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Research Article

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A novel quinone shows activity against MRSA

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ABSTRACT

The emergence of bacterial strains resistant to antibiotics in clinical use stimulates the research for new antimicrobials, especially in the case of multi-resistant strains such as MRSA (Methicillin Resistant Staphylococcus aureus). In the context of medicinal chemistry, the lapachol and derivatives are recognized by different pharmacological actions. This study evaluated the antistaphylococcal activity in vitro by disk diffusion method and determination of minimum inhibitory concentration (MIC) by microdilution of a lapachol semi-synthetic derivative, encoded as LSY. Cytotoxicity was assessed compared to the 2nd instar nauplii of Brine shrimp. The action of LSY against strains of Staphylococcus aureus (ATCC 25923) and MRSA (CCBH 5330) was verified by growth inhibition in the disk diffusion test and MIC values were determined to be 62.5 µg/mL for both microorganisms. The LC50 value corresponded to 14.23 µg/mL. Finally, the compound LSY showed a promising antistaphylococcal activity and relative toxicity in vitro by Brine shrimp bioassay. However, as the possibility of determining the toxicity in vivo should not be ruled out, better toxicological assessment of the molecule (LSY) is essential. A perspective for this study is the evaluation of tissue effects of compound topical use wounds in order that species of Staphylococcus can prevail in this type of injury. Thus, it is expected that the quinone evaluated (LSY) will be validated for therapeutic use in the future.

Keywords: *Brine shrimp*, cytotoxicity, lapachol, medicinal plants, MIC and MRSA.

INTRODUCTION

The excessive use of antibiotics has led to the emergence of resistant microorganisms stimulating a progressive relationship between medicinal chemistry and clinical medicine in the research on alternatives to antimicrobial treatment. Considering the microbial infections, the research is focused on natural bioactive or semi-synthetic molecules with activity against pathogenic bacteria causing human infections, especially when they have mechanisms of resistance to the drugs of choice in the medical routine [1, 2]. The empirical use of medicinal plants in communities stimulates the scientific research to obtain and elucidate the action mechanisms of the naturally active chemical components of such plants; this is achieved together with the medicinal chemistry [3, 4].

The therapeutic properties of medicinal plants result from different secondary metabolites. They have complex structures with a large variety of functional groups such as fatty acids and esters, hydrocarbons, alcohols, aldehydes and ketones, acetylenic, alkaloid and phenolic compounds, coumarins, naphthoquinones, and phenylpropanoid derivatives [5, 6].

Many structures containing quinone nucleus have gained prominence in recent years mainly due to their important physiological functions. In the naphtoquinone groups, Lapachol and derivatives have received special attention. Lapachol was first isolated by E. Paterno from *Tabebuia avellanedae* (Bignoniaceae) in 1882 [7]. His work has encouraged studies that elucidate the action mechanisms of these molecules. Lapachol is one of the most versatile biologically active compounds of the naphthoquinone group and structurally is related to vitamin K. Its spectrum of activity includes, among various biological actions, anti-inflammatory, antimicrobial, antifungal, antiviral, leishmanicidal, and antitumor activities [8].

Among the microorganisms susceptible to lapachol and derivatives are the *Staphylococcus aureus* strains, which are important nosocomial infections because of their prevalence in various infectious processes such as conjunctivitis, keratitis, suppuration, abscess formation, pyogenic infections, and fatal sepsis. However, there is a failure in the treatment of these infections due to the emergence of strains resistant to some antibiotics used in the standard therapy highlighting Methicillin Resistant *Staphylococcus aureus* (MRSA) strains [9].

The increased occurrence, particularly in hospitals, of MRSA strains makes effective therapy more difficult. Although strategies have been proposed for infection control, the search for new ways to treat MRSA infections stimulates the research associated with natural components as an alternative treatment to these infections [10,11].

The aim of this study was to evaluate the activity of a semisynthetic derivative of Lapachol against *Staphylococcus aureus* strains, including a MRSA strain, and to conduct a preliminary test of toxicity as a first step in its validation as a new phytotherapic.

EXPERIMENTAL SECTION

2.1 Obtaining the derivative Lapachol

The evaluated naphthoquinone, coded LSY, was synthesized from Lapachol according to the procedure described in Revista de Proteção Intelectual nº 1921, codes C07C 50/12 (2007.10) and A61P 33/02 (2007.10) [12]. Its solubilization was performed in 5% dimethyl sulfoxide - DMSO (Merck, Darmstadt - Germany) aqueous solution.

2.2 Bacterial strains and culture media

The microorganisms used in this study were Gram-positive *Staphylococcus aureus* (ATCC 25923) and Methicillin Resistant *Staphylococcus aureus* - MRSA (CCBH 5330) strains. The ATCC (American Type Culture Collection) and MRSA CCBH (Coleção de Culturas de Bactérias de Origem Hospitalar da Fundação Oswaldo Cruz - FIOCRUZ) strains were activated by insertion in BHI broth (HIMEDIA, Mumbai - India) and subsequently grown on plates containing nutrient agar (HIMEDIA, Mumbai - India).

2.3 Disk diffusion method

For a qualitative evaluation of microbial growth inhibition, disk diffusion was performed according to Kirby-Bauer [13] with adaptations. Sterile Whatman filter paper disks (6 mm diameter) were impregnated with the sample at 2 mg/mL and placed on Mueller-Hinton Agar surface (HIMEDIA, Mumbai - India) previously inoculated with approximately 1.5 x 10^8 CFU/mL strain tested. The plates were kept at 35ºC for 24 hours in a bacteriological incubator (Nova Ética, São Paulo - Brazil). The reading was performed by measuring the growth inhibition zone formed around the disc. The test was performed in triplicate.

2.4 Minimum inhibitory concentration (MIC)

Quantitative analysis of antibacterial action was performed by minimum inhibitory concentration (MIC) determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute - CLSI (2010) [14]. The BHI broth was distributed in a sterile 96-well microplate and the volume of sample added was adjusted to achieve concentrations of 0.9 to 1.000 µg/mL. The positive control used was gentamicin (0.5 to 64 µg/mL). Subsequently, bacterial suspension (1.5 x 10^8 CFU/mL) was added. After 24 hours (35°C) 0.5% TTC (2,3,5-triphenyltetrazolium chloride) was added and incubated again for 3 hours. The medium color change to red indicated bacterial growth, whereas the keeping of yellow indicated no bacteria activity. The test was performed in triplicate.

2.5 *Brine shrimp* toxicity assay

The Lapachol derivative was evaluated for lethality to *Brine shrimp* larvae (*Artemia salina* Leach) according to the procedures described by Meyer et al. [15]. *Brine shrimp* eggs were hatched and maintained in seawater under artificial illumination (lamp 9W) until reaching the second nauplii. Then, 10-15 *Brine shrimp* were added in each well in a 96-well microplate containing the substance previously diluted in seawater at concentrations of 1, 10, 100, and 1000 µg/mL. After 24 hours, the number of survivors in each well was counted and the percentage of death calculated. The concentration that killed 50% of the *nauplii* (LC50 in µg/mL and confidence intervals 95%) was

determined using the statistical method of PROBIT analysis (POLO-PC). Thymol 1% aqueous solution was used as positive control. Criterion of toxicity was established according to Déciga-campos et al. [16]: LC50 values > 1000 μg/mL (non-toxic), 300 ≤ LC50 ≤ 1000 μg/mL (weak toxicity), and LC50 < 300 μg/mL (toxic).

RESULTS AND DISCUSSION

The measure, in millimeters, of the diameter of growth inhibition zones of *S. aureus* (ATCC 25923) and MRSA (CCBH 5330) strains from the bioactivity derivative Lapachol at concentration of 2 mg/mL were respectively 14 (± 1.73) and 12 (± 2.00) mm. For strain ATCC 25923, the positive controls, oxacillin (OXA01) and vancomycin (VAN30), resulted respectively in zones of 22 (± 1.00) and 16 (± 2.65) mm. The CCBH 5330 strain was resistant to oxacillin and showed a zone of 15.7 (± 0.58) mm for vancomycin (Table 1).

The MICs of ATCC 25923 (Fig. 1) and CCBH 5330 strains were 62.5 μg/mL. Gentamicin, the positive control, inhibited growth at a concentration of 5 μg/mL. For all tests, there was no interference of the negative control, consisting of 5% DMSO aqueous solution.

The performance of the semi-synthetic derivative (LSY), evaluated in the present study, against *S. aureus* ATCC 25923 and CCBH 5330 strains at the MIC of 62.5 μg/mL showed significant potential in inhibiting the growth of *S. aureus*. This compound was effective against a standard strain of *S. aureus*, methicillin-susceptible, and also showed activity in the same concentration against a strain isolated from a clinical sample proved resistant (MRSA), reinforcing the importance of this molecule activity.

In previous steps, LSY showed no activity against Gram-negative strains, *Escherichia coli* and *Pseudomonas aeruginosa*, verified by Andrade et al. (2011) [17]. Similar results were observed by Hussain et al. (2007), who reported no inhibitory activity against these bacteria [8].

An obstacle for the use of Lapachol and derivatives as antimicrobial agents comes from their high toxicity when they are administered for prolonged periods or in high concentrations [8]. Therefore, it is essential to evaluate the median lethal concentration (LC50) before execution of advanced clinical studies.

At the concentration of 100 μg/ml LSY, all *Brine shrimp* were killed, whereas in the well containing 1 μg/ml LSY no significant number of deaths was reported, as compared to controls (seawater). Statistical analysis revealed the following: LC10, 2.562 μg/mL; LC50, 14.230 μg/mL; and LC90, 79.051 μg/mL. According to Déciga-Campos et al. (2007) [16], there is correlation between the median lethal concentration and the rate of toxicity of a sample: LC50 > 1000 μg/mL, nontoxic; LC50 between 300 and 1000 μg/mL, moderately toxic; and LC50 < 300 μg/mL, toxic. Then, LSY shows a significant toxic effect against *A. salina* (LC50: 14.230 μg/mL). Contrary to our results, using the same test, some *Pipper haynanum* extracts were considered nontoxic as reported by Bastos et al. (2009) [18], confirming the sensitivity of this technique.

However, as reported by Lima et al. (2002), who determined the LC50 Lapachol potassium salt at 79.77 ppm for *Brine shrimp*, this toxicity could be an effective option against *Schistosoma mansoni* cercariae and snails (*Biomphalaria* sp.) [19]. A similar evaluation can be conducted for LSY, such as determination of larvicides, molluscicidal, and schistosomicidal activities.

There is also the possibility of more accurate determination of toxicity using *in vivo* assays, in that these can more appropriately simulate the beneficial and adverse effects of chemical components of pharmacological application.

The compilation of the results reveals the need for new molecule elucidation, including structural changes in order to enhance the biological activity for the future validation of new compounds to be used in clinical medicine.

Table 1. Antistaphylococcal activity of LSY by disk diffusion method and determination of minimum inhibitory concentration (MIC).

	Disk diffusion (mm)			MIC (μg/mL)	
Strains	LSY (2mg/mL)	Oxacilin	Vancomicin	LSY	Gentamicin
***Staphylococcus aureus* ATCC 25923**	14 ± 1.73	22 ± 1.00	16 ± 2.65	62.5	5
MRSA CCBH 5330	12 ± 2.00	0	15.7 ± 0.58	62.5	5

Legend: [0] No inhibitory zone.

Finally, the molecule (LSY) showed potential antistaphylococcal activity against an *S. aureus* sensitive strain as well as against an MRSA strain. Despite the relative toxicity observed by *Brine shrimp* bioassay, the molecule should not be invalidated before *in vivo* evaluation, which is essential in the tissue toxicity analysis increasing the toxicological profile of LSY. Besides, LSY can be evaluated for other pharmacological actions not cited in this paper.

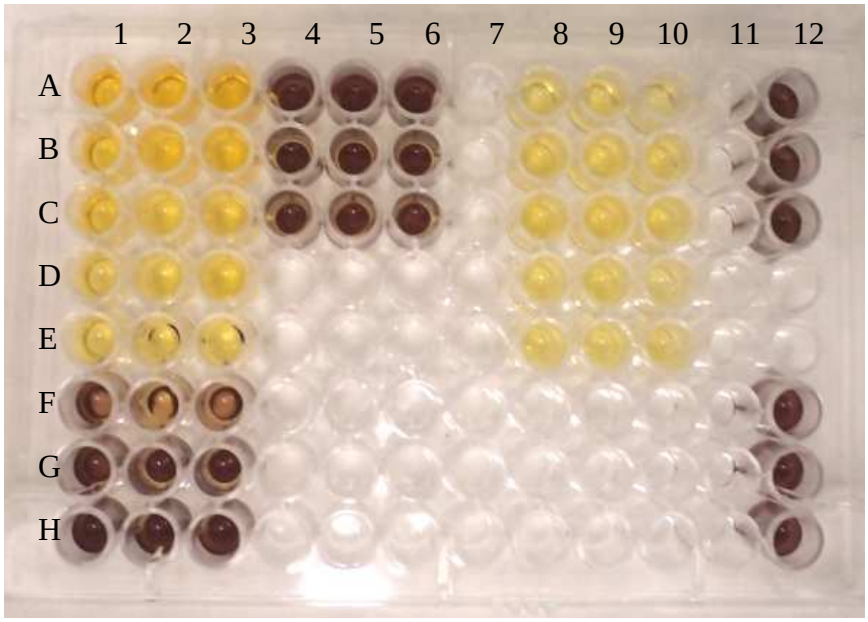

Fig.1. MIC by broth microdilution method. LSY against *S. aureus* ATCC 25923. Lines A1 - H1: LSY at concentrations from 1.000 to 8 µg/mL; A4 - C4: LSY concentrations from 3.9 to 0.9 µg/mL. Lines A8 - E8: gentamicin concentrations from 160 to 10 µg/mL. A12 – C12: Control growth. F12 – H12: negative control. Assays performed in triplicate (columns 1-3, 4-6, 8-10).

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