

ORIGINAL ARTICLE**Susceptibility of Selected Multi-Drug Resistant Clinical Isolates to Leaves of *Carpolobia lutea*****Ibikunle Anibijuwon^{1*}, Ifeoluwa Gbala¹, Jumai Abioye¹****OPEN ACCESS**

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ABSTRACT

Background: *The decline in the production of new effective antibiotics coupled with the constantly evolving antimicrobial resistance remains a public health concern. This study thus evaluated the antibacterial and antifungal effects of the ethanolic, n-hexane and hot aqueous extracts of Carpolobialutea leaves.*

Methods: *The extracts were tested using agar well diffusion method against selected clinical isolates: Pseudomonas aeruginosa, Salmonella typhi, Escherichia coli, Staphylococcus aureus and Candida albicans. Antibiogram profile of the isolates were deduced by disc diffusion method.*

Results: *Multi-drug resistance was confirmed in all bacteria with a notable pandrug resistance in Pseudomonas aeruginosa. Ofloxacin, Erythromycin and Gentamicin were effective on two or three organisms, notably on Salmonella typhi and Escherichia coli. The preliminary antibacterial assay marked the efficacy of the ethanol and n-hexane extracts except on E. coli, with zero activity for hot water extracts at the stock concentration (200 mg/ml). Pseudomonas aeruginosa and Candida albicans were susceptible to lesser concentrations of the ethanol extracts at 5 mg/ml and 25 mg/ml respectively. None of the isolates showed sensitivity to lesser concentrations of n-hexane extract. Carpolobia lutea leaves proved to be effective over the use of antibiotics in inhibiting the activity of Pseudomonas aeruginosa which was resistant to the latter. The Minimum Inhibitory concentration of the ethanolic extract was considerably low (≤ 5 mg/ml for P. aeruginosa and 25mg/ml for C. albicans). However, there was no Minimum Bactericidal concentration for the extracts against the clinical isolates.*

Conclusion: *Carpolobia lutea shelters bioactive components with pharmacological potentials that could show efficiency in the treatment of bacterial infections.*

Keywords: *Carpolobia lutea, antifungal, antibacterial, multi-drug resistance*

INTRODUCTION

Antimicrobials are great resorts in the treatment of bacterial infectious diseases (1). However, over the past few decades, these

public health “guards” have been under threat by the spontaneous emergence of resistant genes among bacteria. Antimicrobial resistance in bacteria has deteriorated so much to multi-, extensive- and pan-drug resistance categories such that a bacterium may exhibit non-susceptibility to two or more or all of the antibiotics used in a study. These phenomena, coupled with the propensity of spread of resistance genes among bacteria, increase the rate of fatality and mortality of bacterial infections. It is therefore pertinent to investigate newer drugs or sources with potentials of lower resistance evolution.

Drugs derived from natural plants play a significant role in the prevention and treatment of human diseases (2). Many drugs presently prescribed by physicians are either directly isolated from plants or are artificially modified versions of natural products (3). According to WHO, about 80 % of the world’s population relies on traditional medicine for their primary healthcare (4).

Carpolobia lutea, shrub usually grown in Asia and Africa (5) is used in the treatment of genitourinary infections, gingivitis and waist pains (6). The plant has been reported to possess anti-inflammatory and anti-arthritis properties (7), gastro-protective effects (8), antinociceptive effects (9), antimicrobial activities (10, 11), antidiarrhoeal and anti-ulcerogenic properties (8), antimalarial activity and moderate toxicity (12).

Based on this background, this study was designed to screen the fractions of *Carpolobia lutea* leaf extracts for antimicrobial activity against selected multi-drug resistant pathogenic bacteria of clinical origin. The assay was carried out using the agar well diffusion method, and the antibacterial activity of the leaf extracts was compared with the effects of the conventional antibiotics.

MATERIALS AND METHOD

Collection and identification of plant materials:

Fresh leaves of *Carpolobia lutea* were purchased from herb sellers at Ipata market in Ilorin, Kwara State. The leaves were identified properly and

authenticated at the herbarium unit of the Department of Plant Biology, University of Ilorin, Kwara State, Nigeria. All other procedures were carried out at the Department of Microbiology laboratory, University of Ilorin.

Collection and maintenance of test organism:

Pure cultures of clinical isolates of selected pathogenic microorganisms were obtained from the Medical Microbiology Laboratory of the University of Ilorin Teaching Hospital (UIH), Ilorin, Kwara State. The organisms obtained were *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* (yeast). They were thereafter tested biochemically to reaffirm their identity before storing in a working refrigerator at 4°C as stock culture. Subcultures of the refrigerated stocks were carried out at every 2 weeks interval to maintain the viability of the test organisms. It was also ensured that 18-hour old cultures of the test organisms were used for the antimicrobial assay.

Preparation of plant material: The leaves were air-dried and pulverized into fine powder using an electric Binatone BLG- 595 blender (Binatone, UK). It was then prepared for extraction by cold maceration in three different solvents: by 95% ethanol, hot water and 95% methanol. Fifty gram of powdered leaves were introduced into 250ml of each solvent in separate flasks, followed by vigorous shaking for five (5) minutes and thereafter left for 48 hours on a rotating shaker. The resulting solutions were filtered using fine muslin cloth, and the filtrates were left on the workbench to settle (decantation). Then the supernatants were sterilized by membrane filtration using Millipore filter paper and afterwards concentrated in hot water bath. The crude extracts were thereafter stored in sterile bottles in a working refrigerator..

Antimicrobial Susceptibility Test (AST)

Standardization of organisms: The cells of the organisms were adjusted to 0.5 McFarland standard which is an estimated value of $1-2 \times 10^8$ CFU/ml or 5×10^5 CFU/ml. The standard was prepared by adding 0.1 ml of 1% Barium chloride (BaCl_2) to 19.9 ml of 1% sulfuric acid (H_2SO_4). The solution was mixed to form a turbid

suspension. The resulting mixture was placed in a foil-covered screw-cap tube. The isolates to be screened were subcultured on nutrient agar prior to the test to get an 18-hour old culture. After incubation, inoculums were picked from each culture and inoculated in 9 ml peptone water by carefully adjusting the turbidity visually with the standard. The turbidity was then observed and measured using a spectrophotometer until the turbidity reached $\geq 0.10D$ at 620 nm (13).

Determination of susceptibility to antibiotics: The antibiotics used for this study (Abtek Biologicals Limited, UK) had been prepared into kit containing multiple discs impregnated with different types of antibiotics. The antibiotics used and their corresponding concentrations were as follows: Gentamicin (10 μ g), Cotrimaxazole (25 μ g), Ofloxacin (30 μ g), Tetracycline (30 μ g), Amoxicillin (25 μ g), Nitrofurantoin (300 μ g), Nalidixic acid (30 μ g) and Augmentin (30 μ g) for the gram-positive bacteria and Ceftazidime (30 μ g), Cefuroxime (30 μ g), Gentamicin (10 μ g), Ceftriaxone (30 μ g), Erythromycin (5 μ g), Cloxacillin (5 μ g), Ofloxacin (5 μ g) and Augmentin (30 μ g) for the gram-negative bacteria isolate.

Solidified Mueller Hinton agar (Rapid Labs, UK) plates were seeded with 100 μ l of the standardized organisms and were spread evenly over the total surface area of the agar using a glass spreader. The plates were left on the laboratory bench for 30 minutes to ensure that the organisms are well absorbed on the agar surface. Multiple antibiotics discs containing the aforementioned antibiotics were carefully and firmly placed on the surface of the agar using sterile forceps. The plates were then left for 1 hour to allow the antibiotics to diffuse. Afterwards, the plates were incubated at 37°C for 18-20 hours. After incubation, the zones of inhibition generated by the antibiotics were measured and recorded in millimeter (mm) (14)(CLSI, 2016). The values for each organism against the antibiotics were interpreted as sensitive, intermediate or resistant using the breakpoints interpretative criteria of Clinical and Laboratory Standard Institutes.

Determination of pH of the Extract of *Carpolobia lutea*: The pH of the extracts was

determined by dipping a pH meter into conical flasks containing the extracts. The constant reading on the pH meter was taken as the pH of the antimicrobial substance in question (15).

Antimicrobial Sensitivity Test of Extract of *Carpolobia lutea*

Antibacterial assay: The antibacterial activity of the extracts against the selected test organism was determined by a modified agar well diffusion assay method as described by Perez *et al.* (16). The solidified MHA plates were then inoculated with the standardized inoculum by transferring 100 μ l of the bacterial suspension onto the agar surface; then using sterile cotton swab to uniformly spread the inoculum on the surface of the agar plate. The plates were left for 20 minutes for the organisms to diffuse into the agar. Five holes were aseptically bored on each of the agar plates, using a sterile 6mm cork borer. The agar wells were then filled with the extracts using sterile micropipette (one concentration per hole). The plates were then allowed to stand for 30 minutes to allow proper diffusion of the extracts into the agar after which they were incubated upright at 37°C for 18-24 hours. Antibacterial activity was determined by observing, measuring and recording the diameter of zone of inhibition to the nearest millimeter (mm) using a metre rule (17).

Evaluation of Antifungal activity: The evaluation of the antifungal activity was done using the diffusion technique described by Sales *et al.* (18). *Candida albicans* cultures in Sabouraud Dextrose agar were incubated for 48 hours at 32°C and transferred to 0.85% saline solution and turbidity adjusted to 10⁶ conidia/ml. Then, 100 μ L of the fungal suspension was spread onto the surface of the agar. After 10 minutes of rest, 6 mm-diameter holes were punched and filled with 100 μ L of the previously prepared leaf extracts. The diameter of zones of inhibition around each well was measured to the nearest diameter.

Minimum Inhibitory/ Bactericidal/ Fungicidal Concentration (MIC/ MBC/MFC): The MIC/MBC/MFC of the organisms was determined following the method described by Willey *et al.* (19). The organisms that were susceptible to the leaf extracts of *Carpolobia*

lutea were inoculated on sterile nutrient broth (Lab M Ltd., UK) and incubated overnight at 37°C. Varying concentrations (150, 100, 75, 50, 25 and 5mg/ml) of the extracts were prepared by serial dilution using Mueller Hinton broth (Rapid Lab, UK) as a diluent. Each of the diluted extracts was then inoculated with 100 µL of the overnight broth culture of the test organisms. The positive control was setup as broth with only the extract while negative control contained broth and organism. The inoculated and control tubes were incubated at 37°C for 24 hours after which they were observed for turbidity. The lowest concentration that showed no turbidity was taken as the MIC while the lowest concentration of the extract which showed no growth on plates after 24 hours of incubation indicates bactericidal/fungicidal effect and was taken as MBC/MFC.

RESULTS

The ranges of zones of inhibition for the AST were interpreted according to the Clinical Laboratory Standard Institute standard. Of all antibiotics used for Gram positive and negative bacteria, ofloxacin, gentamicin and erythromycin showed significant inhibitory activities on two or three of the organisms while the organisms showed resistance to others (Figure 1). *Salmonella typhi* was sensitive to gentamicin (23 mm), ofloxacin (30 mm), and erythromycin (27 mm); *Escherichia coli* was sensitive to ofloxacin (24 mm) and erythromycin (18 mm) but partially sensitive to gentamicin (14 mm). *Staphylococcus aureus* showed an extensive resistance with sensitivity to only ofloxacin (34 mm). *Salmonella typhi* and *Escherichia coli* were both resistant to ceftazidime, cefuroxime, gentamicin, ceftriaxone, erythromycin, cloxacillin and augumentin. *Pseudomonas aeruginosa* showed complete resistance to all the antibiotics tested against it. The ethanol, hot water and n-hexane extracts all had pH values less than 5.0, thus indicating acidity (Figure 2).

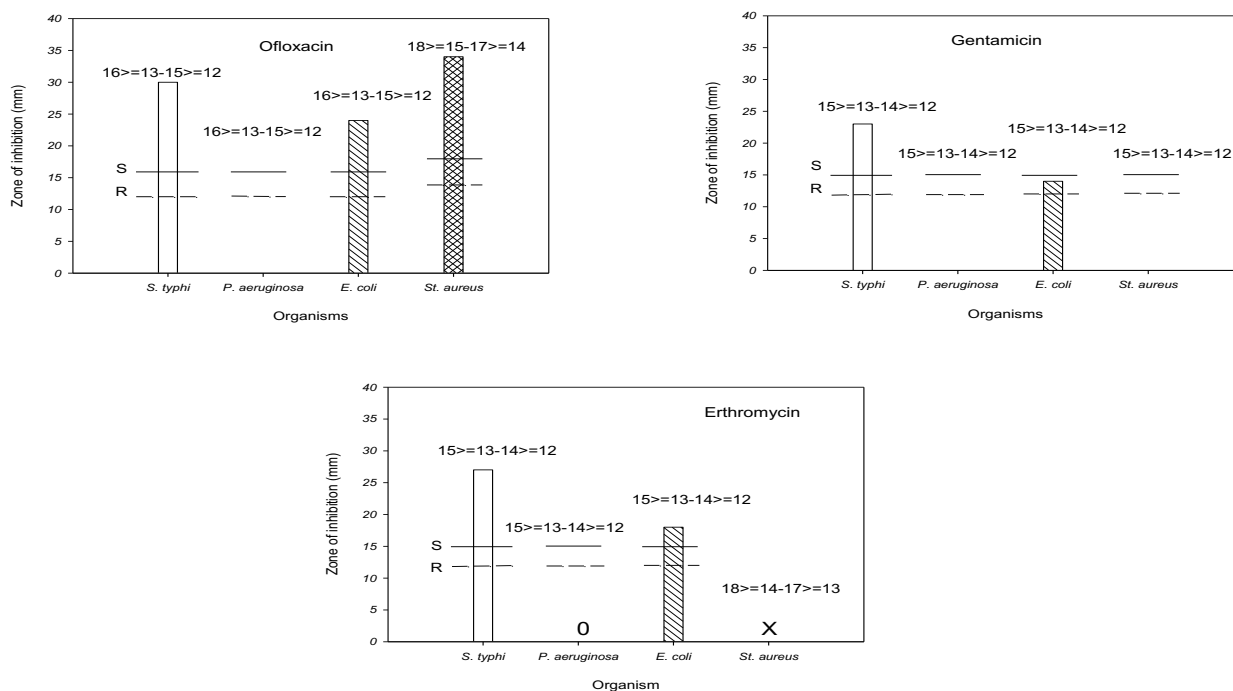


Figure 1: Antibiotic susceptibility test of the organisms. Key: S- Sensitive (solid lines); R- Resistant (broken lines); the ranges indicate $S \geq I \geq R$ (CLSI, 2016) where I (Intermediate) is the region between S and R

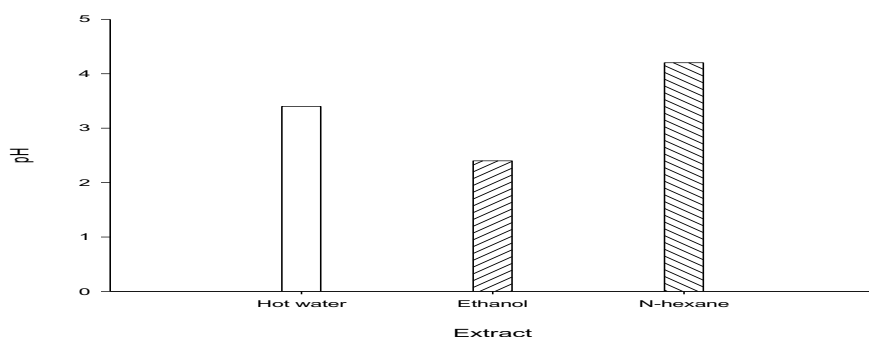


Figure 2: pH of the extracts of hot water, ethanol and n-hexane

From the result of the preliminary assay of the antibacterial efficacy of the extracts at stock concentration (200 mg/ml), it was observed that the hot water extract had no inhibitory effect on any of the isolates. The ethanol and n-hexane extracts, however, showed effects on *Salmonella typhi*, *Pseudomonas aeruginosa* and either *Staphylococcus aureus* or *Candida albicans* with zones of inhibition ranging from 9 – 13 mm (Figure 3). Notably, *Escherichia coli* exhibited resistance to both ethanol and n-hexane extracts. The lower concentrations (150, 100, 75, 50, 25, 5 mg/ml) of the n-hexane extract had no inhibitory activity on any of the isolates while the ethanol extract showed significant inhibitory activities on *Pseudomonas aeruginosa* and *Candida albicans*.

Although *Salmonella typhi*, *Escherichia coli* and *Staphylococcus aureus* were resistant to all the lower concentrations of the ethanol extract, a remarkable sensitivity was observed for *Pseudomonas aeruginosa* and *Candida albicans*. *Pseudomonas aeruginosa* was sensitive to all concentrations (5-150 mg/ml); *Candida albicans* was also sensitive to all concentrations except 5 mg/ml (25-150 mg/ml) (Figure 4). The Minimum Inhibitory Concentration (MIC) for *Pseudomonas aeruginosa* and *Candida albicans* were ≤ 5 mg/ml and 25 mg/ml respectively (Table 1). None of the concentrations of the ethanol extract, however, showed bactericidal activity on either *Pseudomonas aeruginosa* or *Candida albicans* (Table 1).

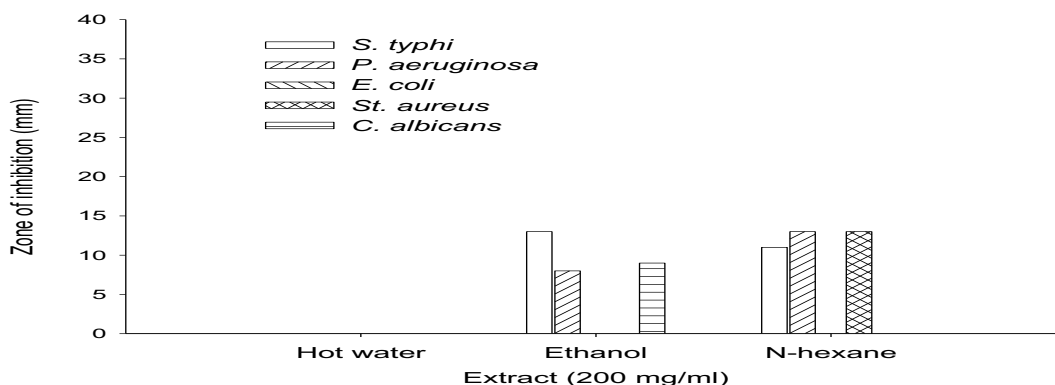


Figure 3: Susceptibility of the organisms to extracts of *C. lutea*

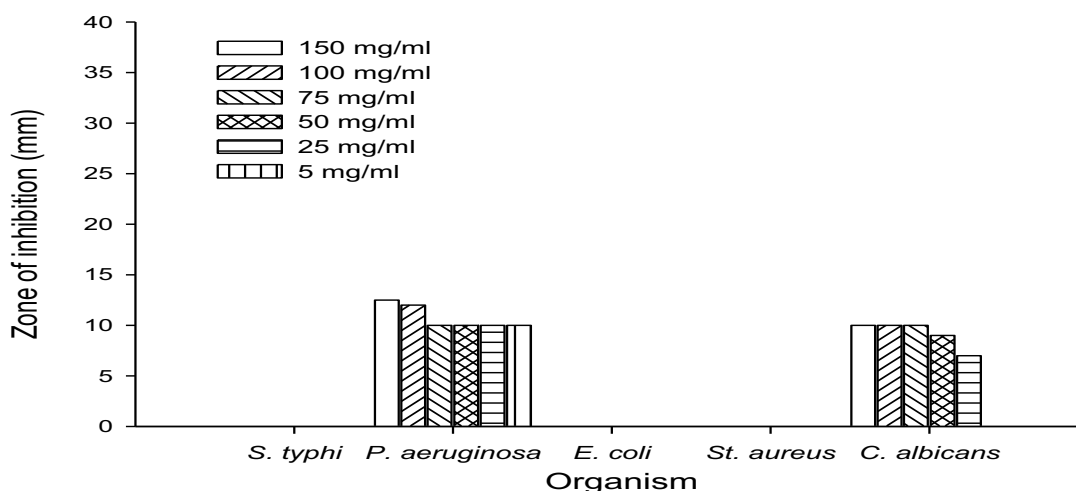


Figure 4: Susceptibility of the organisms to concentrations of ethanol extract of *C. lutea*

Table 1: MIC and MBC of ethanol extract of *C. lutea*

Test organism	Concentrations of Ethanol extract (mg/ml)						Minimum Inhibitory Concentration (mg/ml)	Minimum Bactericidal Concentration (mg/ml)
	50	100	150	70	55	25		
<i>P. aeruginosa</i>	-	-	-	-	-	-	≤5	-
<i>C. albicans</i>	-	-	-	-	-	+	25	-

+ indicates growth - indicates no growth

DISCUSSION

All the bacteria used in this study were multi-drug resistant. *Staphylococcus aureus* proved extensive drug resistance (XDR) with resistance to seven (88%) of the eight antibiotics tested. *Salmonella typhi* and *Escherichia coli* also exhibited a similar resistance pattern with 63% (5 of 8) resistance to the antibiotics. Noteworthy, *Pseudomonas aeruginosa* exhibited pan-drug resistance with resistance to all the antibiotics tested. The classes of antibiotics used in this study include penicillins, quinolones, aminoglycosides, tetracycline, cephalosporins and beta-lactam. The extensive resistance to different classes of antibiotics especially cephalosporins and beta-lactam may suggest the presence of resistance enzymes with inactivating properties in these organisms. Resistance to penicillins

and beta-lactams has been on the increase over the years (20, 21). Some gram negative bacteria have been studied to produce intrinsic chromosomal encoded enzymes that facilitate resistance to penicillins and beta-lactams. These enzymes usually have been classified as beta-lactamases (22, 23, 24). Generally, gram negative bacteria have efficiency in up-regulation or acquisition of genes or mechanisms that code for resistance to antibiotics (25, 26). Multi-drug resistance in *Staphylococcus aureus* has been linked to highly pathogenic strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* with the ability to cause life-threatening diseases (27). For both Gram positive and negative, ofloxacin showed a remarkable antibacterial activity. Ofloxacin is a broad spectrum antibiotic

which is used against both Gram negative and positive bacteria.

It was observed that the pH's of all the extracts were highly acidic with the ethanol extract being the most acidic one. This may support its significant activity on the bacteria especially *Pseudomonas aeruginosa*. Generally, bacteria grow better in slightly acidic to slightly alkaline (6-8.5) medium, and specifically *Pseudomonas aeruginosa* attains optimum grow at a pH of 7.5 (28). Although acidic medium supports the growth of fungi, the ethanol extract showed remarkable inhibitory activity on *Candida albicans*. This could be because *C. albicans* survive optimally at pH of 6.5, and a highly alkaline medium is also required for its morphogenesis and survival in hyphal form (29). The ethanolic extract was the most effective of the three extracts. Previous researches on the evaluation of plants have affirmed that ethanol is a very potent solvent for the extraction of phytochemicals. This could be attributed to its polarity which allows for effective dissolution of the bioactive compounds in it (30).

The aqueous extract was completely non-reactive to any of the test organisms. There could, however, be a probable reactivity at higher concentrations. At the stock concentration (200 mg/ml), *Salmonella typhi*, *Staphylococcus aureus* and *Escherichia coli* were susceptible to both or one of ethanol and n-hexane extracts. However, lower concentrations of the extracts exerted no inhibitory effect on the aforementioned organisms. *Escherichia coli* was, however, resistant to both extracts in contrast to the sensitivity shown to the standard antibiotics. Nwidi *et al.* (31) also reported that lower concentrations (> 770 mg/ml) of ethanol and n-hexane extracts were ineffective on the two strains of *E. coli* used in their study. *Pseudomonas aeruginosa* and *Candida albicans* were susceptible to lower concentrations of the ethanol extract with MIC values ≤ 5 mg/ml and 25mg/ml respectively. This is in contrast with the reports of Nwidi *et al.* (31) that none of the extracts had MIC on *Pseudomonas aeruginosa* and *Candida albicans*. The low MIC recorded for the ethanol extracts of *C. lutea* leaves affirms that the leaves has credible bacteriostatic effects on the bacteria compared to the antibiotics, especially on *P. aeruginosa* which showed pan-drug resistance to the antibiotics. Overall, the effects of the standard antibiotics and the leaf extracts against the bacterial varied per organism. Although with notable bacteriostatic effects, the ethanol extract showed no bactericidal action. Overall, the extracts of the leaves of *Carpolobia lutea* have antibacterial activities against

multi-drug resistant *Pseudomonas aeruginosa* and *Candida albicans*.

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