these arboviruses, but the environmental toxicity of insecticides to non-target organisms, including humans, has spurred interest in alternative plant-based compounds [14].

Plectranthus barbatus Andrews (Lamiaceae), commonly known as "falso boldo" in Portuguese, "forskohlii," or "Brazilian boldo" in English, is native to Africa, Asia, Australia, and South America [15]. The leaves of this aromatic shrub are utilized in traditional medicine through infusion and decoction for various ailments, attributed to their antioxidant, anti-inflammatory, antibacterial, and antifungal properties [15, 16].

Almeida et al. [17] reported that a saline extract of *P. barbatus* leaves effectively killed *A. aegypti* L3 larvae (LC50 of 0.48%, w/v). Treatment for 48 h increased the permeability of the peritrophic membrane and halting larval development before the fourth instar. The extract also reduced the neutral polysaccharide content in larval midgut epithelial cells, stimulated gut microbiota proliferation, and induced intense melanization, suggesting dysbiosis as a larvicidal mechanism. In this study, we aim to investigate the ethanol extract of *P. barbatus* leaves (PbLE) for its chemical constituents and evaluate its antioxidant, antimicrobial, anti-hemolytic, and insecticidal activities.

2. METHODOLOGY

2.1 PLANT MATERIAL AND ETHANOL LEAF EXTRACT

The collection of plant material was authorized (process n°. 36301) by the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio), and the access was recorded under registry A68A2BA in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). The leaves of *P. barbatus* were collected in Paulista city (7°56’29.2”S, 34°53’55.0”W), Pernambuco, Brazil, detached from their petioles, and air-dried at 28 °C for 20 days. Subsequently, the dried leaves were macerated using a blender (model PH900; Philco, Philadelphia, PA, USA). The resulting powder (10 g) was mixed with 100 mL of 99.8% (v/v) ethyl alcohol in the blender for 30 s, with 4 min intervals between each of four mixing cycles. The solvent was evaporated using a low-pressure rotary evaporator, and the residue remaining after evaporation corresponded to the ethanol extract of *P. barbatus* leaves (PbLE).

2.2 CHEMICAL CHARACTERIZATION

The presence of secondary metabolites in PbLE was determined by thin layer chromatography (TLC). PbLE (1:1 v/v) and standards (0.5 mg/mL) were solubilized in methanol using an ultrasound bath (10 min). Aliquots of PbLE and standards were applied onto silica gel 60-F254 chromatography plates (Macherey-Nagel, Germany), which were developed for 15 min at 25 °C using mobile phases listed in Table 1. The plates were dried at 25 °C and treated with specific developing reagents (Table 1). Metabolite classes were visualized under ultraviolet light (254 and 365 nm) or visible light and compared to corresponding standard bands.

Table 1. Conditions employed for phytochemical characterization of the ethanol extract of *P. barbatus* leaves by thin-layer chromatography (TLC).

Metabolite classes	**Mobile phase[1]**	**Revealer**	**Standard**
Cinnamic derivatives		$AlCl_3$	Caffeic acid
Flavonoids		$AlCl_3$	Rutin
Hydrolysable tannins		$FeCl_3$	Ellagic acid
Condensed tannins	90:5:5	Hydrochloric vanillin + Δ	Catechin
Coumarins	50:50:50	KOH	Coumarin
Terpene and steroids	90:10	Lieberman-Burchard + Δ	β-sitosterol
Saponins	16:10:2.5	Lieberman-Burchard + Δ	Escin
Anthracenic derivatives	20:30:15:0,5	HNO_3 + Δ + KOH	Senoside A
Sugars	100:11:11:26	Thymol + H_2SO_4 + Δ	D-fructose
Alkaloids	70:20:10	Dragendorff	Piperine

Δ: heating ; [1]Systems: 90: 5: 5 = Ethyl acetate, formic acid and water; 50:50:50 = Toluene, ethyl ether and glacial acetic acid (saturation); 90:10 = Toluene, ethyl acetate; 100: 11: 11: 26 = Ethyl acetate, glacial acetic acid, formic acid and water; 16: 10: 2.5 = Chloroform, methanol and water; 20: 30: 15: 0,5 = Ethyl acetate, n-butyl alcohol, water and glacial acetic acid; 70:20:10 = Toluene, ethyl acetate, diethylamine.

The contents of phenolic compounds and flavonoids in PbLE were quantified following methods by Li et al. [18] and Woisky & Salatino [19], respectively, with modifications. PbLE (20 µL; 1 mg/mL) was added to a 96-well microplate with Folin-Ciocalteu reagent (100 µL, 10% v/v) and Na_2CO_3 (80 µL, 75 g/L). After 30 min incubation in the dark at 28 °C, the absorbance at 765 nm was measured. Total phenol content in PbLE was calculated using a gallic acid curve ($y = 0.0066x + 0.0273$ µg/mL, $R^2 = 0.9859$) as standard, expressed as milligrams of gallic acid equivalent per gram of extract (mg EAG/g). Flavonoid content was analyzed by incubating PbLE (100 µL, 1 mg/mL) with 20% (w/v) $AlCl_3$ reagent in a 96-well microplate. After 1 h incubation in the dark at 28 °C, the absorbance at 420 nm was measured. Flavonoid content in PbLE was estimated using a quercetin curve ($y = 0.0329x + 0.0333$ µg/mL, $R^2 = 0.9986$) as standard, expressed as milligrams of quercetin equivalent per gram of extract (mg EQ/g).

2.3 ANALYSIS OF ANTIOXIDANT POTENTIAL

PbLE was evaluated for antioxidant activity using three methods. The $ABTS^+$ radical scavenging assay followed Re et al. [20]. An $ABTS^+$ radical solution was prepared by dissolving 7 mM 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 2.45 mM potassium persulfate, incubated for 16 h in the dark at 28 °C. The solution was diluted in ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. PbLE (20 µL) was mixed with 1 mL of $ABTS^+$ solution, incubated for 6 min, and absorbance at 734 nm was measured. $ABTS^+$ elimination percentage was calculated as (Ac - Ae)/Ac x 100, where Ac is control absorbance and Ae is PbLE absorbance, determining the IC50 value.

The phosphomolybdenum reduction assay was performed as per Prieto et al. [21]. PbLE (1 mg) was diluted in 1 mL of 5% (v/v) dimethyl sulfoxide solution, mixed with 100 µL of reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate, 4 mM ammonium molybdate), and incubated in a dry water bath at 90 °C for 90 min. Absorbance at 695 nm was measured, and activity was expressed relative to ascorbic acid activity using the formula (Ae - Ac) / (ascorbic acid - Ac) x 100, where Ae is PbLE absorbance and Ac is control absorbance, determining the IC50 value.

The DPPH assay followed Blois [23]. PbLE (1 mg) was diluted in 1 mL of methanol, subjected to serial dilution (15.625–1000 µg/mL). PbLE aliquots (40 µL) were added to 250 µL of 2,2-diphenyl1-picrylhydrazyl (DPPH) reagent (1 mM) in a 96-well plate, incubated for 30 min in the dark at 20°C, and absorbance at 517 nm was recorded. DPPH radical scavenging activity percentage was calculated as (Ac - Ae)/Ac x 100, where Ac is control absorbance and Ae is PbLE absorbance, determining the IC50 value.

2.4 ANTIMICROBIAL ACTIVITY

PbLE antimicrobial activity was tested against *Escherichia cloacae* (7827), *Enterococcus faecium* (12455 and 17872), *Staphylococcus aureus* (NCTC 12973, ATCC 2913, 5377, 5967), *Acinetobacter baumannii* (7001 and NDW), *Klebsiella pneumoniae* (968.11), *Pseudomonas aeruginosa* (545.6), and *Escherichia coli* (NDW) strains. Antifungal potential was assessed against *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Cryptococcus neoformans* (B3501 and H99), and *Cryptococcus gattii* (R265). All strains were provided by the *Departamento de Antibióticos* at the *Universidade Federal de Pernambuco*.

Bacteria were cultured in solid and liquid Mueller Hinton medium and stored in 100% glycerol and Brain Heart Infusion medium at 4°C. For assays, bacteria were grown in Mueller Hinton broth (MHB) with shaking (200 rpm) at 37°C for 24 h, adjusting optical density at 600 nm with sterile 150 mM NaCl to required cell density. Bacterial suspension (1.5 x 10^8 CFU/mL; 20 µL) was added to 96-well plates containing 160 µL MHB and 20 µL PbLE (2-1024 µg/mL). Plates were incubated for 24 h at 37 °C, and absorbance at 600 nm was measured to determine growth inhibition relative to control (bacterial suspension in 180 µL MHB) (CLSI, 2015).

Fungal strains were cultured in Sabouraud–Dextrose medium at 30°C for 48 h, adjusting turbidity to 106 CFU/mL (optical density at 600 nm). Sabouraud–Dextrose medium (80 µL) was added to all wells, and PbLE (100 µL; 10-5120 µg/mL) was added to the third well, with two-fold serial dilution across the row. Fungal suspension (20 µL) was added to all wells except the first (sterility control) and second (100% growth control). Plates were incubated with shaking at 30 °C for 48 h, and fungal growth inhibition was determined by optical density reduction.

2.5 EFFECT ON HEMOLYSIS BY *S. aureus*

Bacterial hemolysis was assessed following Lee et al. [23]. Human erythrocytes were collected with Ethics Committee approval (process 33550320.1.0000.5208) from a healthy, non-smoking adult volunteer using citrate tubes. Erythrocyte solution in 0.15 M NaCl (500 µL; 10%, v/v) was incubated with *S. aureus* UFPEDA 02 inoculum (500 µL; 1.5 x 10^8 CFU/mL) at 37 °C for 1 h, shaking at 250 rpm. PbLE (128, 64, and 32 µg/mL) was added, and the mixture incubated at 37 °C for 24 h. After centrifugation (3000 rpm, 10 min), supernatant was transferred to a sterile 96-well microplate, and red blood cell lysis

measured at 543 nm. Percentage lysis was calculated relative to control without extract (100% hemolysis).

2.6 EFFECT ON SURVIVAL OF *A. aegypti* LARVAE

Eggs of *A. aegypti* (Rockefeller strain) were obtained from colonies at the *Laboratório de Toxicologia*, *Departamento de Antibióticos*, *Universidade Federal de Pernambuco*. Eggs were placed in plastic containers with 1 L mineral water and 1 g fish food flakes (Friskies), hatched larvae reared at 26 ± 1°C, 12/12 photoperiod, and 38 ± 2% humidity until third instar (L3).

Larvicidal bioassay used semi-acrylic containers (150 mL) with 20 L3 treated with 20 mL PbLE (0.1 to 7 mg/mL) or distilled water (control). Triplicates were performed, and larval survival recorded after 48 h. Larvae were considered dead if unresponsive to mechanical stimuli [24].

2.7 STATISTICAL ANALYSIS

Data were presented as mean ± standard deviation. Statistical analysis and graphing used GraphPad Prism 8.0 software. ANOVA was performed, with $p < 0.05$ considered significant. For antioxidant assays, curves related radical inhibition (%) to sample concentration (mg/mL). IC50 values were determined using linear regression. The analysis of larvicidal activity used the Student's t-test ($p<0.05$).

3. RESULTS AND DISCUSSION

The widespread use of *P. barbatus* as a medicinal plant, coupled with previous reports of its larvicidal activity against *A. aegypti*, prompted the hypothesis that the ethanol extract of its leaves may possess pharmacological and larvicidal potential. Here, we tested this hypothesis. Thin layer chromatography (TLC) analysis of PbLE revealed the presence of cinnamic derivatives, flavonoids, terpenes, and steroids. In contrast, tannins, coumarins, saponins, anthracenes, reducing sugars, and alkaloids were absent. Quantitative analysis showed a total phenolic content of 98.64±0.87 mg EAG/g in PbLE, with flavonoids accounting for 21.84±0.34 mg EQ/g (c.a. 22%).

Similarly, Almeida et al. [17] reported that the saline extract, infusion, and decoction of *P. barbatus* leaves contained cinnamic derivatives, flavonoids, terpenes, steroids, as well as saponins and reducing sugars. Aqueous and acetone:water (7:3) extracts of *P. barbatus* leaves also presented cinnamic derivatives, flavonoids, steroids, and ellagic acid [25].

The antioxidant potential of plant extracts has been well-documented [26]. Given the high phenolic content of PbLE, we investigated its antioxidant properties. The results indicated significant scavenging of $ABTS^+$ and DPPH free radicals, as well as strong reducing ability in the phosphomolybdenum assay (Table 2). The positive controls trolox and ascorbic acid confirmed the suitability of the experimental conditions for assessing antioxidant activity.

Table 2. Antioxidant activity of the ethanol extract of *P. barbatus* leaves (PbLE).

	Antioxidant activity (IC50; µg/mL)		
Samples	**ABTS+**	**DPPH**	**CAT**
PbLE	3246,00±0,97	1371,75±0,94	899,33±0,73
Trolox	270,60±0,59	37,44±0,33	-

Ascorbic acid	-	-	464,05±0,55

IC50: Sample concentration required to reduce the concentration of oxidizing radicals by 50%. Trolox and ascorbic acid were used as positive controls.

The ethanol extract of fresh *P. barbatus* leaves and the aqueous leaf extract demonstrated antioxidant activity (IC50 of 15.87±0.3 µg/mL and 35.87±0.3 µg/mL, respectively) superior to PbLE in the DPPH free radical scavenging assay [27, 28]. This difference may be attributed to variations in extraction protocols or metabolic differences in the specimens collected in São Paulo/Brazil [27] and Pretoria/South Africa [28], as environmental conditions influence secondary metabolite production in plants [29].

Under the conditions employed here, PbLE did not exhibit antimicrobial activity, as it did not affect the growth or survival of bacteria or yeasts. Similarly, the aqueous extract of *P. barbatus* leaves showed no antibacterial activity against clinical isolates, whereas the acetone:water extract inhibited the growth of *E. coli*, *K. pneumoniae* (both susceptible and producing extended-spectrum beta-lactamase or carbapenemase), *P. aeruginosa*, *Acinetobacter baumannii*, and *S. aureus* (Methicillin-sensitive or resistant) with minimal inhibitory concentrations (MIC) ranging from 250 to 500 µg/ml [25].

Many *S. aureus* isolates secrete various virulence factors, including hemolytic enzymes and cytotoxins such as β-toxin, a sphingomyelinase, δ-toxin, and other cytolytic peptides [30]. We selected *S. aureus* strain (UFPEDA 02) to induce erythrocyte lysis and evaluate the antivirulence potential of PbLE. As expected, *S. aureus* caused significant hemolysis of human erythrocytes (Figure 1). However, the addition of PbLE (256 mg/mL) to the erythrocyte and bacterial cell mixture resulted in a substantial reduction (approximately 80%) in hemolysis compared to the control. These findings suggest that PbLE protects human erythrocytes from bacterial action. Furthermore, the absence of hemolysis at higher concentrations of the extract (256 mg/mL; Figure 1) suggests its low toxicity to human cells.

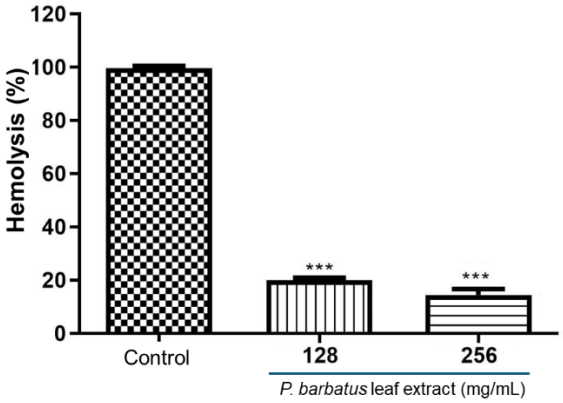

Figure 1. Effect of the ethanol extract of *P. barbatus* leaves on the lysis of human erythrocytes by *S. aureus*. Asterisks indicate significant difference compared to control using ANOVA ($p < 0.05$).

In fact, according to the data reported here, PbLE cannot be considered an antimicrobial tool, including against *S. aureus*. However, its antivirulence effect, which reduces the hemolytic capacity of the bacteria, suggests its pharmacological potential. PbLE may act on toxins, enzymes that degrade the erythrocyte membrane, or other mechanisms that bacteria use to become pathogenic; This property could be valuable in future strategies involving the extract in combination with traditional antibiotics [31]. Such an approach could enhance

antibiotic effectiveness while diminishing the bacteria's pathogenicity, thereby improving therapeutic outcomes and reducing the development of bacterial resistance [32]. The reduction of virulence could prove critical in preventing severe infections, particularly in hospital settings where multiple antibiotic-resistant *S. aureus* strains are prevalent.

Treatment of *A. aegypti* L3 larvae with *P. barbatus* leaf extract for 48 h resulted in larval mortality, with a maximum mortality rate of 40% observed for the extract at 7 mg/mL, as shown in Table 3. Musau et al. [33] reported that water (LC50 of 0.61 mg/mL), acetone (LC50 not reported), and hexane (LC50 of 1.053 mg/mL) extracts of *P. barbatus* leaves were lethal to *A. aegypti* fourth instar larvae. The saline extract of *P. barbatus* leaves also exhibited larvicidal activity against *A. aegypti* L3 (LC50 of 0.48%, m/v), with mechanisms involving increased permeability of the peritrophic membrane, inhibition of digestive enzymes, as well as disruption of midgut morphophysiology and gut microbiota homeostasis [17]. The authors linked the larvicidal effect to the presence of a ribose/galactose-binding lectin and trypsin inhibitory activity in the extract.

Table 3. Effect of the ethanol extract of *P. barbatus* leaves (PbLE) on survival of the *A. aegypti* third instar larvae.

Sample	**Mortality of L3 (%)**
Control	0^{a}
PbLE	
0.1 mg/mL	$5 \pm 5^{a,b}$
1.75 mg/mL	10 ± 7.1^{b}
5 mg/mL	25 ± 5^{c}
7 mg/mL	40 ± 10^{c}

Different letters indicate significant differences ($p<0.05$) from the control by Student's t-test

4. CONCLUSION

The ethanol extract of *P. barbatus* leaves, which contains phenolic compounds, acts as an antioxidant agent that mitigates the virulence of *S. aureus* on human erythrocytes and reduces the viability of *A. aegypti* larvae.

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