



Evaluation of Biofilm Inhibitory Efficacy of *Bacopa monnieri*, *Acalypha indica* and *Calotropis gigantea* Extracts and their Combination Against Wound Colonizing Bacteria *Pseudomonas aeruginosa* and *Staphylococcus Aureus*

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Abstract: Key factors in compromised wound healing primarily include bacterial colonization and infection. Extensive use of systemic antibiotics, preferred choice of treatment for clinically infected wounds, is often accompanied with emergence of bacterial resistance. Quorum sensing (QS), density dependent chemical communication, is emerging as a promising area of research since most infectious microorganisms operate this mechanism to realize their pathogenic potential. Foregoing research indicates the potential of studying medicinal plant extracts/components and their combinations as vital inhibitors of QS regulated virulence factors' production. Our study aims to understand the potential of three plant extracts and their combinations as vital inhibitors of QS regulated virulence factor's production by two wound pathogens and to achieve this our objectives are to perform their violacein inhibitory (on *C. violaceum*) and biofilm inhibitory properties (on *Pseudomonas aeruginosa* and *Staphylococcus aureus*). Minimum inhibitory concentration (MIC) of aqueous plant extracts against *P. aeruginosa* and *S. aureus* was performed (two-fold serial microdilution). Violacein inhibition was assayed using agar well diffusion while biofilm inhibition by crystal violet method. Organisms presented 5mg/ml (*Bacopa monnieri*), 2.5mg/ml (*Acalypha indica*), and 2.5mg/ml (*Calotropis gigantea*) as MIC. Three sets of individual extract concentrations (sub-MIC in μ g/ml) and their corresponding combinations were used for this study. Extracts exhibited more significant ($P<0.0001$) violacein inhibition in combination than individually. Biofilm inhibition by extracts' combination was also significantly ($P<0.001$) higher than that of individual extracts indicating a possible positive herb-herb interaction of phytoconstituents (synergistic or complementary). The relative decrease in response to individual extracts in higher concentrations (set 'c') by both organisms point to possible recalcitrance behavior generally exhibited by bacteria on exposure to higher antibacterial agents. The improved antibiofilm efficacy exhibited by this novel combination may serve as an alternative approach in managing wound colonization by biofilm-producing bacteria and hence faster-wound healing.

Key words: Biofilm, Antibiotic resistance, Plant extract, Quorum sensing, Extract combination, Synergistic effect.

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I. INTRODUCTION

One of the major causes of delayed wound healing is the colonization of wounds by bacteria and if left untreated can lead to a chronic wound.^{1,2} Persistence of infections in chronic wounds is the most common cause that hinders its healing, resulting in increased patient morbidity and mortality.^{3,4} The manifestation of bacterial biofilms in chronic wounds is more critical in delaying the wound healing process than in the more treatable planktonic forms.^{4,5} Biofilm formation, a quorum sensing (QS) regulated mechanism, provides physical protection to the enclosed bacterial colonies from a hostile external environment. This facilitates survival and communication among the bounded bacteria, which enhances their virulence.⁶ Biofilms reduce the uptake of antibiotics leading to resistant bacterial strains. Biofilm-forming bacteria, *P. aeruginosa* and *S. aureus*, are the most frequently isolated pathogens from chronic wounds.⁷ These bacteria are highly invasive and have developed resistance to a wide range of commonly prescribed antibiotics, leading to treatment failure of many infected wounds.^{8,9} Consequently, there is a need to identify alternative therapeutic strategies to manage the wound infections caused by these multidrug resistant pathogens.^{10,11} One of the effective options to tackle the issue of antibiotic resistance is, to reduce the pathogenicity rather than the direct killing of the micro-organisms. This has prompted researchers to identify methods that target specific receptors/activators of QS to combat biofilm-associated infections.¹² Recently, research on the use of medicinal plants in wound care has been given serious importance as new treatment option for acute and chronic wounds.¹³ Phytoconstituents from diverse medicinal plants have been reported to exhibit significant anti-QS activity.^{14,15} Combination of medicinal plants brings together phytoconstituents from individual plants to synergistically and complementarily achieve the desired therapeutic effect/s through the phenomenon of positive herb-herb interaction.^{16,17} The complex nature of QS regulated mechanism requires a composite drug approach such as an herbal combination as an improved option for treating multidrug resistant wound colonizing bacteria.¹⁸ Treatment of wound colonizing bacteria using PHF is gaining acceptance in the field of scientific research. Some of them have been patented, while a few are commercially available.^{17,19} *Bacopa monnieri* (L.) Wettst. also called Brahmi, a plant species belonging to *Plantaginaceae* family, has been used extensively in Ayurveda and Chinese medicine as a memory enhancer.²⁰ It is mostly found in wetlands and swamps. Most of the medicinal uses of *Bacopa monnieri* include treatment of asthma & arthritis,²¹ As antibacterial agent²² It is also known for its anxiolytic effect,²³ anti-depressant effect,²⁴ anticonvulsive action,²⁵ antiulcerogenic and wound healing properties.^{26,27} Bacopasides, basically triterpene saponins, are the most important active compounds in *B. monnieri*.^{28,29} *Acalypha indica* also called Indian acalypha belonging to the family *Eupobiaceae* has been reported to have significant medicinal properties for the treatment of various diseases. Extracts from all parts of this plant have been extensively used in Ayurveda and Siddha for managing several ailments such as rheumatism, diabetes mellitus, skin diseases and wounds.³⁰ It is commonly found in wet, temperate, and tropical areas of Asia, Africa, Europe, Australia and South and North America and used as antimicrobial agent.³¹ *Calotropis gigantea* also known as 'Giant shallow wort' or 'Madar' is a latex producing plant that is found in India, Africa, Bangladesh and Sri Lanka. *C. gigantea* belongs to the family of *Apocynaceae*. This plant is widely used in

Ayurveda to treat various human diseases.³² Screening studies revealed the presence of numerous potent phytoconstituents in this plant which are responsible for its broad pharmacological activities.³³ Medicinal activity of *C. gigantea* include its anti-asthmatic, antibacterial, anti-arthritis, wound healing and antiscarring effects.³⁴ The present study aimed at evaluating the anti-QS activity of *B. monnieri*, *A. indica* and *C. gigantea* extracts and their combinations against wound colonizing bacteria *P. aeruginosa* and *S. aureus*.

2. MATERIALS AND METHODS

2.1 Media and Chemicals

All the media and chemicals used in this research were of analytical grade procured from HiMedia laboratories Pvt. Ltd, Mumbai, India and Thermo Fisher Scientific India Pvt Ltd Mumbai India. Luria Bertani (LB) agar, LB broth, Muller Hinton Broth (MHB) and Tryptic Soy Broth (TSB) are culture media used for experiment and maintenance of bacterial cultures. Ammonium oxalate, ethanol, glycerol, crystal violet, gentamicin, methanol, yeast, cinnamon oil, tryptone, yeast extract, glucose, soy, potassium chloride, sodium chloride, disodium hydrogen phosphate Na_2HPO_4 , and potassium dihydrogen phosphate (KH_2PO_4) are the other chemicals used in this experiment.

2.2 Bacterial Strains and culture conditions

The Bacterial cultures of *C. violaceum* (MTCC2656), *P. aeruginosa* (MTCC3541) and *S. aureus* (MTCC737) were purchased from Microbial Type Culture Collection Centre (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. The lyophilized cultures were activated by inoculating in LB broth and incubated in a shaking condition at 37°C for 24hrs. The 24hr cultures of *P. aeruginosa* and *S. aureus* in (TSB) supplemented with 1% glucose were used for biofilm inhibition assay. The cultures were preserved in glycerol and stored at -80°C for further use.

2.3 Plant Material authentication and voucher reference

Fresh leaves of the selected plants, *B. monnieri* (Brahmi), *A. indica* (Indian acalypha), with voucher number 65 and 50, respectively were procured from Department of Horticulture, University of Agricultural Sciences, Gandhi Krishi Vigyana Kendra (GKV), Bangalore, Karnataka India. They were authenticated by Professor M. Vasundhara, Head, Department of Horticulture, GKV. *C. gigantea*, (Giant shallow wort) with voucher number SMPU/NADRI/BNG/2010-11/490 was obtained from National Ayurveda Dietetics Research Institute, Jayanagar, Bangalore, Karnataka, India. It was authenticated by Dr. Shiddamallaya N. Assistance Research Officer (Botany). The leaves were thoroughly washed, shade dried, and made into a coarse powder using an electric homogenizer.

2.4 Preparation of the Extracts

Crude extracts were prepared by dissolving 25g of the powdered plant sample in a flask containing 250ml of distilled water. The flask was kept in a shaker at room temperature for 24 hours after which the extracts were filtered using Whatman No. 1 filter paper. The filtrates were lyophilized using Labconco Freeze dryer -105°C by LABQUIP India Private limited, Hyderabad. The dried extracts were packed in a closed container and stored at -20°C for further analysis.³⁵

2.5 Minimum Inhibitory Concentration (MIC) Determination

Broth microdilution method was used to estimate the MIC of each extract.^{36,37} 96-well plate was used to prepare a two-fold serial dilution of the extracts. MHB (50 μ L) was added into the 1st – 10th well. Then 50 μ L of 10mg/mL of each plant extract was added to the corresponding first well. A serial two-fold dilution was performed by transferring 50 μ L of the suspension in each well to the subsequent well until the 10th well. The final 50 μ L suspension taken out from the last well was discarded. MHB broth and bacteria alone served as negative control, while gentamicin 10 μ g/ml and bacteria served as a positive control. Bacterial suspension was adjusted to 0.5 McFarland (1-2 $\times 10^8$ CFU/mL). The adjusted broth inoculum was diluted in the ratio of 1:100, and 50 μ L was dispensed into respective wells (1-12) to attain the final inoculum size of 5 $\times 10^5$ CFU/mL per well.^{38,39} The final concentrations of the extracts ranged from 0.0195 to 10mg/mL. The plate was incubated for 24 hrs at 37°C. The MIC for each extract was determined by adding 40 μ L of 200 μ g/mL p-iodonitrotetrazolium chloride (INT) in each well followed by incubation for 1hr 30min. Bacterial growth was indicated by a change of color from yellow to pink. The lowest concentration of the extracts that prevented the appearance of visible bacterial growth was considered as the MIC value.⁴⁰ Experiment was performed in a triplicate of three independent trials.

2.6 Violacein inhibition assay using *C. violaceum* (MTCC2656)

Agar well diffusion assay was used to detect violacein inhibition activity of the plant extract adopting standard method described by Ahmed et al.⁴¹ Overnight cultures of *C. violaceum* (MTCC2656) in LB broth were diluted to 0.5 McFarland. 100 μ L of diluted culture was spread on LB agar plates and was smeared uniformly. Wells were made on the LB agar plates with a standard cork borer of 6 mm diameter. 100 μ L of the

optimized aqueous plant extracts, concentrations as indicated in table 2, were added to each well. Water and 1% DMSO were used as vehicle controls while 2 μ g/mL cinnamon oil was used as a positive control. The plates were incubated at 33°C for 24hrs. A colorless opaque zone with loss of purple pigment around the treated wells indicated violacein inhibition confirming anti-QS activity. The violacein inhibitory activity of the plant extracts were determined by measuring the diameter (in mm) of the zone of pigment inhibition formed around the well. Experiment was performed in a triplicate of three independent trials.

2.7 Biofilm inhibition assay

The anti-biofilm activity against *P. aeruginosa* and *S. aureus* was evaluated using crystal violet assay.⁴² Bacterial cultures were grown in TSB containing 1% glucose overnight. Optimized sub-MIC concentrations of the plant extracts as shown in table 2, were used in this experiment. A combination of these plants' extracts was made for each set of the above concentrations in a respective manner. Bacterial suspension (50 μ L) of 0.5 McFarland was added into 96 well culture plates. Plant extracts (50 μ L) of the respective concentrations were added to the corresponding wells. Bacterial culture and TSB served as positive control while TSB alone served as negative control. The plates were kept in a shaker incubator for 24hrs at 37°C. After 24hrs, the planktonic suspension was aspirated and each well was washed three times with distilled water and stained with 200 μ L of 0.1% crystal violet. The plate was allowed to stand for 20 min. The crystal violet was removed, and excess stain was rinsed three times with tap water. The plates were allowed to dry and 200 μ L of 96% (v/v) ethanol was added to each well to solubilize the crystal violet and the formed biofilm. The plate was read spectrophotometrically at 595 nm using a microplate reader (Varioskan™ lux multimode plate reader Thermo Scientific United states). Experiment was performed in a triplicate of three independent trials. Percentage biofilm inhibition was calculated using the formula below.

$$\text{Biofilm inhibition (\%)} = \frac{\text{OD}_{\text{Control}} - \text{OD}_{\text{Sample}}}{\text{OD}_{\text{Control}}} \times 100$$

2.8 Statistical analysis

All the experimental data of replicates were reported as mean \pm standard error of the mean (SEM). Statistical significance in the difference between various treatments and that of the control was evaluated by applying one-way ANOVA (Tukey's test). Graph pad Prism (Version 8.0) was used and P value \leq 0.05 was considered statistically significant.

3. RESULTS

3.1. Determination of MIC

MIC of the individual plant extracts against *P. aeruginosa* and *S. aureus* are presented in table 1. The three plant extracts exhibited similar MIC values against the two strains of bacteria used in this experiment.

Table 1. MIC (mg/ml) of the plant extracts tested against *P. aeruginosa* and *S. aureus*

S/No	Plant extracts	<i>P. aeruginosa</i>	<i>S. aureus</i>
1	<i>B. monnieri</i>	<5mg/ml)	<5mg/ml)
2	<i>A. indica</i>	<2.5mg/ml)	<2.5mg/ml)
3	<i>C. gigantea</i>	<2.5mg/ml)	<2.5mg/ml)

Table I shows the MIC of the three plant extracts (*B. monnieri*, *A. indica*, *C. gigantea*) used in this study on *P. aeruginosa* and *S. aureus*. The results indicated that all the three plant extracts showed similar MIC on both the organism tested. This served as the basis for determining the extract concentrations that has been used in this research.

3.2. Preparation of plant extracts' combinations

Different sub-MIC concentrations of each plant extract were optimized and three sets of individual plant extract concentrations and their corresponding combinations were prepared as per Table 2.

Table 2. The concentration of plant extracts used for the study

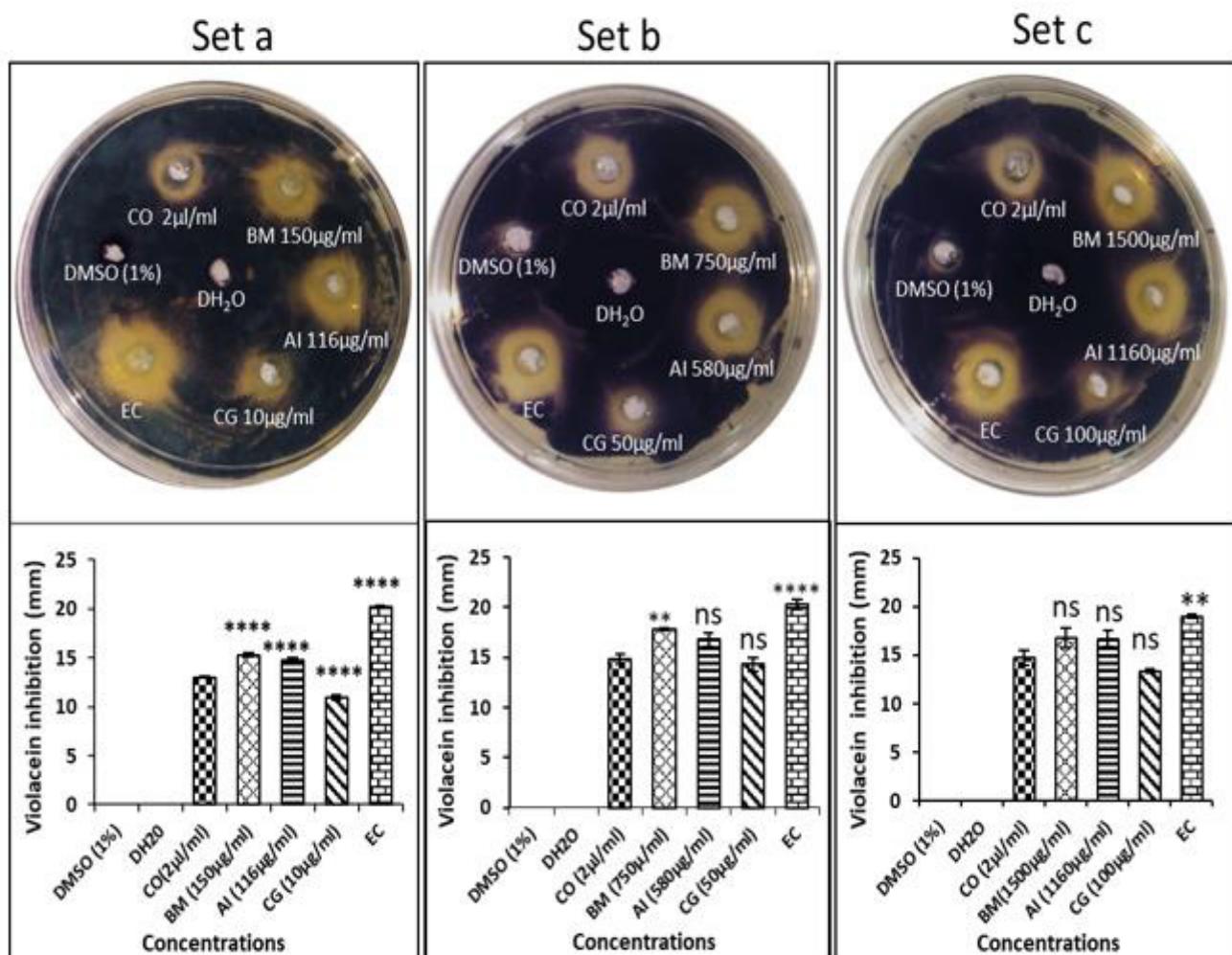
	<i>B. monnieri</i> ($\mu\text{g}/\text{ml}$)	<i>A. indica</i> ($\mu\text{g}/\text{ml}$)	<i>C. gigantea</i> ($\mu\text{g}/\text{ml}$)	Extract combination ($\mu\text{g}/\text{ml}$)
Set a	150	116	10	150+116+10
Set b	750	580	50	750+580+50
Set c	1500	1160	100	1500+1160+100

Based on the MIC results (Table 1), three sets of sub MIC concentrations labelled set 'a', set 'b' and set 'c' for each of the plant extracts and their respective combinations were chosen for this study. *B. monnieri*, *A. indica* and *C. gigantea* have initial concentrations of 150, 116 and 10 $\mu\text{g}/\text{ml}$, respectively (set 'a'). These concentrations were increased 5 times (set 'b') and 10 times (set 'c') to test for the anti-quorum sensing activity of lower, middle, and higher sub MIC concentrations.

3.3. Violacein inhibition

All tested concentrations of the plant extracts and their

combinations (Table 2) showed varying levels of violacein inhibition without affecting the organism's growth (Figure 1). No pigment inhibition was observed around the vehicle control wells containing DMSO (1%) and sterile distilled water. Extracts' combination showed more significant ($P<0.001$) violacein inhibition in sets 'a' and 'b' than in set 'c' ($P<0.01$) compared to the positive control. The observed violacein inhibition appears to be concentration independent as an increase in the extract dose didn't contribute to any incremental growth in violacein inhibition.



ns=not significant, ** $P<0.01$, **** $P<0.0001$

Fig 1: Violacein inhibition activity of different sets of sub-MIC concentrations of aqueous plant extracts against biosensor strain *C. violaceum* by agar well diffusion assay.

CO=Cinnamon oil, BM=Bacopa monnier, AI=Acalypha indica, CG=Calotropis gigantea, EC=Extract combination, DMSO=Dimethyl sulphoxide, DH₂O=Distilled water. Mean values of triplicate independent experiments and standard error of mean are shown. DH₂O and 1% DMSO serve as vehicle controls while CO serves as a positive control. Set a, Set b and Set c represent lowest, intermediate and highest concentrations of the optimized aqueous plant extracts' concentration chosen for this experiment. The upper panel in

this figure shows the representative images of agar well diffusion method showing inhibition of violacein pigment in *C. violaceum* while the lower panel shows measurement of corresponding diameter of zone of violacein inhibition.

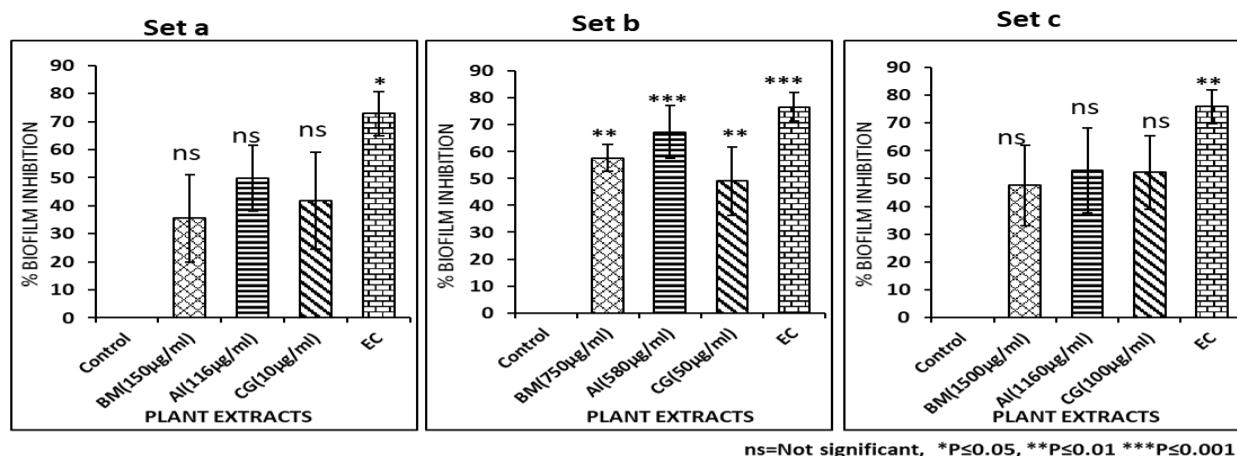
3.4. Biofilm inhibition

Different optimized sub-MIC concentrations of plant extracts were used for this study (Table 2). Percentage biofilm

inhibition activity of different sets of sub-MIC concentrations and their respective combinations was performed (Figure 2). Results from this experiment showed varying degrees of percentage biofilm inhibition exhibited by individual plant extracts and their combinations (Table 3). In *S. aureus* (Figure 2A), the percentage biofilm inhibition of extracts' combinations was shown to be significantly higher ($P<0.001$) in all three tested concentrations compared to the control. For individual plant extracts, there was a general tendency of increase in biofilm inhibition from set 'a' to 'b' and then a slight reduction as the concentrations rose in set 'c'. However, CG does not seem to follow this pattern in set 'c' though this was not statistically significant. BM showed percentage biofilm inhibition of 35.49 ± 15.72 , 57.63 ± 5.07 and 47.57 ± 14.48 in set 'a', 'b' and 'c', respectively, showing slight concentration-dependent pattern between set 'a' and set 'b' while the biofilm inhibition drops in set 'c'. The same is observed in AI exhibiting percentage biofilm inhibition of 49.82 ± 11.74 , 67.36 ± 9.81 and 52.94 ± 15.17 in set 'a', 'b' and 'c' respectively. On the other hand, CG with biofilm inhibition of 41.78 ± 17.17 , 49.00 ± 12.72 and 52.18 ± 13.15 in set 'a', 'b' and 'c' respectively demonstrated a slight disproportionate concentration dependent increase in percentage biofilm inhibition. Despite these variations in percentage biofilm inhibition across the different individual plant extracts' concentrations, the EC of set 'a' (72.87 ± 7.85), set 'b' (76.61 ± 5.29) and set 'c' (75.85 ± 6.02) did not show any significant change in the percentage biofilm inhibition corresponding to increase in

concentrations. In *P. aeruginosa* (Figure 2B), extracts' combinations of the three sets of concentrations exhibited significant increase ($P<0.001$) in percentage biofilm inhibition compared to individual plant extracts and control. Percentage biofilm inhibition of the individual extract did not follow concentration dependent nature. BM exhibited percentage biofilm inhibition of 44.22 ± 13.68 , 48.65 ± 9.48 and 62.76 ± 5.68 in set 'a', 'b' and 'c', respectively indicating a slight concentration dependency which is not maintained with further increase in concentration. The opposite was observed for CG which show percentage biofilm inhibition of 70.29 ± 9.30 , 51.97 ± 11.72 and 47.51 ± 12.20 in set 'a', 'b' and 'c' respectively indicating a decrease in percentage biofilm inhibition with increase in extract concentrations, though the reduction was non incremental. AI showed percentage biofilm inhibition of 72.33 ± 9.05 , 62.09 ± 17.59 and 67.10 ± 9.61 in set 'a', 'b' and 'c' respectively indicating a slight increase in biofilm inhibition in set 'c' compared to set 'b'. Percentage biofilm inhibition of EC in set 'a' (87.96 ± 4.73), set 'b' (80.85 ± 4.58) and set 'c' (78.53 ± 6.06) did not vary significantly despite the variations in the percentage biofilm inhibition exhibited by individual plant extracts' concentrations. The improved violacein and percentage biofilm inhibition activities exhibited by the extracts' combinations can be attributed to a synergistic, reinforcement, potentiation or complementary interaction of the phytoconstituents present in them and not additive.⁴³

A. *Staphylococcus aureus*



B. *Pseudomonas aeruginosa*

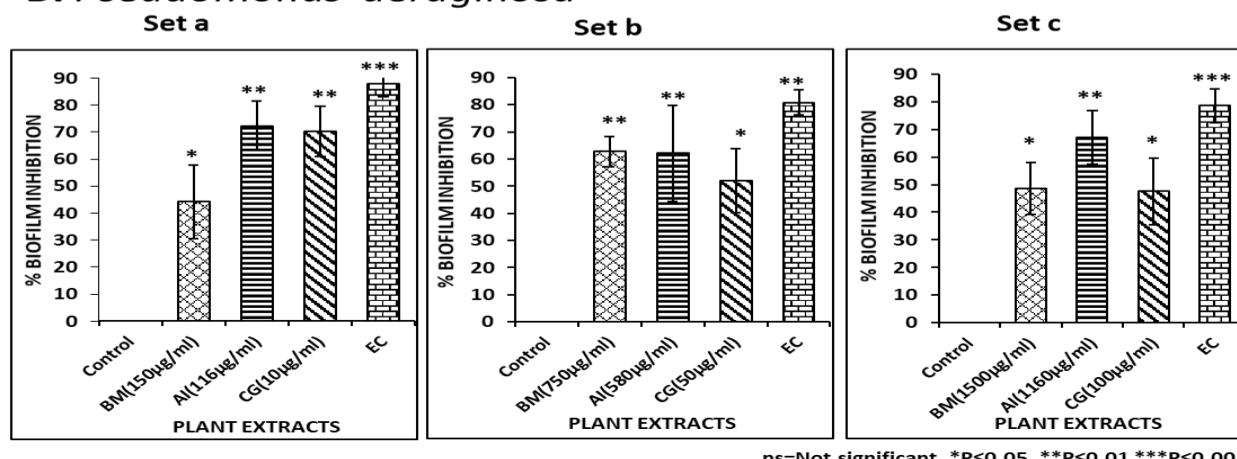


Figure 2. Biofilm inhibitory activity of different sets of sub-MIC concentrations of aqueous plant extracts and their combinations against (A) *P. aeruginosa* and (B) *S. aureus*, using crystal violet staining assay.

BM=*Bacopa monnieri*, AI=*Acalypha indica*, CG=*Calotropis gigantea*, EC=Extract combination, Bacterial culture and MHB without plant extract served as a control. Mean values of triplicate independent experiments and standard error of mean are shown. Set 'a', Set 'b' and Set 'c' represent lowest, intermediate and highest concentrations respectively of the optimized aqueous plant extracts chosen for this experiment.

Table 3. Percentage biofilm inhibition of different sets of sub-MIC concentrations and their respective combinations

Plant extracts	set a		set b		Set c	
	<i>P.aeruginosa</i> %	<i>S.aureus</i> %	<i>P.aeruginosa</i> %	<i>S.aureus</i> %	<i>P.aeruginosa</i> %	<i>S.aureus</i> %
BM	44.22±13.68*	35.49±15.72 ^{ns}	62.76±5.68**	57.63±5.07**	48.65 ±9.48*	47.57±14.48 ^{ns}
AI	72.33±9.05**	49.82±11.74 ^{ns}	62.09±17.59**	67.36±9.81***	67.10±9.61**	52.94±15.17 ^{na}
CG	70.29±9.30**	41.78±17.17 ^{ns}	51.97±11.72**	49.00±12.72**	47.51±12.20*	52.18±13.15 ^{ns}
EC	87.96±4.73***	72.87±7.85*	80.85±4.58**	76.61±5.29***	78.53±6.06***	75.85±6.02**

Table 3 showing percentage biofilm inhibition ± standard error of mean of the plant extracts selected for this study on *P. aeruginosa* and *S. aureus*. BM=*B. monnieri*, AI=*A. indica*, CG=*C. gigantea*, EC=Extract combination. Results of the three sets of individual plant extracts concentration (set 'a', set 'b' and set 'c') and their corresponding combinations are presented. It is observed that biofilm inhibitory properties of the individual plant extracts is generally higher in *P. aeruginosa* than in *S. aureus* with varying degree of significance. Percentage biofilm inhibition of extract combinations did not vary significantly with each other despite the large variations in the individual extracts concentration in them.

4. DISCUSSION

One of the major causes of delayed wound healing is an infection of the wound area by opportunistic pathogenic bacteria.⁴⁴ The two opportunistic bacteria most commonly isolated from chronic wounds are *S. aureus* and *P. aeruginosa*. These bacteria are also reported to co-exist within the chronic wound environment making them more dangerous than if found separately.⁴⁵ These bacteria are increasingly gaining resistance to commonly used antibiotics due to their ability to produce multiple virulence factors such as biofilm, LasB Elastase, hemolysins etc making them multidrug resistant. Most of these virulence factors are QS regulated; hence, interrupting the QS regulated mechanism of these bacteria using suitable inhibitors from plant extracts is an emerging approach to tackle the menace of wound-related infections.^{46,47} There is a growing need for novel antimicrobial and anti-biofilm treatment strategies to tackle this problem.⁴⁸ QS-regulated attribute of *C. violaceum* is an easily noticeable and measurable indicator trait and therefore, this bacterium has been extensively used as a model organism for studying QS inhibitory activity of natural and synthetic compounds.⁴⁹ Hence, medicinal plants which inhibit violacein production without affecting bacterial cell viability can be considered as potential QS inhibitors.⁵⁰ Data suggest that bacterial biofilm was reported to be 1000 times more tolerant to wide range of antibiotics than their planktonic counterpart, which makes them persist in wound causing delay in its healing.^{44,51} Before testing for biofilm inhibitory efficiency of the optimized sub-MIC concentrations of the plant extracts on *S. aureus* and *P. aeruginosa*, biosensor strain of *C. violaceum* was used to test their anti-QS activity. Inhibition of violacein production by the three plants under investigation from this study validates the potent QS inhibitory role associated with them individually and in combination compared to the control. The same extract concentrations were used to test for their anti-biofilm activity on *S. aureus* and *P. aeruginosa*. The non-incremental

pattern observed in the percentage biofilm inhibition exhibited by plant extracts used in this study can be attributed to the recalcitrance behavior of *S. aureus* and *P. aeruginosa* as reported by Lebeaux et al.,⁵² when exposed to higher concentration within sub-MIC. Results from this experiment revealed that the treatments in all three sets of concentrations inhibit bacteria biofilm formation and reduce the violacein production compared to the control, which is suggestive of the *in vitro* anti-QS activity of the extracts and their combinations. A great deal of research focuses on the newer concept of antipathogenic compounds, focusing largely on controlling different pathogens by not exerting a sharp killing effect.^{53,54} The significant percentage biofilm inhibition exhibited by the EC in all the three sets of extracts' concentrations has pointed to the advantage of polyherbal combination in treating wound infection.⁵⁵ The same pattern was reported by Jose et al.⁵⁴ where they showed a promising percentage of biofilm inhibition of a herbal combination against toxigenic *Vibrio cholerae* at a relatively lower concentration compared to the individual plants extracts. Different phytoconstituents in herbal combinations have been reported to simultaneously inhibit diverse QS-regulated virulence factors' production. This is an important attribute of herbal combinations because it renders the bacteria susceptible to the body immune system and/or any mild antibiotic.^{53,56} One of the advantages of targeting virulence factors in an attempt to combat bacterial resistance is that inhibitors of QS-regulated virulence factors usually target the adaptation and not the survival mechanisms of bacteria making them less likely to generate resistance against antibiotics.⁴⁶ To avoid the ever-increasing prevalence of multidrug resistance tendencies of bacteria, it is necessary to shift away from the conventional antimicrobial approach where antibiotics are produced by looking at bacteria as individual free moving (planktonic form) rather than as multicellular tissue-like structures (Biofilm) that are attached to a particular surface.⁵⁷

5. CONCLUSION

Even with the availability of diverse antibiotics and ongoing research to develop novel ones, bacterial multidrug resistance is still a serious medical concern. One of the mechanisms by which *P. aeruginosa* and *S. aureus* become multidrug-resistant strains is by the formation of biofilm, which make them persist in wounds causing a serious threat to individuals and public health. *B. monnieri*, *A. indica* and *C. gigantea* are plants known for various medicinal properties in traditional medicinal systems. The recent past has witnessed an escalating interest in studying plants for diverse medicinal properties. Advanced scientific techniques have led to the isolation of a variety of

bioactive compounds from many such candidates, some of which have been taken even to the level of pharmacological validation. Poly Herbalism is a concept that is gaining an extensive acceptance now than ever before and an increasing interest can be witnessed among the scientific community to develop and validate novel polyherbal combinations/formulations. Several phytoconstituents in herbal combinations have been reported to be effective in treating wound colonizing bacteria by simultaneously inhibiting the production of diverse virulence factors such as biofilm and exhibit significantly higher bactericidal activity in comparison to the use of a single plant. Results from this study validates the quorum modulatory potential of these three plants individually and in combination. Extracts of these plants are suitable for curbing the infection by *S. aureus* and *P. aeruginosa* in wounds. The study can be further extended towards the identification of active components of these extracts, their purification and subsequently understanding their mechanism of action on biofilm assemblies. The major findings of this study are as follows (i) All the individual plant extracts exhibit various degrees of violacein and percentage biofilm inhibition (ii) The inhibitory property of these plant extracts doesn't seem to be fully concentration-dependent (iii) The tendency of increase in the inhibitory activity in set 'b' and decreases in set 'c' indicate the possible recalcitrant behavior of bacteria in higher concentration of antibacterial agents (iv) Extract combinations showed more significant violacein, and percentage biofilm inhibition than the individual plant extracts (v) Extract combinations did not show significant variation from each other despite the changes in concentrations of individual plant extracts in them. This indicates one of the

9. REFERENCES

- Dong D, Thomas N, Ramezanpour M, Psaltis AJ, Huang S, Zhao Y, et al. Inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms by quatsomes in low concentrations. *Exp Biol Med (Maywood)*. 2020;245(1):34-41. doi: 10.1177/1535370219896779, PMID 31903777.
- Wu SC, Marston W, Armstrong DG. Wound care: the role of advanced wound healing technologies. *J Vasc Surg*. 2010;52(3):Suppl:59S-66S. doi: 10.1016/j.jvs.2010.06.009, PMID 20804934.
- Bass S, Sheetu A. Herbal advancements in the treatment to accelerate wound healing. *Mod Phytomorphology*. 2021;15:76-7.
- Mir M, Permana AD, Tekko IA, McCarthy HO, Ahmed N, Rehman AU et al. Microneedle liquid injection system assisted delivery of infection responsive nanoparticles: A promising approach for enhanced site-specific delivery of carvacrol against polymicrobial biofilms-infected wounds. *Int J Pharm*. 2020;587:119643. doi: 10.1016/j.ijpharm.2020.119643, PMID 32702455.
- Rhoads DD, Wolcott RD, Percival SL. Biofilms in wounds: management strategies. *J Wound Care*. 2008;17(11):502-8. doi: 10.12968/jowc.2008.17.11.31479, PMID 18978690.
- De Kievit TR, Iglesias BH. Bacterial quorum sensing in pathogenic relationships. *Infect Immun*. 2000;68(9):4839-49. doi: 10.1128/IAI.68.9.4839-4849.2000, PMID 10948095.
- Doern GV, Jones RN, Pfaller MA, Kugler KC, Beach ML. Bacterial pathogens isolated from patients with skin and soft tissue infections: frequency of occurrence and

advantages of extract combination over individual plant extracts. Study observations suggest that the meticulous combining of these plant extracts may serve as a promising alternative to inhibit quorum sensing regulated virulence factors' production, thereby combating multidrug-resistant wound colonizing bacteria.

6. AUTHORS CONTRIBUTION STATEMENT

The study was designed and executed under the guidance of Dr. Bindhu O.S. Plant extract preparation, pigment and biofilm inhibition assays as well as data analysis were performed by Habibu Tanimu. MIC experiment and bacterial culture maintenance was performed by Nadasha Koonath Vijayan. Conceptualization, supervision, review and editing was done by Dr. Bindhu O.S. Manuscript writing was also initiated under the guidance of Dr. Bindhu O.S. All authors critically reviewed the manuscript, contributed to its revision, and approved the final version submitted.

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8. CONFLICT OF INTEREST

Conflict of interest declared none.

- antimicrobial susceptibility patterns from the SENTRY Antimicrobial Surveillance Program (United States and Canada, 1997). SENTRY Study Group (North America). *Diagn Microbiol Infect Dis*. 1999;34(1):65-72. doi: 10.1016/s0732-8893(98)00162-x, PMID 10342110.
- Poole K. *Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol*. 2011;2(65):65. doi: 10.3389/fmicb.2011.00065, PMID 21747788.
- Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev*. 2015;28(3):603-61. doi: 10.1128/CMR.00134-14, PMID 26016486.
- Gilmer DB, Schmitz JE, Euler CW, Fischetti VA. Novel bacteriophage lysis with broad lytic activity protects against mixed infection by *Streptococcus pyogenes* and methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2013;57(6):2743-50. doi: 10.1128/AAC.02526-12, PMID 23571534.
- Manzoor-ul-Haq VR, Singh D, Singh AK, Ninganagouda S, Hiremath J. Dried Mushroom *Agaricus bisporus* mediated synthesis of silver nanoparticles from Bandipora District (Jammu and Kashmir) and their efficacy against methicillin resistant *Staphylococcus aureus* (MRSA) strains. *Nanosci Nanotechnol Int J*. 2014;5(1):1-8.
- Gupta K, Singh SP, Manhar AK, Saikia D, Namsa ND, Konwar BK, et al. Inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm and virulence by active fraction of *Syzygium cumini* (L.) Skeels Leaf extract: in-vitro and in silico studies. *Indian J Microbiol*.

2019;59(1):13-21. doi: 10.1007/s12088-018-0770-9, PMID 30728626.

13. Sharma A, Khanna S, Kaur G, Singh I. Medicinal plants and their components for wound healing applications. *Futur J Pharm Sci.* 2021;7(1). doi: 10.1186/s43094-021-00202-w.

14. Qaralleh H, Al-Limoun MO, Khlaifat A, Khleifat KM, Al-Tawarah N, Alsharafa KY, et al. Antibacterial and antibiofilm activities of a traditional herbal formula against respiratory infection causing bacteria. *Trop J. Nat Prod Res.* 2020;4(9):527-34.

15. Patel P, Joshi C, Kothari V. Antipathogenic potential of a polyherbal wound-care formulation (Herboheal) against certain wound-infective gram-negative bacteria. *Adv Pharmacol Sci.* 2019;2019:1739868. doi: 10.1155/2019/1739868, PMID 30833966.

16. Meunier B. Hybrid molecules with a dual mode of action: dream or reality? *Acc Chem Res.* 2008;41(1):69-77. doi: 10.1021/ar7000843, PMID 17665872.

17. Karole S, Shrivastava S, Thomas S, Soni B, Khan S, Dubey J, et al. Polyherbal formulation concept for synergic action: a review. *J Drug Delivery Ther.* 2019;9(1-s):453-66. doi: 10.22270/jddt.v9i1-s.2339.

18. Sulaiman CT, Anju K, Anandan EM, Balachandran I. Synergistic interactions of phytochemicals in polyherbal formulation enhance the chemical transformations of active constituents. *J Appl Spectrosc.* 2021;88(1):181-6. doi: 10.1007/s10812-021-01156-w.

19. Patel S, Srivastava S, Singh MR, Singh D. Mechanistic insight into diabetic wounds: pathogenesis, molecular targets and treatment strategies to pace wound healing. *Biomed Pharmacother.* 2019;112:108615. doi: 10.1016/j.bioph.2019.108615, PMID 30784919.

20. Aguiar S, Borowski T. Neuropharmacological review of the nootropic herb *Bacopa monnieri*. *Rejuvenation Res.* 2013;16(4):313-26. doi: 10.1089/rej.2013.1431, PMID 23772955.

21. Viji V, Helen A. Inhibition of lipoxygenases and cyclooxygenase-2 enzymes by extracts isolated from *Bacopa monnieri* (L.) Wettst. *J Ethnopharmacol.* 2008;118(2):305-11. doi: 10.1016/j.jep.2008.04.017, PMID 18534796.

22. Moskwa J, Naliwajko SK, Markiewicz-Żukowska R, Gromkowska-Kępka KJ, Nowakowski P, Strawa JW, et al. Chemical composition of Polish propolis and its antiproliferative effect in combination with *Bacopa monnieri* on glioblastoma cell lines [sci rep] [internet]. *Sci Rep.* 2020;10(1):21127. doi: 10.1038/s41598-020-78014-w, PMID 33273550.

23. Singh D, Nag MK, Patel S, Shrivastava S, Daharwal SJ, Singh MR. Treatment strategies in burn wounds: an overview. 2013;5(6):341-52.

24. Mani JS, Johnson JB, Steel JC, Broszczak DA, Neilsen PM, Walsh KB, et al. Natural product-derived phytochemicals as potential agents against coronaviruses: a review. *Virus Res.* 2020;284(April):197989. doi: 10.1016/j.virusres.2020.197989, PMID 32360300.

25. Shanmugasundaram ER, Akbar GK, Shanmugasundaram KR. Brahmighritham, An Ayurvedic herbal formula for the control of epilepsy. *J Ethnopharmacol.* 1991;33(3):269-76. doi: 10.1016/0378-8741(91)90088-u, PMID 1921425.

26. Murthy S, Gautam MK, Goel S, Purohit V, Sharma H, Goel RK. Evaluation of in vivo wound healing activity of *Bacopa monnieri* on different wound model in rats. *BioMed Res Int.* 2013; 2013:1-9.

27. Sharath R, Harish BG, Krishna V, Sathyanarayana BN, Kumara Swamy HM. Wound healing and protease inhibition activity of bacoside-A, isolated from *Bacopa monnieri* Wettst. *Phyther Res.* 2010; 24. Vol. 8; 1217-22.

28. Nuengchamnong N, Sookying S, Ingkaninan K. LC-ESI-QTOF-MS based screening and identification of isomeric jujubogenin and pseudojujubogenin aglycones in *Bacopa monnieri* extract. *J Pharm Biomed Anal.* 2016;129:121-34. doi: 10.1016/j.jpba.2016.06.052, PMID 27423009.

29. Zhou Y, Shen Y-H, Zhang C, Zhang W-D. Chemical constituents of *Bacopa monnieri*. *Chem Nat Compd.* 2007;43(3):355-7. doi: 10.1007/s10600-007-0133-y.

30. Chekuri S, Lingfa L, Panjala S, Bindu KCS, Anupalli RR. *Acalypha indica* L. - an Important Medicinal Plant: A Brief Review of Its Pharmacological Properties and Restorative Potential. *Eur J Med Plants.* 2020 (July):1-10. doi: 10.9734/ejmp/2020/v3i11130294.

31. Nag A, Anoop M, Sharma K, Verma K. *Acalypha indica* L.. An Important Medicinal Plant with antimicrobial agents: a review. *Int J Res Anal Rev.* 2018;5(4):304-9.

32. Chakre MB, Gaikwad PN, Medpalli KJ, Birru SB. Centre W, biotechnology F. Medicinal properties of *Calotropis gigantea*. *Int J Res Anal Rev.* 2019;6(1):175-81.

33. Biswas TK, Srikanta Pandit S, Shrabana C. Regulation of herbal medicinal products. *Pharmaceutical journal Mukherjee PH, P, editors.* Vol. 282; 2009. p. 228-40.

34. Deshmukh PT, Fernandes J, Atul A, Toppo E. Wound healing activity of *Calotropis gigantea* root bark in rats. *J Ethnopharmacol.* 2009;125(1):178-81. doi: 10.1016/j.jep.2009.06.007, PMID 19539020.

35. Gupta A, Naraniwal M, Kothari V. Modern extraction methods for preparation of bioactive plant extracts. *Int J Appl Nat Sci.* 2012;1(1):8-26.

36. Clinical and Laboratory Standards Institute. M07-A10: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically [internet]. Vol. 35(2). p. 1-87; 2015. Available from: <http://www.clsi.org> [cited 14/9/2022].

37. Ngadino SA, Setiawan, Koerniasari, Ernawati, Sudjarwo SA. Evaluation of antimycobacterial activity of *Curcuma xanthorrhiza* ethanolic extract against *Mycobacterium tuberculosis* H37Rv in vitro. *Vet World.* 2018;11(3):368-72. doi: 10.14202/vetworld.2018.368-372, PMID 29657431.

38. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc.* 2008;3(2):163-75. doi: 10.1038/nprot.2007.521, PMID 18274517.

39. Kebede T, Gadisa E, Tufa A. Antimicrobial activities evaluation and phytochemical screening of some selected medicinal plants: A possible alternative in the treatment of multidrug-resistant microbes. *PLOS ONE.* 2021;16(3):e0249253. doi: 10.1371/journal.pone.0249253, PMID 33770121.

40. Famuyide IM, Aro AO, Fasina FO, Eloff JN, McGaw LJ. Antibacterial and antibiofilm activity of acetone leaf extracts of nine under-investigated South African *Eugenia* and *Syzygium* (Myrtaceae) species and their selectivity indices. *BMC Complement Altern Med.* 2019;19(1):141. doi: 10.1186/s12906-019-2547-z, PMID 31221162.

41. Ahmed SO, Zedan HH, Ibrahim YM. Quorum sensing inhibitory effect of bergamot oil and aspidosperma extract against *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. *Arch Microbiol*. 2021;203(7):4663-75. doi: 10.1007/s00203-021-02455-8, PMID 34175964.
42. Jantorn P, Heemmamad H, Soimala T, Indoung S, Saising J, Chokpaisarn J, et al. Antibiotic resistance profile and biofilm production of *staphylococcus pseudintermedius* isolated from dogs in Thailand. *Pharmaceutics (Basel)*. 2021;14(6). doi: 10.3390/ph14060592, PMID 34203050.
43. Che CT, Wang ZJ, Chow MSS, Lam CWK. Herb-herb combination for therapeutic enhancement and advancement: theory, practice and future perspectives. *Molecules*. 2013;18(5):5125-41. doi: 10.3390/molecules1805125, PMID 23644978.
44. Dev SK, Choudhury PK, Srivastava R, Sharma M. Antimicrobial, anti-inflammatory and wound healing activity of polyherbal formulation. *Biomed Pharmacother*. 2019;111:555-67. doi: 10.1016/j.biopha.2018.12.075, PMID 30597309.
45. Hotterbeekx A, Kumar-Singh S, Goossens H, Malhotra-Kumar S. In vivo and in vitro interactions between *Pseudomonas aeruginosa* and *Staphylococcus* spp. *Front Cell Infect Microbiol*. 2017;7:106. doi: 10.3389/fcimb.2017.00106, PMID 28421166.
46. Patel P, Joshi C, Kothari V. Anti-pathogenic efficacy of a polyherbal wound-care formulation (Herboheal) against *Staphylococcus aureus*, and identifying its molecular targets. *Infect Disord Drug Targets*. 2018;18. doi: 10.2174/1871526518666181022112552.
47. Doğan Ş, Gökalsın B, Şenkardeş İ, Doğan A, Sesal NC. Anti-quorum sensing and anti-biofilm activities of *Hypericum perforatum* extracts against *Pseudomonas aeruginosa*. *J Ethnopharmacol J*. 2019;235:293-300.
48. Alves TF, Chaud MV, Grotto D, Jozala AF, Pandit R, Rai M, et al. Association of silver nanoparticles and curcumin solid dispersion: antimicrobial and antioxidant properties. *AAPS PharmSciTech*. 2018;19(1):225-31. doi: 10.1208/s12249-017-0832-z, PMID 28681332.
49. McKenna SR, Latenser BA, Jones LM, Barrette RR, Sherman HF, Varcelotti JR. Serious silver sulphadiazine and mafenide acetate dermatitis. *Burns*. 1995;21(4):310-2. doi: 10.1016/0305-4179(94)00023-q, PMID 7662136.
50. Ibrahim YM, Abouwarda AM, Nasr T, Omar FA, Bondock S. Antibacterial and anti-quorum sensing activities of a substituted thiazole derivative against methicillin-resistant *Staphylococcus aureus* and other multidrug-resistant bacteria. *Microb Pathog*. 2020;149:104500. doi: 10.1016/j.micpath.2020.104500, PMID 32926996.
51. Hrynyshyn A, Simões M, Borges A. Biofilms in surgical site infections: recent advances and novel prevention and eradication strategies. *Antibiotics (Basel)*. 2022;11(1). doi: 10.3390/antibiotics11010069, PMID 35052946.
52. Lebeaux D, Ghigo JM, Beloin C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol Mol Biol Rev*. 2014;78(3):510-43. doi: 10.1128/MMBR.00013-14, PMID 25184564.
53. Moradi F, Hadi N, Bazargani A. Evaluation of quorum-sensing inhibitory effects of extracts of three traditional medicine plants with known antibacterial properties. *New Microbes New Infect*. 2020;38:100769. doi: 10.1016/j.nmni.2020.100769, PMID 33194208.
54. Jose D, Lekshmi N, Goel AK, Kumar RA, Thomas S. Development of a novel herbal formulation to inhibit biofilm formation in toxigenic *Vibrio cholerae*. *J Food Prot*. 2017;80(11):1933-40. doi: 10.4315/0362-028X.JFP-17-091, PMID 29053421.
55. Singh D, Sharma D, Agarwal V. Screening of antimicrobial, anti-biofilm activity, and cytotoxicity analysis of a designed polyherbal formulation against shigellosis. *J Ayurveda Integr Med*. 2021;12(4):601-6. doi: 10.1016/j.jaim.2021.06.007, PMID 34772585.
56. Saito ST, Trentin Dda S, Macedo AJ, Pungartnik C, Gosmann G, Silveira Jde D et al.. Bioguided Fractionation Shows *Cassia alata* Extract to Inhibit *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* Growth and Biofilm Formation. *Evid Based Complement Alternat Med*. 2012;2012:867103. doi: 10.1155/2012/867103, PMID 22548121.
57. Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM. Microscopic and physiologic evidence for biofilm-associated wound colonization *in vivo*. *Wound Repair Regen*. 2008;16(1):23-9. doi: 10.1111/j.1524-475X.2007.00303.x, PMID 18211576.