Research Article

Antimicrobial Efficacy and Molecular Docking of Ouabain Derived from *Cordia macleodii* against *Klebsiella pneumoniae*

Minarbha Patel, Ankita Agrawal, Amiya Kumar Patel*
Department of Biotechnology and Bioinformatics, Sambalpur University, At/po- Jyoti Vihar, Burla- 768019, Odisha, India.
***Corresponding author's E-mail:** amiya.gene@gmail.com

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ABSTRACT

Rapid emergence of antibiotic-resistant pathogenic bacteria has limited the effective life span of commercial drugs. Naturally derived complex bioactive compounds with diverse structure, antimicrobial potency and minimal toxicity are more effective against microbial infections. In the present study, phytochemical screening of the methanolic leaf extract of medicinal plant, *Cordia macleodii* revealed existence of bioactive compound, ouabain, which was evaluated for antimicrobial and antibiofilm activity against *Klebsiella pneumoniae* and reference strain (MTCC 109). The MIC value of ouabain against test bacterium was found to be 50mM. The study reported that the ouabain at sub-MIC value significantly mitigated the biofilm formation in *K. pneumoniae* with a reduction percentage of 62.26 ± 5.29 % compared to untreated culture confirmed by quantitative biofilm assay. Further, *in silico* molecular docking analysis of ouabain against the targeted protein (bssS and metE) responsible for biofilm formation in *K. pneumoniae* was examined to predict possible binding site. The ouabain showed lowest minimum docking score of -5.187 Kcal/mol with bssS and -9.826 Kcal/mol with met E respectively. This study signifies that isolated bioactive compound, ouabain derived from the methanolic leaf extract of *C. macleodii* with varying degrees of antimicrobial properties might be considered as potent antimicrobial agent to combat the infections caused by the biofilm producing bacteria.

Keywords: Antibiofilm, *Cordia macleodii, K. pneumoniae*, Molecular docking, Ouabain.

INTRODUCTION

Bacterial antimicrobial resistant emerged as an imminent pandemic that has critically threatened the healthcare sector with increased mortality rate globally[1]. Presently, the world is confronted with conundrum of a drop in the number of novel therapeutic drugs and an increase in death rates (7 lakh annual death) due to resistance[2]. The pathogenic bacteria have diverse resistance mechanisms, including biofilm formation to counteract the action of antibiotics. Biofilm is a complex matrix of microbial communities which develop strong attachments to biotic and abiotic surfaces to persist in extreme conditions. These biofilms are responsible for over 60 % of microbial infections. To tackle these problem, novel and potent antimicrobial products from different sources (especially natural source) are explored to control these pathogens.

Medicinal plants are an abundant source of bioactive compounds with structural diversity, biological and pharmacological properties and less harmful effect[3]. Further, plant derived compounds remain predominant alternative to conventional medicines due to low cost, accessibility, efficiency, historical preferences, and high public acceptance[4]. Hence, the plant derived compounds are explored continuously with a hope to develop new antimicrobial agents with diverse mechanism of action which might be potential alternatives to antibiotics in the battle against the biofilm and related microbial infections[5].

The genus *Cordia* belonging to *Boraginaceae* family with more than 300 species are widely investigated for their ethnobotanical and pharmacological properties[6]. *Cordia macleodii* is an endangered plant found in Gandhamardhan hills of Odisha[7], which possess anti-inflammatory, wound healing, antivenom, antimicrobial, antioxidant and hepatoprotective activities[8]. Analysis of leaves of *C. macleodi* reported the presence of several phytochemicals with antimicrobial properties[9]. However, the phytochemicals constituents of leaf extract of *C. macleodi* have not been fully assessed. The present study was designed for phytochemical screening of methanolic leaf extract of *C. macleodii* for the isolation of bioactive compounds. I*n vitro* antimicrobial assay of the leaf extract and isolated compound against the pathogenic bacterial strain *Klebsiella pneumoniae* and its reference strain was carried out to determine its antibacterial and antifilm efficacy. Further, *in silico* molecular docking of isolated compound (Ouabain) was performed to predict the possible binding site in the targeted protein of *K. pneumoniae* responsible for pathogenesis.

MATERIALS AND METHODS

Sampling and Extraction

Freshly plucked leaf samples of *Cordia macleodii* were collected from the Gandhamardhan hill range, Odisha, India (Location: 20°42' - 21°00' North latitude and 82°41' - 83°05' East longitude), washed properly, chopped into smaller pieces, dried and grinded into fine powder. About 500 gm of powdered sample was soaked with methanol for 72 hrs. The supernatant was filtered, then rota-evaporated to remove the solvent, air dried and stored at 4°C.

HRMS analysis

Phytochemical composition in methanolic leaf extract of *C. macleodii* was performed using Exactive™ Plus Orbitrap HRMS integrated with tandem ultimate 3000 high-performance LC (Thermo Scientific, USA). About 0.5 ml extract was suspended in 1.5 ml of methanol: water (1:1), centrifuged at 10000 rpm for 10 min at 4°C and filtered by 0.22 µM syringe. Filtrate of 0.5 ml was aspirated into DP ID vial for mass spectrometry analysis using Hypersil BDS C18 (250x2.1 mm, 5 µm) column. Sample was injected at 3 µl/min with 30°C column temperature and 700 bar pressure. Electrospray ionization was used to ionise molecule at 3eV for 15 mins and the positive and negative polarity were detected within (50-750) m/z range. Mass peak intensities were examined against five standard compounds with mass range of (50-500) m/z for converting peak intensities to concentration. Using python program and PubChem library parse the mass peaks were annotated having error range of (± 0.01) m/z.

Test microorganisms and culture media

Bacterial strain (*Klebsiella pneumoniae*) was clinically isolated and identified by 16S rRNA sequencing (SF-1). Reference strain (MTCC 109) was received from IMTECH, Chandigarh. All the culture media was purchased from Himedia laboratory.

Minimum inhibitory concentration (MIC)

Microdilution method using 96-well plates, was carried out to measure the MIC value of leaf extract and isolated compound[10]. Samples were dissolved in DMSO and serially diluted (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 mM) using Mueller Hinton broth. Then, 10 µl of 0.5 McFarland bacterial culture was inoculated and incubated for 16 hrs at 37°C. Well having culture without sample was treated as negative control. The microbial growth was detected by optical density at 600nm. MIC value was determined by taking the lowest concentration of sample that completely inhibited the microbial growth, which was subsequently utilized to compute the sub-MIC value. The MIC and sub-MIC value was expressed in microgram per millilitre. All experiments were conducted in triplicates.

Antibacterial assay

Antibacterial assay of the leaf extract and isolated compound (Ouabain) against the test bacterium (*K. pneumoniae*) and its reference strain (MTCC 109) was evaluated by well diffusion assay, which was performed following CLSI guidelines[11]. For these purpose, bacterial culture was swabbed over solidified Muller-Hinton agar plates and wells (8 mm diameter) was prepared. MIC and sub-MIC of leaf extract and ouabain were added to wells and incubated at 37°C in BOD incubator overnight. The diameter of zone of inhibition was measured to evaluate the antibacterial efficacy[12].

Antibiofilm assay

Qualitative Method

Congo red agar (CRA) method was performed to evaluate the antibiofilm activity[13]. Bacterial culture treated with sub-MIC level of leaf extract and ouabain were streaked over the solidified CRA plates and incubated for 48 hrs at 37°C for the development of colonies. Untreated bacterial culture was regarded as control.

Additionally, test tube method was performed to detect biofilm producing microbes based on the occurrence of visible biofilm[14]. Bacterial isolates (untreated and treated at sub-MIC level of extract and ouabain) were inoculated with tryptic soya broth (TSB) and incubated for 24 hrs at 37°C. Then, test tube was PBS washed (twice), stained by crystal violet, again PBS washed (twice) to remove excess stain and air dried. Occurrence of rings on the walls and bottom of the test tube indicates the biofilm formation.

Quantitative method

Polystyrene based 24-MTP (microtiter plate) was performed for quantitative biofilm activity. For the purpose, bacteria culture (with or without sub-MIC level of leaf extract and ouabain) was inoculated in Muller-Hinton broth and incubated for 24 hrs at 37°C. After incubation, media was discarded, attached biofilm was washed (twice) to remove cell debris and stained with 0.1% crystal violet (w/v) for 20 mins. The stained biofilm was PBS washed (thrice) and air dried. Further, the biofilms lining the wall of microplate is resolubilized with 95% ethanol and optical density at 540 nm was measured[15].

Microscopic studies

Microbial culture (0.5 McFarland standard) was added to 24-MTP containing sterile coverslip immersed in Luria-Bertani medium with or without the sample (leaf extract and ouabain) and subjected to incubation for 16 hrs at 37°C. Planktonic debris was removed via PBS wash and biofilm matrix was microscopically examined. For light microscopic studies, adhered biofilms were stained with 0.4% crystal violet (w/v) for 10 mins. Further, the biofilm was dyed with 0.01% acridine orange for 10 mins in dark for fluorescence microscopic studies[16].

In silico prediction and molecular docking

Protein preparation

Two proteins, bssS (biofilm regulatory protein) and metE (transferase activity; PDB ID: 3L7R) were used as targeted protein in this study. The PDB structures of these proteins were preprocessed using the multistep procedures of protein preparation wizard (Schrödinger Inc., NY). Besides, the missing hydrogen atoms were added using protein preparation wizard. The missing side chain atoms of the amino acids were subsequently identified using the Prime side-chain prediction tool and repaired using Prime (Schrödinger, Inc., NY). Further, the structures of the bioactive proteins were refined through energy minimization using Macromodel (Schrodinger) and OPLS

2005 force field. In the present study, the energy was minimized using Polak-Ribiere Conjugate Gradient (PRCG) algorithm with energy gradient of 0.01 kcal/mol.

Preparation of molecular structure

Chemical structure of ouabain was drawn by ChemDraw, which was then loaded into Maestro (Schrödinger). Energy minimization was performed using Macromodel and OPLS 2005 force field with Polak-Ribiere conjugate gradient algorithm. DFT (hybrid density functional theory) with Becke's three-parameter exchange potential along with the Lee-Yang-Parr correlation functional (B3LYP) with basis set 6-31G** using Jaguar (Schrödinger, package) was used for the geometric optimization of the structure[17]. Ligprep (Schrödinger) was used to generate various conformations of Ouabain.

Molecular docking

Blind docking strategy was adopted to investigate molecular interactions of ouabain with different proteins in absence of co-crystal structures. Protein binding sites was predicted by SiteMap (Schrodinger) and receptor grid boxes having dimension (12Å x 12Å x 12Å) were created for each projected site using Glide grid-receptor program (Santoshi and Naik, 2014). Several conformations of ouabain so generated were docked onto each of the predicted binding site using Glide XP algorithm and their binding poses were evaluated using Glide XP_{Score} function[18]. The single best conformation of ouabain with minimal docking score was selected for further study.

RESULT AND DISCUSSION

HRMS analysis

HRMS analysis of methanolic leaf extract of *C. macleodii* revealed the occurrence of nineteen different compounds. The bioactive compounds were identified based on their fragmentation patterns of mass spectra (SF-2), total ion chromatogram (Figure 1) and direct comparison of spectra with NIST database and documented spectra (SF-3). HRMS result reveals the presence of prime bioactive compound (Ouabain) with the peak area (0.63%) in methanolic extract of *C. macleodii*.

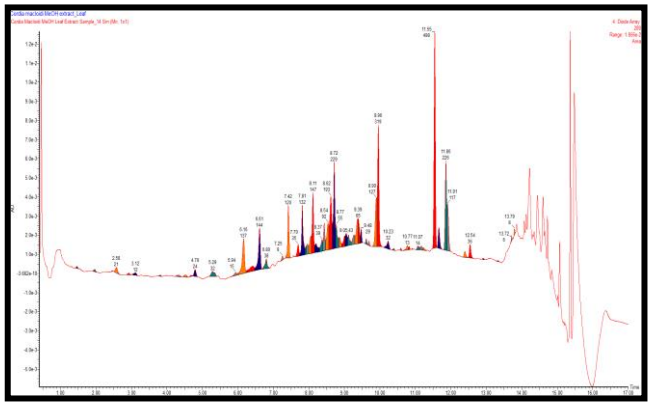

Figure 1: Chromatogram of the crude methanolic leaf extract of *Cordia macleodii* indicating presence of several major phytochemicals.

Antibacterial activity (MIC and Zone of inhibition)

MIC value of leaf extract was found to be 5% whereas isolated compound (ouabain) has MIC value of 50 mM against the clinical isolate (*K. pneumoniae*) and reference strain (MTCC 109) (Figure 2). Well diffusion assay revealed the leaf extract at the MIC value have diameter of zone of inhibition measuring 18 mm and 20 mm against *K. pneumoniae* and MTCC 109 respectively. The diameter of zone of inhibition of isolated compound (ouabain) at MIC value was found to be 16 and 18 (in mm) against clinically isolated *K. pneumoniae* and reference strain MTCC 109 respectively. The present study evident that ouabain derived from the methanolic leaf extract have promising antibacterial at MIC and sub-MIC value against the pathogenic bacterial strains *K. pneumoniae* and MTCC 109.

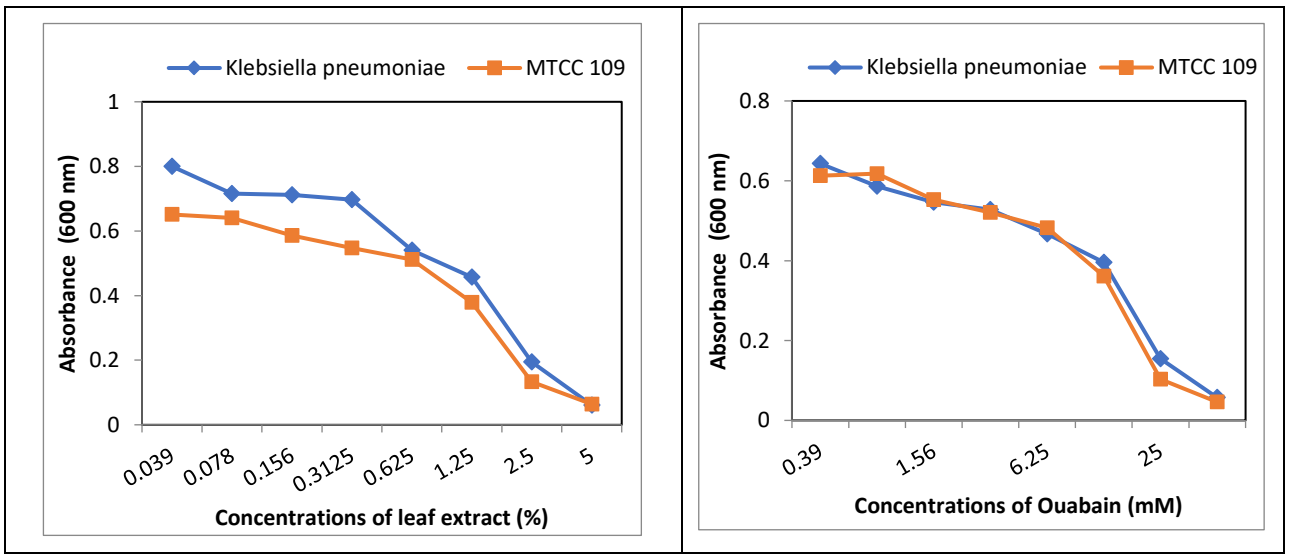

Figure 2: MIC of (a) methanolic leaf extract of *C. macleodii* and (b) Ouabain against *K. pneumoniae* and reference strain (MTCC 109).

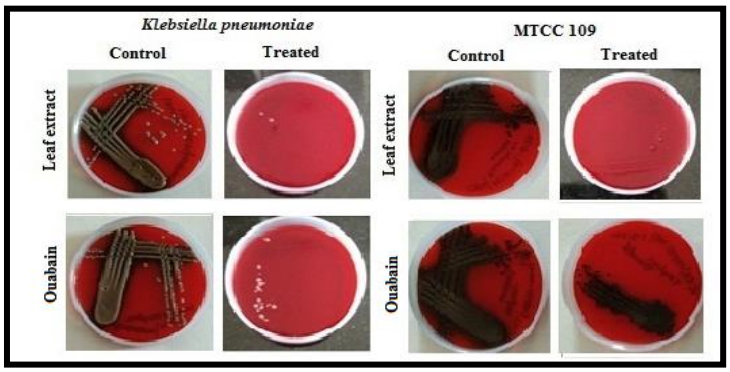

Figure 3: Antibiofilm activity of ouabain derived from *C. macleodii* at sub-MIC against *K. pneumoniae* and reference strain (MTCC 109) based on Congo red agar method.

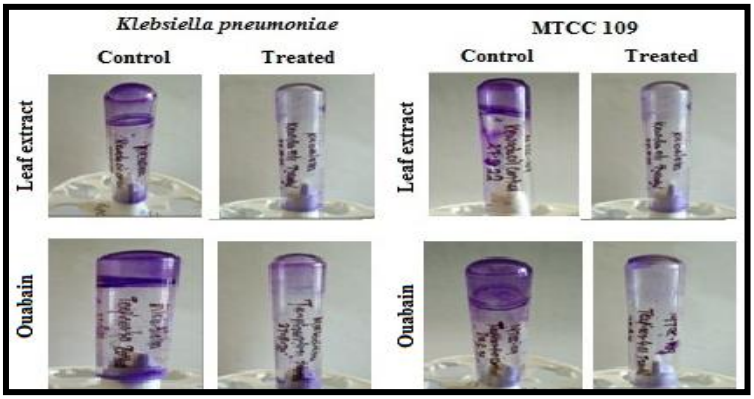

Figure 4: Antibiofilm activity of ouabain derived from *C. macleodii* at sub-MIC against *K. pneumoniae* and reference strain (MTCC 109) based on the Test tube method.

Antibiofilm assay

Qualitative method

CRA method showed significant reduction in the number of the black crystalline colonies and occurrence of white colonies in the treated bacterial strain of *K. pneumoniae* and MTCC 109, when supplemented with sub-MIC value of leaf extract and ouabain (Figure 3). In contrast, the production of dark black colonies by the untreated isolates of *K. pneumoniae* and MTCC 109 indicates their biofilm forming ability (Figure 3). Subsequently, reduction in the biofilm rings in the wall and bottom of the test tube in treated strain as compared to untreated strains was evident from test tube method (Figure 4).

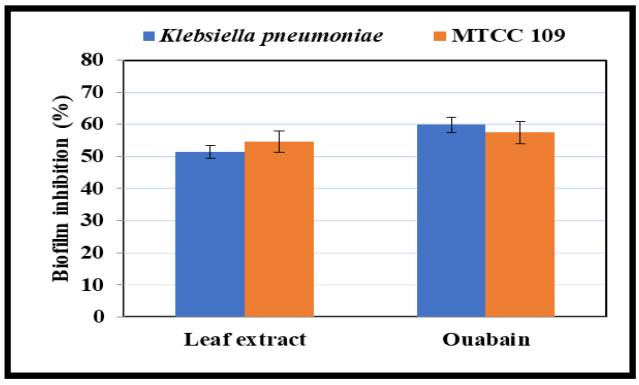

Figure 5: Antibiofilm activity of methanolic leaf extract of *C. macleodii* and Ouabain at sub-MIC on biofilm formation in *Klebsiella pneumoniae* and MTCC 109.

Quantitative assay

The leaf extract as well as the isolated compound (ouabain) at sub-MIC value found to have promising biofilm inhibitory ability against the pathogenic strains. Biofilm inhibition percentage of ouabain against the pathogenic strain *K. pneumoniae* and MTCC 109 was found to be 62.26 ± 5.29 % and 59.65 ± 4.68 % respectively. However, leaf extract exhibits relatively less biofilm inhibitory effect as compared to the isolated compound (Figure 5).

Microscopic studies

The biofilm inhibitory activity of leaf extract and ouabain at sub-MIC value was further evident through microscopic studies. Both the light and fluorescence microscopic studies showed significant decline in the biofilm formation with scattered biofilm matrix in the treated strains of *K. pneumoniae* and MTCC 109 as compared to thickened and aggregate biofilm in untreated isolates (Figure 6a and 6b).

Ouabain is regarded as one of the important bioactive compounds derived from plant origin with therapeutic value. All three antibiofilm assay (qualitative, quantitative, and microscopic analysis) indicated that both the leaf extract as well as isolated compound ouabain significantly declines exopolysaccharide matrix production by the test bacterium when treated with sub-MIC value. These finding are in accordance to earlier studies carried out by several workers. Studies reported the role of ouabain that synergistically increased the antibacterial activity of aminoglycosides and inhibit biofilm formation in pathogenic strains of *Staphylococcus aureus*[19,20].

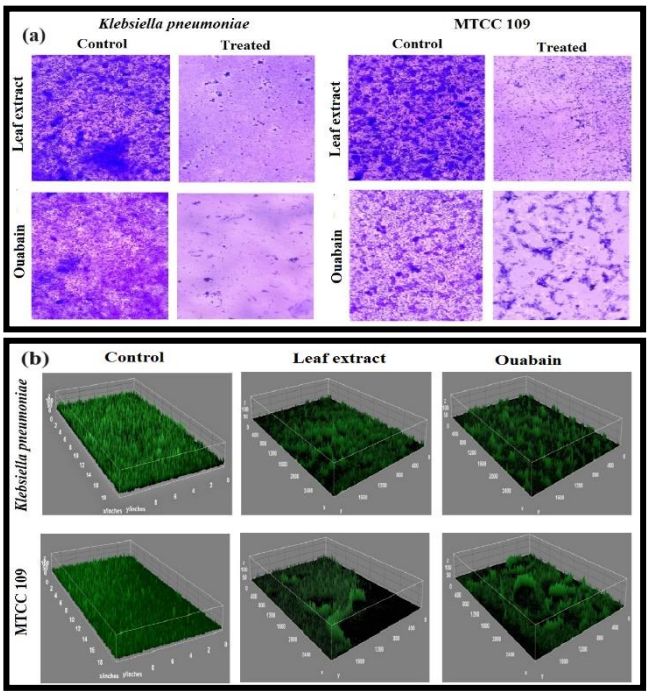

Figure 6: Microscopic antibiofilm assay of methanolic leaf extract of *C. macleodii* and ouabain at sub-MIC against *K. pneumoniae* and MTCC 109 by (a) light microscopic and (b) fluorescence microscopic studies.

Subsequently, study reported the existence of ouabain in leaf extract of *Holothuria parva* (Sea cucumber) shows antibacterial activity against various pathogenic bacterial strains like *Enterococcus faecalis*, *P. aeruginosa* and *E. coli*[19]. The present result along with previous finding clearly suggested the antibacterial properties of ouabain derived from the methanolic leaf extract as novel antimicrobial agents.

Molecular docking of Ouabain

Docking results of ouabain with different binding sites of targeted proteins in *K. pneumoniae* were presented (Table 1). It is evident from the study that ouabain showed lowest minimum docking score of -5.187 Kcal/mol with bssS and -9.826 Kcal/mol with metE. Binding of ouabain involved only one hydrogen bond with three binding site amino acids (Arg 58A, Thr 54A and Trp 16A) of bssS protein (Figure 5B) whereas three hydrogen bonds with amino acids (Asp 588A, Ser 422A and His 683A) of metE protein (Figure 5.6D). The study suggested that binding of Ouabain involved several hydrophobic interactions with the binding site amino acids (Figure 7).

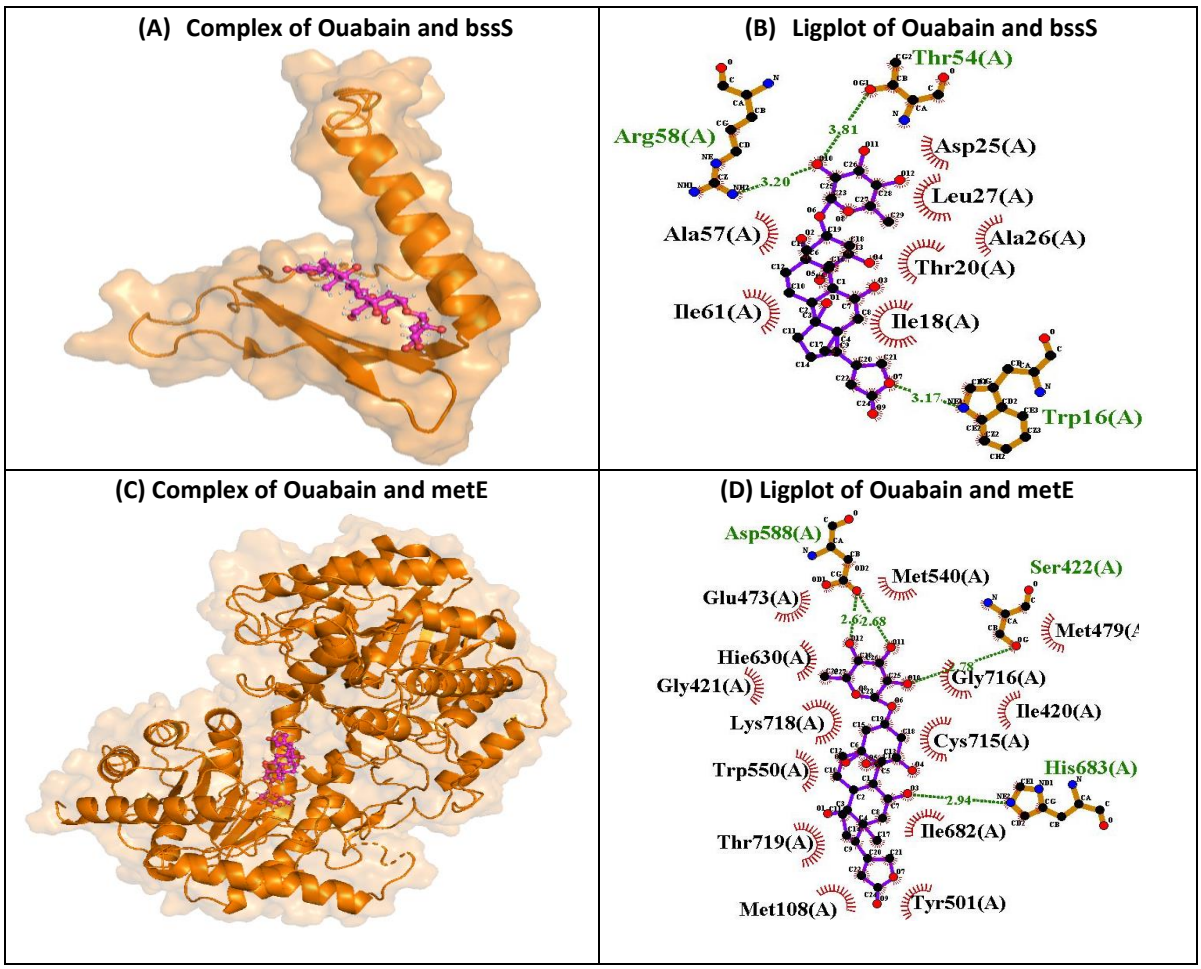

Figure 7: Molecular docking of ouabain onto proteins responsible for biofilm production in *K. pneumoniae*. Ouabain was well accommodated inside the binding site of (A) bssS and (C) metE protein. Ligplot analysis with binding site amino acids of (B) bssS and (D) metE protein. Hydrogen bonds were represented as dotted (green) lines and hydrophobic interactions with curved (red) lines.

Table 1: Docking results of Ouabain with respect to different binding sites onto the proteins involve in biofilm production exhibited by *Klebsiella pneumoniae*.

Site ID	Site score	Volume (Å)^3	Glide XP score (Kcal/mol)
(a) bssS protein			
1	0.539	52.822	-5.187
(b) metE protein (PDB ID: 3L7R)			
1	1.073	260	-9.826
2	1.047	232	-8.642
3	1.069	141	-8.472
4	1.095	99	-8.532
5	1.086	73	-7.627

Molecular docking analysis reported that the binding affinity of isolated bioactive compound, ouabain onto the different binding sites of proteins (bssS and metE) which are responsible for the biofilm production and pathogenesis in *K. pneumoniae.* The present study evident that ouabain showed high binding affinity towards bssS and metE with a Glide XP score of -5.187 Kcal/mol and -9.826 Kcal/mol respectively. These potential interaction between isolated compound and proteins influencing pathogenesis indicates the potency of ouabain as a suitable candidate for development of novel and potent antimicrobial compounds alternative to commercial drugs to combat microbial infection.

CONCLUSION

Increased antimicrobial resistances necessitates rapid identification and isolation of novel therapeutic agents from natural resources. Plant derived compounds are regarded as promising alternative drug as pathogenic microbes is less likely to acquire resistance against these compounds due to structural and functional diversity. In this regard, the bioactive compounds, ouabain derived from leaf extract of the medicinal plant *Cordia macleodii* with significant antibacterial and antibiofilm potentials and high binding affinity for the targeted protein in the pathogenic bacteria might be considered as potent antimicrobial agents to fight against the antimicrobial resistance and biofilm infections.

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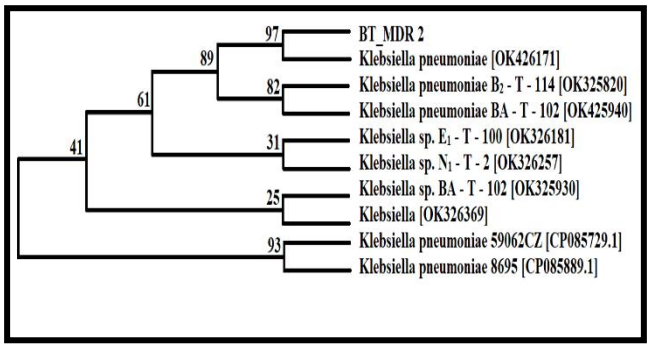
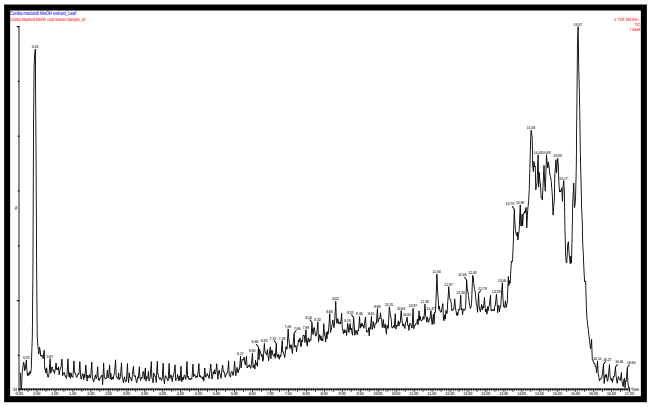
Conflict of Interest: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

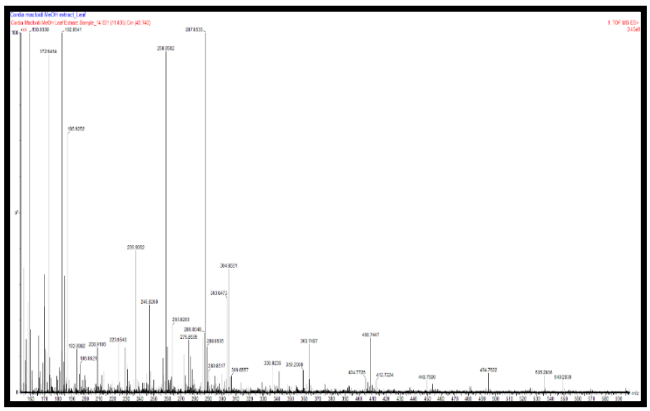
Supplementary Data



Supplementary Figure 1: Phylogenetic analysis of the bacterial isolates (BT_MDR2) that was identified as *Klebsiella pneumoniae* using Maximum Likelihood method based on the Tamura-Nei model.



Supplementary Figure 2: Total ion chromatogram (TIC) of the crude methanolic leaf extract of *C. macleodii*.



Supplementary Figure 3: Combined spectra indicating molecular mass of phytochemicals present in crude methanolic leaf extract of *Cordia macleodii*.

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