



CLINICAL ISOLATES OF STAPHYLOCOCCUS AUREUS (R.): BIOFILM CHARACTERISATION AND INHIBITORY ASSAY USING HAMAMELIS VIRGINIANA (L.).

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ABSTRACT

In this study, 75 clinical isolates of *Staphylococcus* species which included 45 isolates of *S.aureus* and 30 isolates of other *Staphylococcus* species were initially screened for biofilm production. The prevalence of Multidrug Resistant *Staphylococcus aureus* (MDRSA) and its biofilm production efficacy was assayed. Further biofilm inhibition using *Hamamelis virginiana* (Witch Hazel) extract was analysed. The findings showed that the clinical isolates are multidrug resistant. Antibacterial activity of *Hamamelis virginiana* (Witch Hazel) extract has been proved in this study. The infections caused by *Staphylococcus aureus* can be minimized by using this extract formulation as an alternative treatment instead of antibiotics. The minimum inhibitory concentration (MIC) was found to be at 10^{-6} dilution (0.0001 μ l/ml) of *Hamamelis virginiana* extract against *Staphylococcus aureus*. From various experiments, the inhibitory activity of *Hamamelis virginiana* extract was proved and from the recorded results, the potential use of the extract as an inhibitory agent in controlling Staphylococcal infections is suggested.

KEYWORDS: Biofilms, *Staphylococcus aureus*, Biofilm inhibition, *Hamamelis virginiana*



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INTRODUCTION

Staphylococci are Gram-positive bacteria, with diameters of 0.5 – 1.5 μm and characterized by individual cocci, which divide in more than one plane to form grape-like clusters. . The species are non-motile, non-spore forming facultative anaerobes that grow by aerobic respiration or by fermentation. Pathogenic *staphylococci* are commonly identified by their ability to produce coagulase, and thus clot blood¹. This distinguishes the coagulase positive strains, *S. aureus* (a human pathogen), and *S. intermedius* and *S. hyicus* (two animal pathogens), from the other staphylococcal species such as *S. epidermidis*, that are coagulase-negative (CoNS). *Staphylococcus aureus* is a major pathogen of increasing importance due to the rise in antibiotic resistance². *Staphylococcus aureus* and *Staphylococcus epidermidis* are the two most characterized and studied strains. *Staphylococci* are also a common cause of infections related to bacterial biofilm formation on implanted devices. Infections may result in longer hospitalization time, or need for surgery, and they can even cause death³. Biofilms are highly resistant to antibiotic treatment³⁻⁷. The spread of drug-resistant strains of *Staphylococci* and ineffectiveness of treatments in cases of biofilm-related infections underscore the necessity to find new modes of prevention and effective alternatives to antibiotic treatment. A novel way would be to interfere with bacterial cell-to-cell communication that leads to virulence. The excessive use of antibiotics has led to the emergence of multiple drug resistant *S. aureus* strains². *Staphylococci* are very capable of evolving resistance to the commonly used antimicrobial agents, such as, erythromycin⁸, ampicillin⁹, and tetracycline¹⁰. In most cases, resistance to antibiotics is coded for by genes carried on plasmids, accounting for the rapid spread of resistant bacteria¹¹. Biofilm helps the bacteria to form stable communities of protection rather than live as free cells¹². Biofilms can resist antibiotic concentration 10 – 10,000 folds higher than those required to inhibit the growth of free floating bacteria¹³. These infections are generally associated with

the use of catheters and other medical devices. *Staphylococcus aureus* cause disease through the production of virulence factors. *S. aureus* are part of our normal flora, but they can cause fatal diseases as a result of the expression of multiple virulence factors. These factors include adhesins, exotoxins, enterotoxins, hemolysins and leukocidin, as well as proteases that enable bacteria to spread within the host^{2, 14, 15}. Strains defective in their ability to form a biofilm or produce toxins show diminished virulence; suggesting that a novel approach for therapy development would be to interfere with production of virulence factors or to inhibit biofilm formation¹⁶.

Quorum sensing (QS) refers to the molecular mechanism of regulation of gene expression in response to fluctuations in cell density. 2,5-di-O-galloyl-D-hamamelose (Hamamelitannin) has been discovered as a nonpeptide analog of RNA inhibiting peptide (RIP) that effectively prevents biofilm formation and RNAPIII production in vitro as well as device-associated infections in vivo. Instead of killing bacteria, as is done with antibiotics, *Staphylococci* are rendered harmless by inhibiting their quorum-sensing mechanisms¹⁷. They have previously shown that the peptide RIP acts as an inhibitor of quorum sensing¹⁸. Studies have shown that hamamelitannin is analogous to RIP. Hamamelitannin is considered a polyphenol and polyphenols have been shown to have multiple activities. The bioactive component belongs to the family of tannins, which are plant polyphenols that are used in tanning animal hides into leather. It is a natural product found in bark and the leaves of *Hamamelis virginiana* (Witch hazel), a deciduous shrub native to damp woods in eastern North America and Canada. Witch hazel extracts were used by Native Americans for pain relief, colds, and fever. They are currently used in skin care products and in dermatological treatment of sunburn, irritated skin and atopic eczema¹⁹ as well as to promote wound healing via anti-inflammatory effects²⁰. The present study embarks to prove the biofilm

inhibition and bactericidal activity of Hamamelitannin against some clinically isolated multi drug resistant strains of *Staphylococci*.

MATERIALS AND METHODS

Bacterial isolates

The investigation was carried out on 75 clinical isolates isolated from nasal swabs, pus, wound, throat swab and urine specimens. The isolates were identified by their cultural characteristics, microscopic appearance in Gram stained preparations, coagulase test with rabbit plasma by tube test. Hemolysis was detected on sheep blood agar after 20 h incubation at 35°C.

Identification of *Staphylococcus aureus* - Vitek Automated system was used for identification and antibiotic sensitivity of *Staphylococcus aureus*²¹.

Antibiotic assay

Antibiotic susceptibility test (Agar diffusion method) of biofilm producing bacteria was done on Mueller Hinton agar²² using antibiotic discs of a wide spectrum of clinically used antibiotics: Imipenem, Meropenem, Tobramycin, Nalidixic acid, piperacillin, Cefaperaxone/Sublactam, Cefixime, Cefuroxime, Norfloxacin, Netilmycin, Azithromycin, Vancomycin, Oxacillin, Chloramphenicol, Ofloxacin, Methicillin, Erythromycin, Streptomycin, Gentamicin, Clindamycin, Penicillin, Levofloxacin, Linezolid, Ciprofloxacin, Doxycycline, Rifampicin, Sulfamethoxazole, Moxifloxacin, Quinopristin & Tetracycline. Biofilm characterization; three methods were adopted namely Congo red agar method²³, Tube method²⁴ and Microtiter plate method²⁵. Biofilm formation was classified and interpreted based on the method proposed by Mathur et al²⁶ (Table 1)

Table 1
Classification of *Staphylococcus aureus* biofilm based on OD values

Mean OD value	Biofilm formation
< 0.120	Non/weak
0.120-0.240	Moderate
>0.240	Strong

The alcoholic extract of *Hamamelis virginiana* was subjected to various estimation methods to estimate the amount of Tannins, Flavonoids, Total phenols and Gallic acid respectively (Table 2).

Table 2
Characterization of *Hamamelis virginiana* alcoholic extract.

S.No	Components	Method Adopted
1.	Tannins	Spectrophotometry; Mole S and Waterman PG ²⁷
2.	Flavonoids	Spectrophotometry; Woisky R and Salatino, A ²⁸
3.	Total Phenols	Spectrophotometry; Karen Slinkard and Vernon L. Singleton ²⁹
4.	Gallic acid	HPLC; Pawar et.al ³⁰

Biofilm inhibition assay, Antibacterial activity assay & Bacterial attachment invitro-24hr Bacterial culture (*S. aureus*) in LB broth was used for the assay and the method proposed by Madanahally et al¹⁷ was adopted. 96-well plate was read at OD₆₃₀ nm in an ELISA plate reader (Stat Fax 2100)

Determination of Minimum Inhibitory Concentration (MIC) of crude extract of *Hamamelis virginiana*- An aliquot of serial dilution of the extract (100µl) was prepared in the 96 well microtitre plate containing TSBGlc and MIC was determined using the method proposed by Das et al³¹.

RESULTS

Identification of *Staphylococcus aureus* species

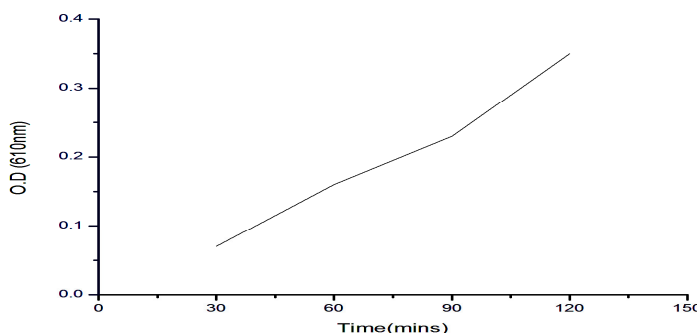
All isolates were initially evaluated by Vitek- 2 with additional tests (coagulase production was detected by aggregation of rabbit plasma by the tube method). Haemolysis was detected on sheep blood agar after 20 h incubation at 35°C. Of the 75 specimens studied, 45 specimens (*Staphylococcus aureus*), 12 specimens (*Staphylococcus epidermidis*), 11 specimens (*Staphylococcus haemolyticus*), 3 specimens

(*Staphylococcus lentus*) and one specimen (Each of *Staphylococcus warneri*, *Staphylococcus sciuri*, *Staphylococcus xylosus* and *Staphylococcus lugdunensis*) were identified.

Determination of Bacterial growth curve

The bacterial growth curve was determined to identify the log or exponential phase of the organism. The generation time was calculated for the test organism and the log phase or exponential phase for the test organism was recorded at 60 minutes after inoculation. (Fig 1).

Figure 1
Growth curve of *Staphylococcus aureus* in Tryptic Soy broth media



Determination of susceptibility (Agar disc diffusion assay)

A wide range of antibiotics (Agar diffusion method) were placed in Mueller-Hinton agar plates to identify multidrug resistant *Staphylococcus aureus*. In each set of experiment, bacterial control tubes showed no growth inhibition. The Susceptibility pattern of isolated *Staphylococcus aureus* with clinically prescribed antibiotic spectrum was identified and the multi drug resistant strains were successfully screened.

Biofilm Characterization

Production of biofilm was detected by three phenotypic methods: Congo red agar, microtitre plate and tube method. Congo red agar method could detect 5 (11%) strong biofilm producers, 28 (62%) moderate biofilm producers, 5 (11%) very low biofilm producers and 6 (13%) non-biofilm producers (Fig 2). Four clinical isolates of *S.aureus* that showed strong, moderate, weak and non- biofilm producer were selected based on the results of Congo red agar method of microtiter plate method (Fig 3). Tube method could detect strong, moderate, weak and non biofilm producer which was identified by visible stained film on the wall & bottom of the tube in positive biofilm formation (Fig 4).

Figure 2
Biofilm characterization by CRA method
A) Positive- Strong biofilm producer and B) Negative- Nonbiofilm producer

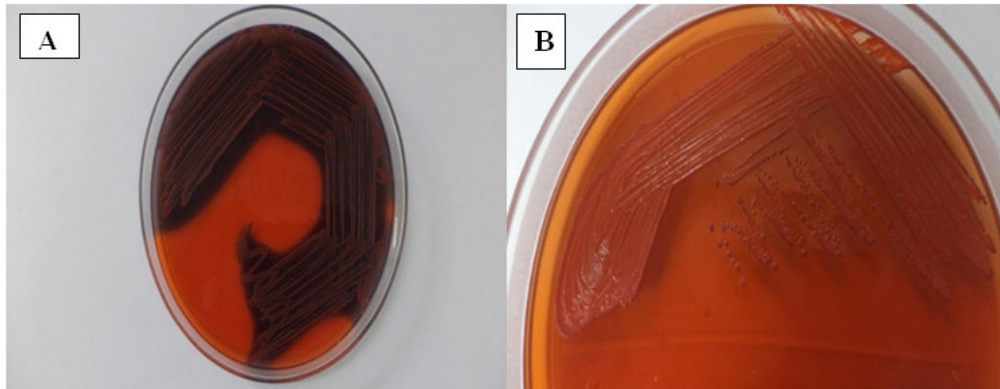


Figure 3
Biofilm characterisation by TCP method: A) Positive- Strong producer, B) Negative- Non biofilm producer, C) Moderate- Intermediate biofilm producer

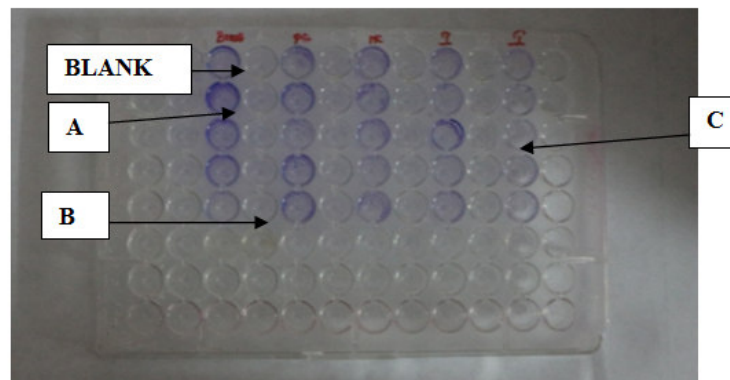


Figure 4
Biofilm characterization by TM
A) Strong biofilm producer(PC), B) Nonbiofilm producer (NC) and C) Intermediate biofilm producer(I)

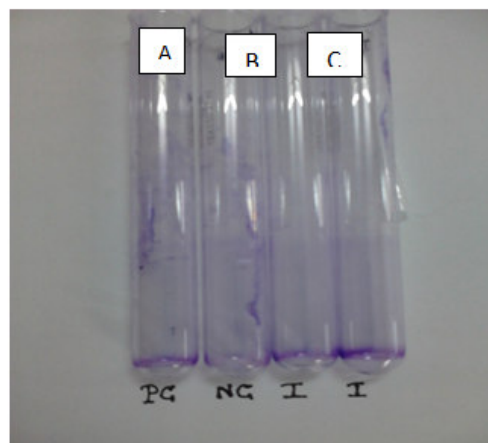
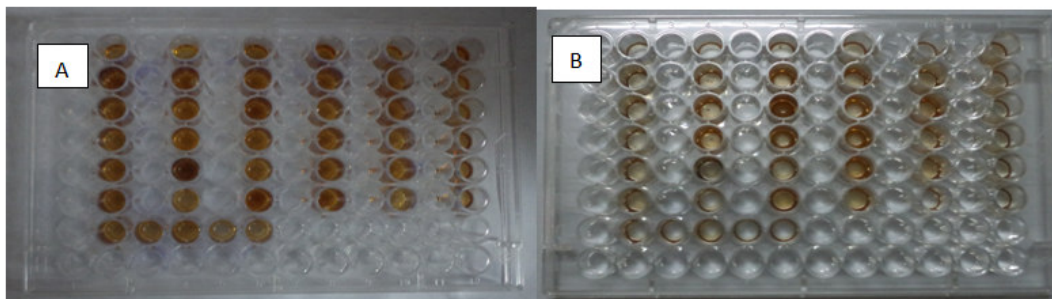


Figure 5

**Inhibition of pre-formed biofilm by *Hamamelis virginiana* Alcoholic extract by TCP method
A- initial (0hr) and B- After incubation(24hr)**



Characterization of alcoholic extract of *Hamamelis virginiana* -TLC analysis of the fraction

The extract was checked with Thin Layer Chromatography (TLC) on analytical plates over Silica gel 60F₂₃₄ (Merck). The solvent system used was Toluene: Methanol: Ethyl acetate: Hexane: Formic acid (1:2:3:3:1). Some spots were observed in vanillin sulphuric acid reagent and UV light at 254nm. UV light (254nm) identifies that secondary metabolite present in the extract are alkaloids and flavonoids. The reports of condensed tannin estimation indicated no specific presence of condensed tannin but the previous estimates show a presence of 10.11% tannin. Therefore, we can conclude that the tannin present is hydrolysable tannin (Hamamelitannin).

UV-VIS Spectrophotometer analysis

The extract was analyzed by UV-VIS spectrophotometer and 0.01ml of sample (*Hamamelis virginiana* extract) was reported to contain the components enlisted in the described percentage-Bitters-0.61%, Gallic

acid-0.89%, Quercetin-Absent, Condensed tannin-Absent, Total tannins-10.11, Flavonoids-14.08 & Total phenols-10.47%.

Biofilm inhibition assay

Hamamelis virginiana extract was used to inhibit biofilm producing strains of *S.aureus*. The assay was done in the preformed biofilm in microtiter plate and inhibition of biofilm growth was observed with addition of extract as shown in (Fig 5).

In Vitro inhibition of cell attachment by *Hamamelis virginiana*

To test the effect of *Hamamelis virginiana* on bacterial attachment in-vitro, *S.aureus* cells incubated with 0.1 ml of bacteria was inoculated in polystyrene 96-well plates with 5 µl of the extract of *Hamamelis virginiana* for 2h at 37°C. Adherent bacteria were stained and OD was determined. The extract *Hamamelis virginiana* showed a positive effect in the reduction of biofilm formation by *Staphylococcus aureus* indicated by the reduction in O.D values recorded at 630nm. (Table 3)

Table 3
Biofilm inhibition profile of *Hamamelis virginiana* extract.

	Control	Isolate- 1	Isolate- 2	Isolate- 3	Isolate- 4	Isolate- 5
OD at 630nm	0.036±0.017	0.033±0.003	0.071±0.044	0.033±0.002	0.048±0.026	0.031±0.006

The *Hamamelis virginiana* extract showed effective degree of antibacterial activities against multidrug resistant strains of *Staphylococci* species. The minimum inhibitory concentration (MIC) of the extract corresponds to 1µg/ml at 10⁻⁶ dilution.

DISCUSSION

Staphylococci are among the most commonly recovered bacteria in the clinical microbiology laboratory. Out of 75 specimens studied- 60 % (*S.aureus*), 16% (*S.epidermidis*), 14.6% (*S. hemolyticus*), 4% (*S. lentus*) and 1.3% (each of *S. warneri*, *S.scuri*, *S.xylosus* and *S.lugdunensis*) were identified. Coagulase test was done to confirm *Staphylococcus aureus* species. Out of 75 isolates studied - 60% *S.aureus* (coagulase positive). Of the remaining 30 clinical *Staphylococcal* isolates-16% (*S. epidermidis*), 15% (*Staphylococcus haemolyticus*) were identified and 5% consisted of a number of other coagulase negative *Staphylococcus* species. In *S. aureus*, the synthesis of surface proteins occurs in early growth and is down-regulated in post-exponential and stationary phase^{32, 33}. The generation time was calculated for the test organism and the initiation of exponential phase for the test organism was found to be in 60 minutes (1hr) unlike 2hrs as reported in previous studies by Madanahally et.al¹⁷. In recent years, *Staphylococcus aureus* have become more resistant to antibiotics to both traditional and synthetic. The development and spread of bacterial strains that are resistant to antibacterial drugs has emerged as a global problem. The result of the study suggests that among the 75 clinical isolates of *Staphylococcus aureus*, 45 were resistant to the following antibiotics: Penicillin, Cefexime, Gentamicin, Norfloxacin, Piperacillin, Ampicillin, Methicillin, Netilmicin, Cefaperaxone & Tazobactam and Nalidixic acid whereas *Staphylococcus epidermidis* were resistant to Penicillin, Cefexime, Methicillin, Azithromycin and Nalidixic acid whereas isolated *S.aureus* strains were resistant to kanamycin and oxacillin³⁴ as well. Biofilm formation is reported to be an important characteristic of all *Staphylococcal* species associated with the infection of biomedical devices^{35- 37}. Biofilm production in *Staphylococci* isolated from other clinical samples are also of clinical significance as biofilm constitutes reservoir of pathogens and are associated with resistance to

antimicrobial agents and chronic infections. The isolates were tested by three in-vitro screening tests for biofilm production namely TCP, TM and CRA methods. It was found that although the formation of biofilm on implanted medical devices is generally associated with coagulase negative *Staphylococci*, *S. aureus* strains are also capable of production of biofilm as reported by the studies of Christensen, Freeman, Kloos WE and Bose S^{1,38- 40}. The CRA method was found to be easier and faster to perform than other phenotypic methods but could probably identify only the strong biofilm producers. It is not a precise method for identification of moderately biofilm producing strains. Hence, it could detect the least number of biofilm producers. In this study, CRA method was performed with 45 isolates only five were found to be strong biofilm producers (Black colonies with dry crystalline consistency), 28 isolates were found to be moderate biofilm producers (Black smooth colonies/red colonies with dry crystalline consistency), 5 isolates were found to be weak biofilm producers (Black colonies were formed in the initial streak region) and 6 isolates were non biofilm producers (Red smooth colonies). Similar trend was reported by Milanov Dubravka⁴¹. Four proto type isolates of strong, intermediate and non biofilm producers each were selected and used for TM & microtiter plate method. The modified microtiter plate method with extended incubation period of 24h with Trypticase soy broth and 1% glucose medium was adopted. This method was claimed superior to other methods by various researchers using Trypticase soy broth without glucose and Brain heart infusion broth with sucrose²⁶.

Tube method detected less number of strong biofilm producers as compared to other two methods. It could discriminate between strong and moderate biofilm producers. However, the interpretation is observer dependent and there are chances of subjective errors. Further it was hard to differentiate between moderate and non-biofilm producers. This is in agreement with previous reports of

Christensen's et al., 1985 and Mathur et al., 2006 and hence cannot be recommended as a general screening test to identify biofilm producing isolates. Biofilm producing strains were found to be more resistant to almost all the groups of antibiotics as compared to biofilm non-producing strains. In TCP method biofilm formation was observed in 3 isolates which was confirmed by CRA method as Strong, intermediate and non biofilm producers. These results are in cocurrence to the observation made by Mathur et al. and Bose et al.^{26, 40}, which reported that 54.19% of *Staphylococci* were biofilm producers whereas Mathur et al., 2006 reported 53.9% of *Staphylococci* as biofilm producers. The bark and leaves of *Hamamelis virginiana* (Witch hazel) of the family *Hamamelidaceae* were used by Native Americans in the treatment of external inflammations. The plant extract has been used to treat skin ulcers, sores, and tumors, to treat colds and cough, as an astringent and blood purifier & eye inflammation, hemorrhoids, bites, stings and skin sores, diarrhea and dysentery. The crude extract of *Hamamelis virginiana* was analysed by UV-VIS spectrophotometer and 0.01ml of sample was reported to contain 10.11% tannin, 10.47% total phenols, and 14.08% of flavonoids which is in accordance to the European medical agency. The leaf contains 3-10% tannins (a mixture of catechins, gallotannins, plus cyanidin and delphinidin type proanthocyanidins), mainly hamamelose; catechins, mainly (+)-catechin, (+)-gallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate; phenolic acids (caffeic and gallic acids); flavonoid galactosides and glucuronides; flavonoids such as kaempherol, quercetin, quercitrin, and isoquercitrin⁴³. Microtiter plate method was adopted for antibacterial activity assay of the extract *Hamamelis virginiana*. To a microtiter plate with preformed biofilm growth, alcoholic extract of *Hamamelis virginiana* was added to screen preformed biofilm inhibition efficacy. The readings recorded at 492nm before and after addition of extract with incubation for 24h indicated a decrease in the OD values (0.186 to 0.051). This substantiates the hypothesis that *Staphylococcus aureus* infections could be

minimized to an extent by using this extract. In order to confirm this antibacterial activity and Bacterial attachment *in vitro* were performed in Luria broth (pH 7.5±0.2). Unlike direct *agr* inhibitors that suppress disease in vivo but enhance biofilm formation in vitro, hamamelitannin down-regulate *agr* expression and biofilm formation. Hamamelitannin, a nonpeptide analog of RNA inhibiting peptide (RIP) effectively prevents biofilm formation and RNAIII production in vitro instead of killing bacteria, as is done with antibiotic. It inhibits staphylococcal virulence by acting as a quorum-sensing inhibitor. Hamamelitannin also inhibit cell attachment in vitro at a minimal effective concentration of <10 nM/1000 bacteria²⁶. In this study the MIC of the extract was found to be at 10⁻⁶ dilution of 1µg/ml. Most importantly, hamamelitannin can be an excellent candidate for inhibition of device-associated infections in vivo. Inhibition of infection is concentration-dependent. These findings may have important and far-reaching benefits for prevention and treatment of nosocomial infections caused by multidrug resistant *S. aureus* and *S. epidermidis* strains.

CONCLUSION

Staphylococcus species is a major pathogen of increasing importance due to antibiotic resistance. They are most often associated with chronic infections of implanted medical devices. They are also a common cause of infections related to bacterial biofilm formation on implanted devices. Biofilms are highly resistant to antibiotic treatment. A novel way to antibiotic treatment is to interfere with bacterial cell-cell communication that leads to virulence. The *Hamamelis virginiana* extract (Witch Hazel family) is a nonpeptide analogue of RIP and inhibits the quorum sensing regulator – RIP. The MIC of *Hamamelis virginiana* was determined. The antibacterial activity of the extract has been proved. The infections caused by *Staphylococcus aureus* can be minimized by using this extract as an alternative treatment against antibiotics. From various experiments, the inhibitory activity of the extract *Hamamelis*

virginiana was studied and from the recorded results, the potential use of the extract as an inhibitory agent was understood. Hence it can be used as a potential inhibitory agent against multi drug resistant strains of *Staphylococcus aureus*.

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