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Research Article

BIOFILM INHIBITION POTENTIAL OF AQUEOUS LEAF EXTRACT OF *SARACA ASOCA* LINN. AGAINST *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

About 95-99% of microorganisms exist in nature as biofilms. The homogenous or heterogeneous communities of pathogenic bacteria constituting microbial biofilms exert anti-microbial resistance. Several natural anti-biofilm agents have been recognized, like phytochemicals, biosurfactants, anti-microbial peptides, and microbial enzymes. The present study investigates the effect of aqueous extract of *Saraca asoca* Linn. on the *in vitro* formation of *pseudomonas aeruginosa* biofilm. The quantitative assessment of biofilm formation was done by crystal violet staining assay. Various factors such as temperature, pH, and concentration were optimized for enhanced effect in inhibiting the biofilm by aq. *Saraca asoca* leaves extract. Aq. *Saraca asoca* leaves extract was characterized using GC-MS analysis. Brine shrimp lethality bioassay was also carried out to investigate the cytotoxicity of aq. extract. *Saraca asoca* was observed as a potential source of anti-biofilm molecules and can be further harnessed to maximum potential.

Keywords: Biofilm, *Saraca asoca*, Anti-microbial resistance, GC-MS, Crystal violet staining, Brine shrimp lethality assay.

1. INTRODUCTION

Anti-microbial resistance (AMR) has become a global concern as resistance is reported within a wide range of infectious agents. World Health Organization has expressed alarm to a fast-approaching post-antibiotic era, where common infections and minor injuries could lead to mortality. Anti-microbial resistance has been observed across various infectious agents like bacteria, fungi, viruses and parasites, thus jeopardising the effective prevention and treatment of infectious diseases. World Health Organization states that bacteria responsible for common ailments have a high degree of resistance, and AMR emerges as a public health threat of the 21st century [1].

Tolerance to antibiotics, host defense systems and other external stresses has been observed as a common phenomenon in microbial biofilms. Microbial consortium colonised on solid surfaces with the aid of self-synthesized extracellular polymeric substances are called biofilms. Biofilm development is a well-synchronized event involving adsorption of macro and micro molecules onto solid surfaces, bacterial adhesion and subsequent release of extracellular polymeric substances followed by colony formation and biofilm maturation [2]. Biofilm matrix protects microbial communities from antibiotics and harsh environments with altered pH, osmolarity, nutrients scarcity, mechanical and shear forces [3]. The development of biofilms on medical implants such as sutures, catheters and dental implants is widespread and leads to pathological conditions. Nosocomial infections have been reported to be the fourth leading cause of death in the United States, and about 65% of these infections are due to biofilms on implanted devices. Bacteria in biofilm cause chronic infections [4] characterized by persistent inflammation and tissue damage [5]. Bacterial biofilms are also notorious for causing human health problems like cavities, periodontitis, endocarditis and prostatitis [6].

About 95-99% of microorganisms exist in nature as biofilms [7]. In contrast to colonies, biofilms show immune response and pathological persistence [8]. The homogenous or heterogeneous communities of pathogenic bacteria constituting microbial biofilms exert AMR via three mechanisms. Firstly, the entry of

antibiotics into the innermost microbial community is inhibited by the thick layer of the extracellular biofilm matrix. The second mechanism involves sequestering antibiotics by the biofilm components, and finally, the metabolically inactive microbial community inside the biofilm poses resistance to antibiotics.

Biofilms exhibit different mechanisms of tolerance on various anti-microbial agents. Several studies have reported the AMR of biofilms against various anti-biotics. Antibiotics like tobramycin, ciprofloxacin, and tetracycline are effective on metabolically active bacteria present in the outer region but possess no action on bacteria residing in the interior location. However, other anti-microbials like chlorhexidine, SDS, colistin, and EDTA are effective in internally locating bacteria but ineffective on actively growing bacteria on the outside [9]. β-lactam antibiotics are partially diffusible into biofilms, and they are reported to have no action on the metabolically inactive cells. Similarly, quinolones and aminoglycosides have impaired action on biofilms [10]. The lack of functionality of conventional antibiotic therapies indicate that biofilm treatments need auxiliary up-gradation. Hence, there is a constant need for new bioactive lead molecules with the antibiofilm property.

Several natural anti-biofilm agents have been recognized, and studies have been carried out to elucidate their mechanism of action. Phytochemicals, biosurfactants, anti-microbial peptides, and microbial enzymes have been on the top of the list with the anti-biofilm property. Phytochemicals include phenolics, essential oils, terpenoids, lectins, alkaloids, poly-peptides, and polyacetylenes with diverse mechanisms of action like substrate deprivation, membrane disruption, binding to adhesin complex and cell wall; protein binding; interaction with eukaryotic DNA etc. On the other hand, bio-surfactants exert their anti-biofilm property via decreased cell surface hydro-phobicity, membrane disruption, and inhibited electron transport chain, thus restricting cellular energy demand. Anti-microbial peptides have demand in the treatment of both fungal and bacterial biofilms [11]. The present study investigates the effect of aqueous extract of *S. asoca* Linn. on the *in vitro* formation of *Pseudomonas aeruginosa* biofilm.

2. MATERIAL AND METHODS

2.1. Collection of bacterial strains

Pseudomonas aeruginosa strains were obtained from Marian centre for advanced research, St. Mary's College, Thrissur. The bacteria were subcultured on nutrient agar and stored at 30°C until required for the study.

2.2. Collection and extraction of *Saraca asoca* Linn. Leaves

Fresh leaves of *Saraca asoca* Linn. were collected from the Thrissur locality of Kerala, India. The plant was identified and authenticated by Dr Sr. Meena K Cheruvathur, Botanist, Department of Botany, St. Mary's College, Thrissur and deposited in the herbarium with an accession number SMC-011. The plant material was shade dried at ambient temperature, finely powdered and stored for future use.

The crude aqueous extract of leaves of *S. asoca* Linn. was prepared by the hot decoction method. Powdered plant material was boiled in 250 ml of distilled water for 30 minutes until the sample was reduced to 50 ml crude extract, cooled to 37°C, filtered using Whatman filter paper No: 1 and collected for the experiments.

2.3. Chemicals and reagents

Gentamicin, crystal violet, sodium acetate, acetic acid, disodium hydrogen phosphate, sodium chloride and, in some formulations, potassium chloride and potassium dihydrogen phosphate, cefepime and all other reagents used in this study were of analytical reagent grades from Merck India Pvt. Ltd.

2.4. Screening of bacterial biofilm inhibition

Aq. extract of *S. asoca* Linn. leaves were evaluated for their efficiency in preventing initial cell attachment through the biofilm inhibition assay as per the protocol of Sandasi M *et al.*, 2008 [12]. Briefly, 100 µl of standardized concentration of *pseudomonas aeruginosa* culture with an OD value of 0.02 at 560nm was added into 96 well microtitre plates and incubated at 37°C for 4 hrs without shaking. The plates were removed, and 100 µl of plant extracts at a 2mg/ml concentration were added in twelve replicates to a final concentration of 1 mg/ml and further incubated at 37°C for 24 hrs without agitation. Gentamicin (Virbac) served as the positive control, while acetone and sterile distilled water served as negative controls. The biomass was quantified using the modified crystal violet staining method [13].

2.5. Crystal violet staining assay

Crystal violet staining assay was done as per the protocol of Djordjevic *et al.*, 2002 [13] with some modifications [12]. Briefly, 96-well microtitre plates were washed five times with sterile distilled water, air-dried and subsequently oven-dried at 60°C for 45 min. The wells were then stained with 100 µl of 1% crystal violet and incubated at room temperature for 15 min.

The plates were further washed thrice with sterile distilled water to remove the unabsorbed stain, and at this point, biofilms could be visualized as purple rings at the side of the wells.

The semi-quantitative assessment of biofilm formation was done by adding 125µl of ethanol to destain the wells. 100 µl of the destaining solution was transferred to a new sterile plate, and absorption was measured at 590 nm in a microplate reader (BioTek Synergy). The mean absorbance of the samples was calculated, and percentage inhibition of biofilm was determined using the equation.

Percentage (%) inhibition = $\{(OD_{\text{Negative control}} - OD_{\text{Positive control}}) / OD_{\text{Negative control}}\} X100$

2.6. Optimising the effect of limiting factors in biofilm inhibition

Various factors such as temperature, pH, and concentration were optimized for enhanced effect in inhibiting the biofilm by aq. *Saraca asoca* leaves extract. Varying concentration of aq. plant extract ranging from 0-40 mg/ml was evaluated. The test tubes were exposed in groups of five for 24 to 48 hours to 10 ml of each concentration of *Saraca asoca* leaf extract. Similarly, the effect of pH was analyzed by varying pH from 5 to 9 at a constant concentration (20mg/ml) of *Saraca asoca* leaf extract. Three different buffers maintained pH; acetate (3.6-5.6), phosphate (5.8-7.4), bicarbonate (9.2-10.6). The effect of temperature was also studied by experimenting at a range of 20-60°C.

2.7. Characterisation of aq. *Saraca asoca* leaves extract using GC-MS analysis

The bioactive constituents present in aq. extract of *Saraca asoca* leaves was determined by Gas Chromatography (Agilent 6890 series) equipped with HP-5MS column mass spectrometer operated at an initial column temperature of 30°C and heated up to 300°C/5min. Chromatographic conditions were the flow of 1.0 ml /min of high purity helium as the carrier gas in split mode. The identification of the compounds in spectra was made based on retention time and integral area of peaks. The similarity of compounds matched with > 70% is listed based on NIST library search.

2.8. Determination of cytotoxicity by brine shrimp lethality assay

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of aq. extract of *Saraca asoca* leaves. *Artemia salina* was hatched using brine shrimp eggs in an IL conical-shaped vessel, filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48h. After hatching, active nauplii free from eggshells were collected from the brighter portion of the hatching chamber and used for the assay.

Tennauplii were collected using a pasteur pipette and placed in a test tube containing 2 ml of brine solution. To this, 0.5ml of plant extract was added and incubated at room temperature for 24 hrs under the light. The live larvae were counted. 0.5 ml distilled water was kept as the control. The per cent of the lethality of the naupli was calculated as:

Mortality rate=(No. of dead Naupli/total No. of Naupli)×100

3. RESULTS AND DISCUSSION

3.1. Collection and extraction of plant material

The collected plant identified as *Saraca asoca* Linn. was dried and preserved as herbarium with Voucher No: SMC011 in the herbarium collection, Department of Botany, St. Mary's College, Thrissur. Extractof *Saraca asoca* leaves prepared by hot decoction method was filtered and heated moderately to achieve a dried formof extract.

3.2. Screening of bacterial biofilm inhibition

The leaves extracts of *S. asoca* Linn. showed a positive anti-adherence effect when subjected to the *P. aeruginosa* biofilm formed on the glass surface. It was observed to inhibit the biofilm formation on the glass surface and developed turbidity due to bacterial strains.

3.3. Optimising the effect of limiting factors in biofilm inhibition

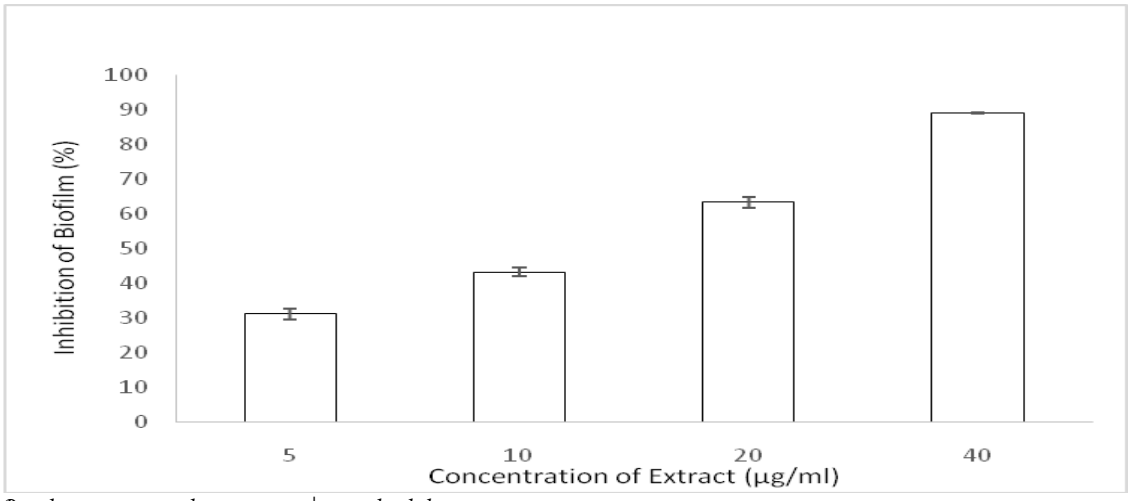
3.3.1. Analysis of the effect of concentration

To understand the effects of *Saraca asoca* Linn. leaf extract concentration, the bacterial biomass was treated with varying concentrations of extract ranging from 5 to 40 µg/ml. The results indicated an increased inhibition in the formation of biofilm with an increased concentration of the extract (Fig: 1). The maximum inhibition was obtained at a concentration of 40µg/ml.

3.3.2. Analysis of the effect of temperature

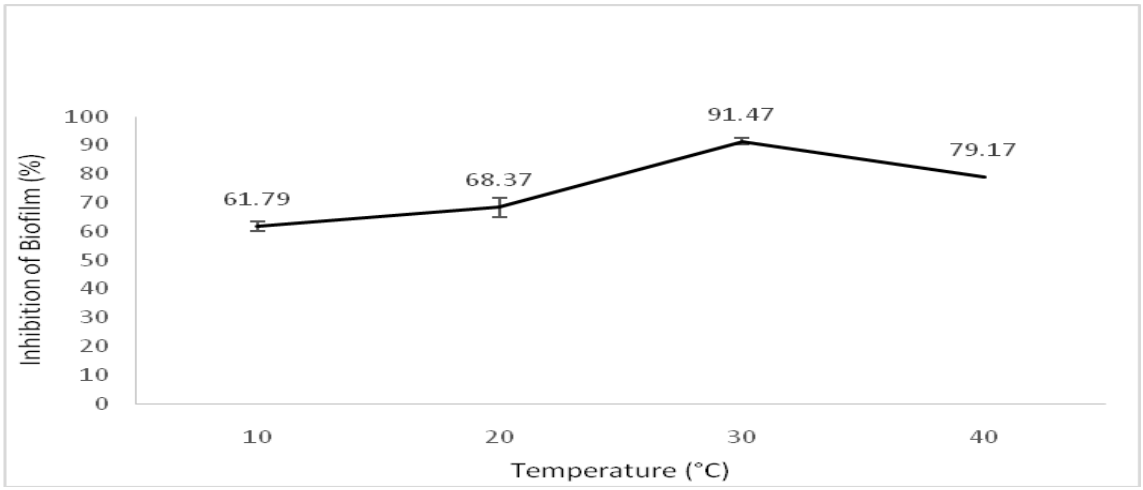
To understand the effect of temperature on inhibition of *Pseudomonas aeruginosa* biofilm formation by *asoca* Linn. leaf extract, the treated bacterial biomass was incubated at varying temperatures ranging from 20°C to 50°C. The results indicated an increased inhibition

in biofilm formation with the increase in temperature up to 40°C. The inhibitory action was reversed with a further increase in the temperatures (Fig: 2). The optimum temperature for the activity was 40°C with 91.47±1.157 % of inhibition.



Results are expressed as average ± standard deviation

Fig. 1: Effect of concentration of *Saraca asoca* Linn. leaf extract on the inhibition of *Pseudomonas aeruginosa* biofilm formation.



Results are expressed as average ± standard deviation

Fig 2: Effect of temperature on the inhibition of *Pseudomonas aeruginosa* biofilm formation by treating with *Saraca asoca* Linn. leaf extract

3.3.3. Analysis of the effect of pH

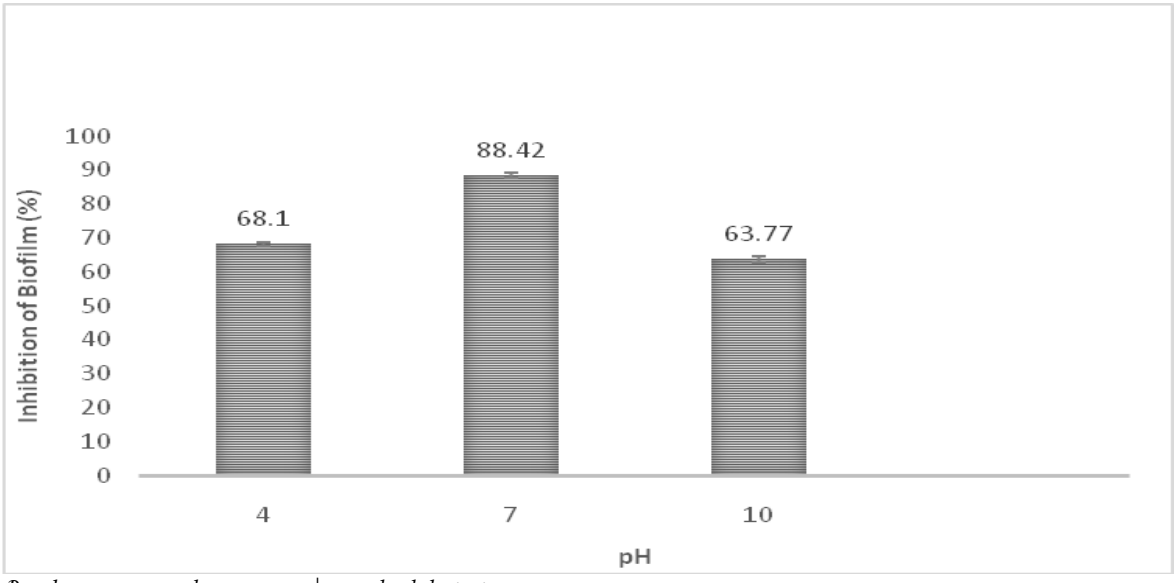
To understand the effect of pH on inhibition of *Pseudomonas aeruginosa* biofilm formation by *Saraca asoca* Linn. leaf extract, the treated bacterial biomass was incubated at varying pH ranging from 4 to 10. The results indicated an increased inhibition in the formation of biofilm with an increase in pH up to 7. The inhibitory action was reversed with a further increase in the pH (Fig: 3). Hence, the optimum pH for the activity was observed to be 7 with 88.42±0.56 % of inhibition.

3.4. Characterisation of the bioactive fragment using GC-MS analysis

GC-MS analysis of *Saraca asoca* Linn. was carried out to determine the possible chemical compounds in it. It was stated that water-soluble plant compounds had been determined by liquid chromatography, while GC-MS has been used to analyze the volatile substances [14]. GC-MS chromatogram had revealed various peaks indicating different phytochemical compounds in the extract (Fig.4). The spectra disclosed the highest

relative abundance of organic molecules like Hematoporphyrin and Anethole occurring at about 4.27 and 13.33 min of the chromatogram. Hematoporphyrin was the most abundant photo component observed, and it could be the most contributing to the potential activity of the extract.

Hematoporphyrin is a porphyrin prepared from hemin (Fig 5). It is a derivative of protoporphyrin IX, where the two vinyl groups have been hydrated (converted to alcohols). It is a deeply coloured solid that is usually encountered as a solution. Its chemical structure was determined in 1990.



Results are expressed as average ± standard deviation

Fig 3: Effect of pH on the inhibition of *Pseudomonas aeruginosa* biofilm formation by treating with *Saraca asoca*Linn. leaf extract

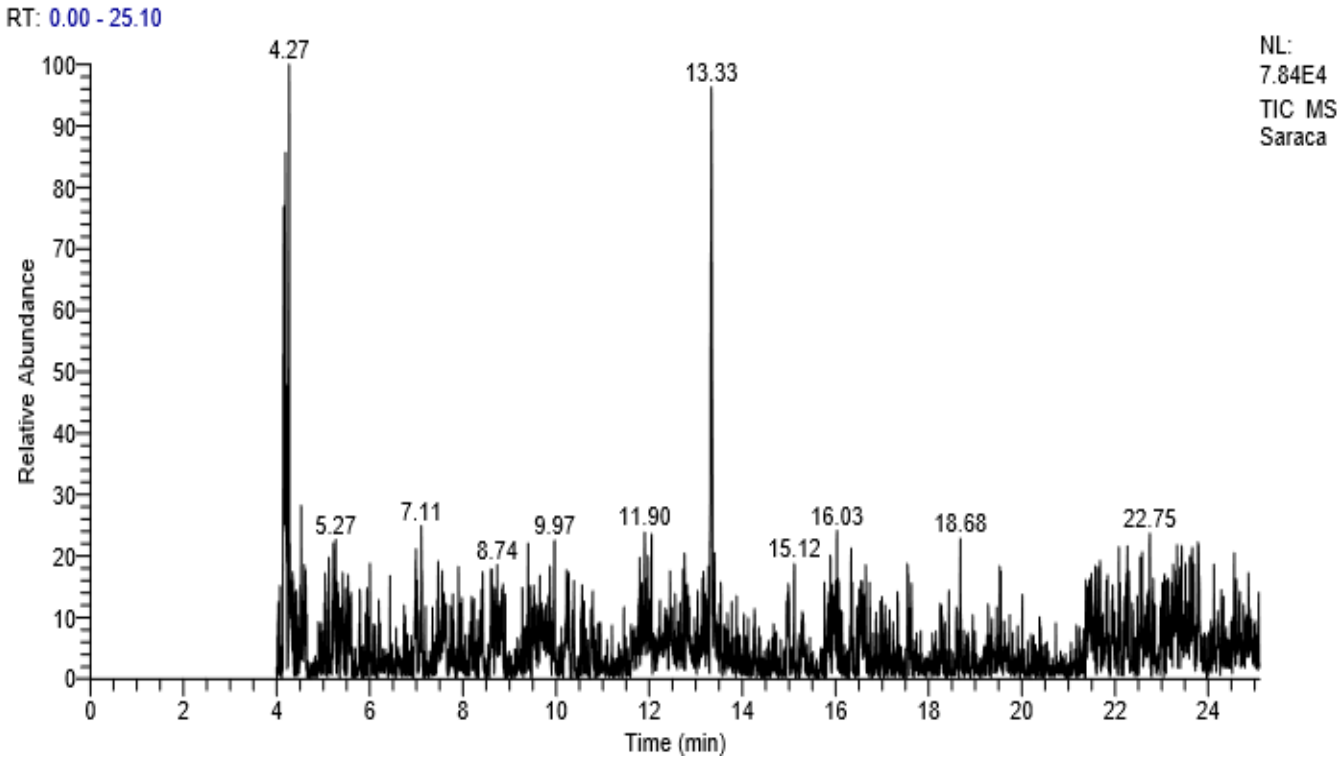


Fig. 4: GC-MS Chromatogram of aqueous extract of *Saraca asoca* Linn. Leaves

Fig. 5: Structure of Hematoporphyrin

3.5. Determination of cytotoxicity by brine shrimp lethality assay

Toxicity testing was done by exposing the nauplii to the plant extract, and cytotoxicity was calculated. It was observed that with control, the mortality rate was 0% and all naupli were surviving and healthy. However, when treated with the plant extract, 1.11±0.033% naupli were dead.

3.6. Discussion

Pseudomonas aeruginosa is the leading cause of nosocomial infections in immune-compromised patients, including patients with malignancy, cystic fibrosis, burns, etc. Due to many pathogenic factors, this bacterium shows high resistance to the most common antibiotics. The plant extract of *Saraca asoca* was found to significantly inhibit biofilm formation. The active plant extracts showed a positive anti adherence effect on the *pseudomonas aeruginosa* biofilm formation on the glass surface. Further, analyzing factors such as temperature, concentration and pH revealed that biofilm inhibition is dependent on these parameters.

The significant decrease observed in the amount of biofilm at high temperatures could be a result of the detachment of matured biofilm [6]. In contrast, biofilm detachment was not observed at low temperatures (Fig. 2). It has been suggested that the increased hydrophobicity at high temperatures (37°C) may enhance the initial cell adherence, contributing to a higher biofilm density. Further, it is not surprising that bacteria form more biofilm at 37°C than in other temperatures tested since, at this temperature, bacterium grow best and consequently, the cells number increase. As a result, the cell mass facilitates the sedimentation, resulting in a higher initial attachment.

While considering the effect of concentration of plant extracts on biofilm formation, the best biofilm reduction is observed in a higher concentration of the extracts (Fig. 1). Maximum biofilm inhibition shows at pH 7 (Fig. 3). Characterization of the bioactive compound using GC-MS analysis reveals the compounds present in the plant extract and the possible compound to inhibit the biofilm formation. *Artemia salina* bioassay was used to evaluate the toxicity of plant extracts and has the advantage of being inexpensive, reliable and reproducible. Using the plant extracts of *Saraca asoca*, the survival percentage of *A. salina* was unaffected.

The excellent ability of the plant extracts to interfere with the initial stage of biofilm formation of the bacterial isolates may be attributed to interfering with forces such as Brownian, sedimentation, VanderWaals and electrostatic interaction forces that favour the deposition and adherence of bacteria to surfaces [15]. Also, since specific organic and inorganic molecules and other nutrients are essential for cell growth and hence cell adhesion [12], the plant extracts may inhibit the availability of nutrients. The active plant extracts may hold promise for reducing colonisation surfaces and various epithelial of the body, thereby preventing infections.

4. CONCLUSION

Biofilm and multidrug resistance have been identified as virulence factors of great magnitude in clinical infections. Due to the increase in complexity of most microbial infections and the resistance to conventional therapy, researchers have been prompted to identify alternatives for treating infections. Plant extracts and other biologically active compounds isolated from plants have gained widespread interest in this regard as they have been known to cure diseases and illnesses since ancient times. The present study put forward *Saraca asoca* is a potential source of anti-biofilm molecules that needs to be harnessed to maximum potential.

5. ACKNOWLEDGEMENT

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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