DOI: 10.15415/jptrm.2013.11003

In Vitro Anti-Staphylococcal Activity of Alkaloids from the Leaves of Callistemon Rigidus R.Br.

Charu Gomber and Sanjai Saxena

Department of Biotechnology and Environmental Sciences,
Thapar University, Patiala, INDIA
Email: sanjaibiotech@yahoo.com;
ssaxena@thapar.edu

Abstract

Multidrug resistant *Staphylococcus aureus* poses a severe global threat worldwide due to their prevalence, genomic plasticity and limited therapeutic options being refractory to most antibiotic classes. This necessitates the discovery of new anti-staphylococcal interventions. *Callistemon rigidus* R.Br. (Myrtaceae) has been found to possess antibacterial potential against clinical isolates. Thied me study was a isolate and evaluate the antibacterial alkaloids from the leaves of *Callistemon rigidus* and assess their *in vitro* anti-staphylococcal potential. Alkaloid isolation was carried out by modified method for plant alkaloid extraction. The *in vitro* anti-staphylococcal potential of the alkaloid bioactive fraction was assessed by using micro broth dilution and plate count assay methods. Pus and wound isolates had MIC_{50} of 80 μg/mL and IC_{50} of 27.22 μg/mL respectively. Burn isolates showed MIC_{50} of 320 μg/mL and 13.57 μg/mL respectively. Urine and vaginal isolates exhibited a MIC_{50} of 80 μg/mL and IC_{50} of 13.15 µg/mL respectively. Cefixime had MIC_{50} values was 320 μg/mL, 160 μg/mL and 160 μg/mL for pus and wound, burn, urine and vaginal isolates indicating the refractory behaviour when compared to alkaloid bioactive fraction from the leaves of *Callistemon*. The alkaloid bioactive fraction exhibits immense activity compared to standard antibiotic Cefixime. This work is the first report of alkaloids and their antimicrobial activity from *Callistemon rigidus* leaves. The results suggest further isolation of individual alkaloids from alkaloid bioactive fraction and assessment their anti-staphylococcal activity as leads for development of anti-staphylococcal drugs.

Key Words: *Callistemon rigidus, Staphylococcus aureus, MIC50, alkaloid bioactive fraction, Cefixime.*

Key words: MRSA, *Staphylococcus aureus*, Microbroth dilution assay, MIC, IC_{50}

1 INTRODUCTION

Multidrug resistant *Staphylococcus aureus* has created a critical situation for the clinicians due to their refractory behaviour against the current armamentarium of antimicrobials. Methicillin resistant *Staphylococcus aureus* (MRSA) infections complicate the therapy in the immunocompromised, the elderly, the infants and patients in surgical and burn units (Saxena and Gomber, 2010). Hence there is a need to develop therapeutic alternatives to overcome the refractory behaviour in treating chronic infections caused by these multidrug resistant pathogenic microbes. Plant extracts possess

Journal of Pharmaceutical Technology, Research and Management
Vol. 1
May 2013
pp. 31–42

a variety of specialized chemicals and biochemicals as a response to various environmental stresses like competition for nutrients, space, allelopathic interactions and pathogenesis (Saxena and Kumar, 2000). These specialized chemicals and biochemicals are largely underexplored and possess antimicrobial properties which may be directly or indirectly used in development of new drugs to overcome the multidrug resistant pathogenic microbes.

We have earlier reported the potential antimicrobial activity of crude leaf extract of *Callistemon rigidus* R.Br. (Myrtaceae) against multidrug resistant *Staphylococcus aureus*; ESBL (extended spectrum of β-lactamase) producing *E. coli* and multidrug resistant *Pseudomonas* species (Saxena and Gomber, 2006; Gomber and Saxena; 2007; Gomber and Saxena, 2010).

Phytochemical investigations previously have revealed aromatic nature of the plant owing to the higher content of α-pinene, 1, 8-cineol and α-terpineol (Jirovitz *et al.*, 1997). The crude leaf extract possesses higher concentration of alkaloids whose antimicrobial properties have not been reported in the literature so far. In the present study we report the isolation of alkaloids from leaves of *Callistemon rigidus* and their assessment for anti-staphylococcal potential using a test panel of reference and clinical isolates.

2 MATERIALS AND METHODS

2.1 Leaf Collection

Fresh and healthy leaves (no visible contamination) of *Callistemon rigidus* were collected in month of October from the Thapar University campus by Ms. Charu Gomber and confirmed with the herbarium sample deposited at the Department of Biotechnology and Environmental Sciences, numbered as #7 San 03. Samples were thoroughly washed under running water and air-dried. The fresh weight was noted, and the samples were subjected to drying at 37°C and pulverised into a fine powder.

2.2 Isolation of crude alkaloid extracts

Microwave assisted extraction was employed for direct extraction of the alkaloids from the dried pulverized leaves of *Callistemon rigidus* by cellular destruction initially and then steeping with 5% acetic acid at 120 rpm, 28 °C for 2 h. The insoluble material was removed by filtration and the aqueous phase was washed with dichloromethane (CH_2Cl_2) so as to remove chlorophylls followed by extraction with ethyl acetate for removal of fats. The aqueous phase was further taken up for basification with sodium carbonate as it exhibited the antimicrobial activity. It was re-extracted with dichloromethane to obtain

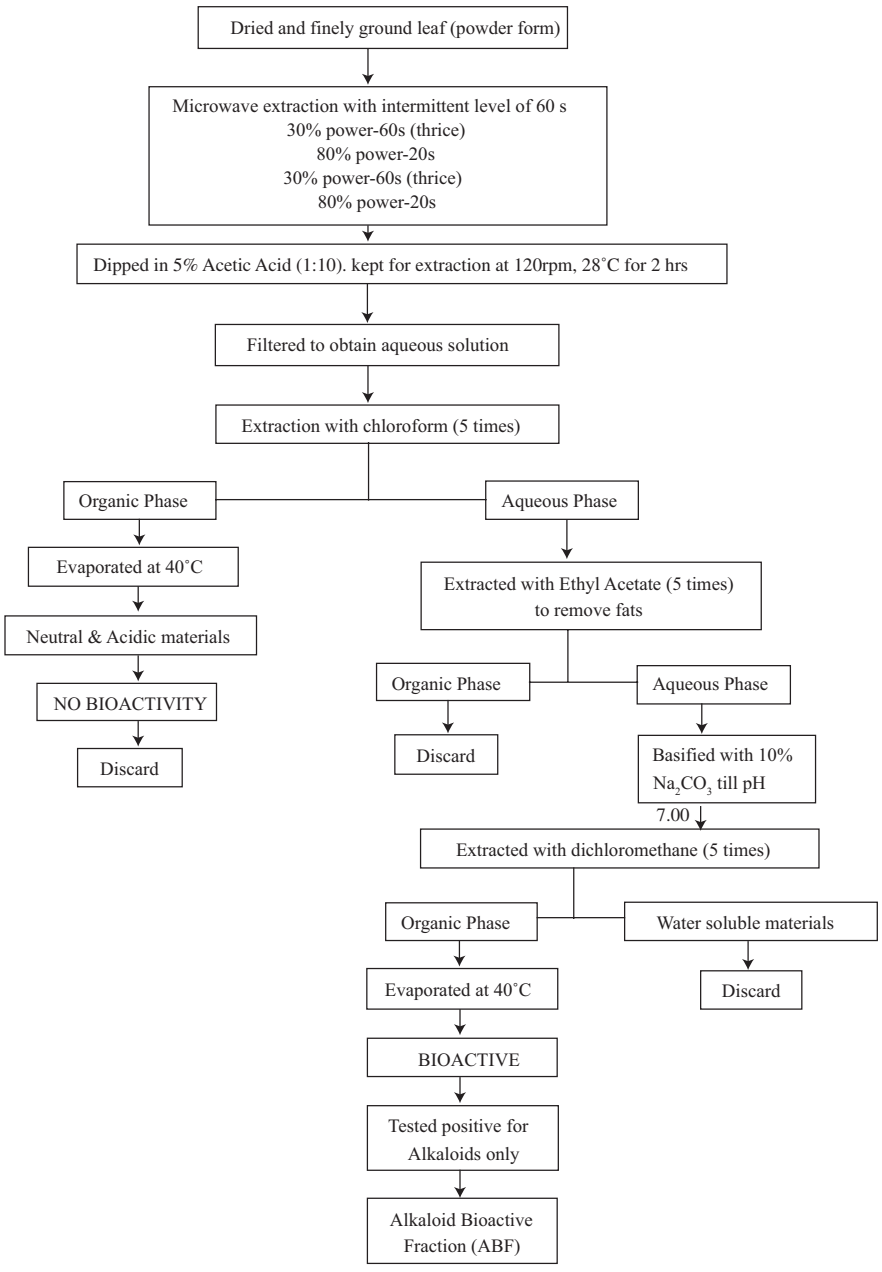

Figure 1: Schematic representation of extraction of Alkaloid Bioactive Fraction from C. *rigidus* leaves

organic and aqueous fractions which were tested for antimicrobial activity (Hadi and Bremner, 2001) (Fig.1). Subsequently phytochemical tests using Dragendroff's, Mayer's and Marquis Reagents were carried out to confirm the presence of alkaloids.

2.3 Evaluation of antimicrobial activity of crude Alkaloid Bioactive Fraction (ABF)

The bioactivity of the crude alkaloid bioactive fraction was evaluated by *in vitro* microbroth dilution method using 96-well microtitre plate and plate count assay.

2.3.1 Test Panel of Microorganisms

The test panel comprised of reference and clinical isolates of *Staphylococcus aureus*. The resistance patterns of these isolates have been predetermined and have been grouped as VRSA, VISA, MRSA and MARSA (Table 1). The maintenance medium of these cultures was Trypticase Soya Agar and these were activated on cation adjusted Mueller Hinton (MH) broth 18- 24 h prior to the test.

2.3.2 In vitro microbroth dilution assay

The *in vitro* microbroth dilution assay using 96 well microtitre plate and 3-(4, 5-dimethyl-2-thiazolyl - 5-Diphenyl-2H)-tetrazolium bromide (MTT) assay was performed to establish to MIC and MIC_{50} of the alkaloid bioactive fraction (O' Shea *et al*., 2009). Since visual MIC of each organism varies in the test panel of microorganisms being tested, MIC_{50} is the term used for the minimum MIC which is inhibiting 50% of the microbes in the test panel. The alkaloid bioactive fraction (ABF) was evaluated between 640-10 μg/mL. 50 μl of the bacterial suspension in saline was added to 125 μl of MH broth to achieve a final bacterial cell concentration of 10^5 cells in the test and control wells on the titre plate. Subsequently the plates were incubated for 2.5 hours at 37°C after which 25 μl of the test extract at different concentrations was added. These were then incubated for 24 hours at 37°C. After 24 hours 20 μl of 0.02% MTT was added to each well. The MIC value was taken as the lowest concentration of the ABF where no colour change occurred. The assay was performed in triplicates. Cefixime served as a positive control.

2.3.3 Plate count assay

The surface plate count assay was used to estimate the viable counts of the test panel bacteria at MIC concentration of ABF (Miles and Mishra, 1938; Slack and Wheldon, 1978). Briefly 10 µl aliquots were withdrawn using a sterile tip

Table 1. Test panel isolates of *Staphylococcus aureus*

Species	Culture Id	Repository	Resistance pattern
Pus and Wound Isolates			
Staphylococcus aureus	Sau A1	AIIMS*	MRSA
Staphylococcus aureus	Sau A2	AIIMS	MRSA
Staphylococcus aureus	Sau A4	AIIMS	----
Staphylococcus aureus	Sau G1	GMCP	VISA (MIC= 16μg/ ml)+
Staphylococcus aureus	Sau G2	GMCP	MRSA, MARSA
Staphylococcus aureus	Sau G3	GMCP	MARSA
Staphylococcus aureus	Sau G10	GMCP	MRSA, MARSA
Staphylococcus aureus	Sau G14	GMCP	MARSA
Staphylococcus aureus	Sau G23	GMCP	MARSA
Staphylococcus aureus	Sau G24	GMCP	MARSA
Staphylococcus aureus	Sau G25	GMCP	MARSA
Staphylococcus aureus	Sau G27	GMCP	MARSA
Staphylococcus aureus	Sau G28	GMCP	MARSA
Burn Isolates			
Staphylococcus aureus	Sau G9	GMCP	MRSA
Staphylococcus aureus	Sau G15	GMCP	MARSA
Staphylococcus aureus	Sau G16	GMCP	MRSA
Staphylococcus aureus	Sau G17	GMCP	VISA (MIC= 16μg/ ml)
Staphylococcus aureus	Sau G18	GMCP	MRSA, MARSA
Staphylococcus aureus	Sau G19	GMCP	MRSA, MARSA
Staphylococcus aureus	Sau G 26	GMCP	MRSA
Urine and Vaginal Isolates			
Staphylococcus aureus	Sau A3	GMCP	----
Staphylococcus aureus	Sau G4	GMCP	MRSA, MARSA
Staphylococcus aureus	Sau G5	GMCP	MARSA
Staphylococcus aureus	Sau G7	GMCP	VISA (MIC= 16μg/ ml), MARSA
Staphylococcus aureus	Sau G11	GMCP	MRSA, MARSA
Staphylococcus aureus	Sau G13	GMCP	
Standard Culture			
Staphylococcus aureus	Sau NCTC	LHMC	Antibiotic sensitivity testing strain

**AIIMS: All India Institute of Medical Sciences, New Delhi, INDIA; GMCP: Government Medical College, Patiala, INDIA, LHMC: Lady Harding Medical College, New Delhi, INDIA.*

+VISA (Vancomycin Intermediate S. aureus); VRSA (Vancomycin resistant S. aureus), MRSA (Methicillin resistant S. aureus); MARSA (Multi- antibiotic resistant S. aureus)

Table 2. Minimal Inhibitory Concentration (MIC) of ABF against test isolates by *in vitro* microbroth dilution assay**.**

Test Isolate Groups	Average Values	Culture Id	MIC (µg/ ml) of ABF	MIC(µg/ml) of Cefexime
Pus and Wound Isolates	MIC_{ABF} = 210 ± 58.86 µg/ml [a]$MIC_{Cefexime}$ = 193.8±30.67µg/ ml	Sau A1	320	40
		Sau A2	640	160
		Sau A4	10	80
		Sau G1	320	320
		Sau G2	80	320
		Sau G3	80	320
		Sau G10	160	320
		Sau G14	80	320
		Sau G23	80	80
		Sau G24	640	160
		Sau G25	160	80
		Sau G27	80	160
		Sau G28	80	160
Burn Isolates	MIC_{ABF} = 277.1 ± 74.12 µg/ml [a]$MIC_{Cefexime}$ = 133.1 ±36.89 µg/ml	Sau G9	640	320
		Sau G15	160	160
		Sau G16	320	160
		Sau G17	320	40
		Sau G18	320	80
		Sau G19	20	40
		Sau G26	160	160
Urine and Vaginal Isolates	MIC_{ABF} = 213.3 ± 93.9 µg/ml [a]$MIC_{Cefexime}$=160 ± 35.78 µg/ml	Sau A3	640	160
		Sau G4	80	80
		Sau G5	80	80
		Sau G7	80	160
		Sau G11	320	160
		Sau G13	80	320
Standard isolate	MIC_{ABF} = 80µg/ml $MIC_{Cefixime}$ = 80µg/ ml	Sau NCTC	80	80

All the values are means of triplicate readings
[a]-Non-significant difference in mean MIC values of ABF and Cefixime by unpaired t-test

micropipette from the test samples containing MIC concentrations of ABF at different time intervals viz. 2, 4, 6, 8, 10, 22, 24 h from the 96-well titre plates and placed as a single drop on MH agar plates divided into number of sectors and observed for the growth of the bacteria as pinhead colonies without the presence of any confluence after incubation of 12-18 h at 37 °C.

2.4 Statistical analysis

All the data presented in the tables and figures are Mean ± SD values of triplicate readings. Further Graph pad Prism ver. 5.1 has been used to assess the difference in IC_{50} values in different groups of test microbe's viz. pus-wound, burn and urine-vaginal isolates by one way ANOVA and Bonferroni's multiple comparison test.

3 RESULTS

The minimal inhibitory concentration (MIC) of ABF as observed by *in vitro* micro broth dilution assay using visual method using MTT ranged between 10-640 μg/mL for Pus and wound isolates. The MIC_{50} of these isolates was 80 μg/mL. Burn isolates exhibited a MIC range of 20-640 μg/mL with a MIC_{50} of 320 μg / mL. Vaginal and Urine isolates exhibited MIC range of 80-640 μg/mL with MIC_{50} of 80μg/mL. The MIC for the reference strain was 80 μg/mL. The positive control Cefexime exhibited a MIC in the range of 40-320 μg/mL (Table 2). A non-significant difference between average MIC values of ABF and Cefixime was observed in different groups of test isolates.

The viable count reduction by plate assay indicated that at MIC concentrations the ABF induced a bactericidal action in SauA4 and SauG2. Bacteriostatic activity of ABF at MIC concentration was exhibited by SauG3 and SauG10. Rest pus and wound isolates exhibited a reduction in viable count when compared to the control (Fig 2).

Among the burn isolates both Sau G15 and Sau G17 exhibited reduction in viable counts by 1 log at MIC concentration of the ABF when compared to initial inoculums count indicating a bactericidal action (Fig.3).

Sau G7 and Sau G11 were the only among urine and vaginal isolates exhibiting bactericidal activity at MIC concentrations of ABF. There was 2-log and 3-log reduction in the viable count in Sau G7 and Sau G11 respectively when compared to the initial inoculum count (Fig.4)

IC_{50} range of ABF for pus and wound isolates was between 3.422-189.9 µg/ml. Sau A4 exhibited the least IC_{50} of 3.42 µg/ml. The burn isolates exhibited IC_{50} in range of 6.413- 80.9 µg/ml. Sau G16 exhibited the least IC_{50} of 6.4 µg/ml. The urine and vaginal isolates were having an IC_{50} in the range of 4.21-

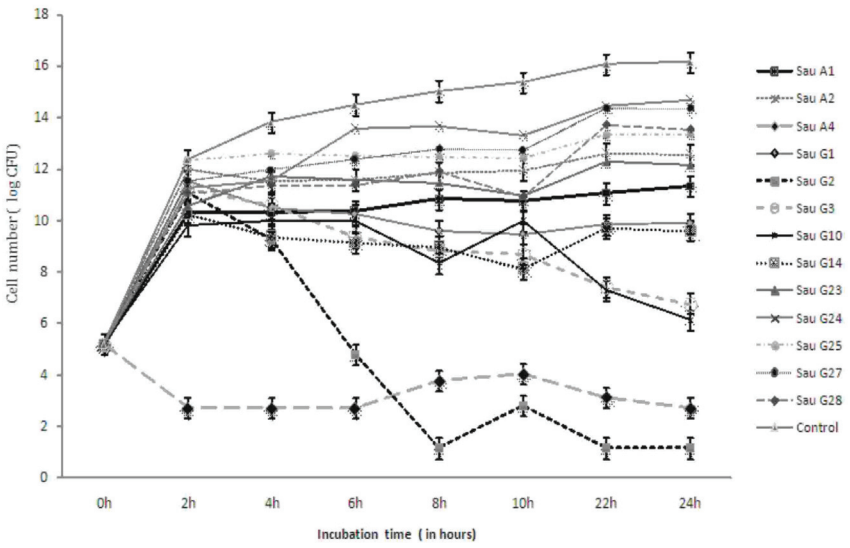

Figure 2: Kill pattern of pus and wound isolates by alkaloid bioactive fraction at MIC

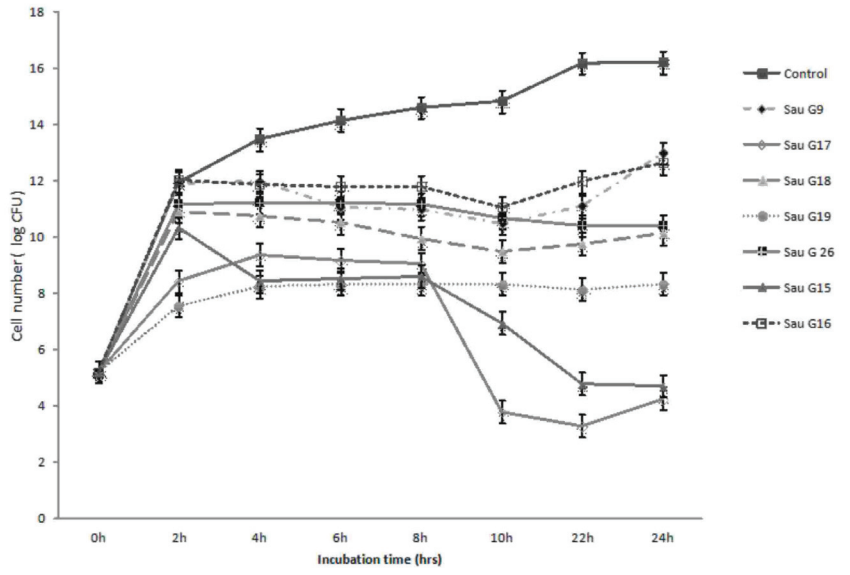

Figure 3: Kill pattern of burn isolates by alkaloid bioactive fraction at MIC

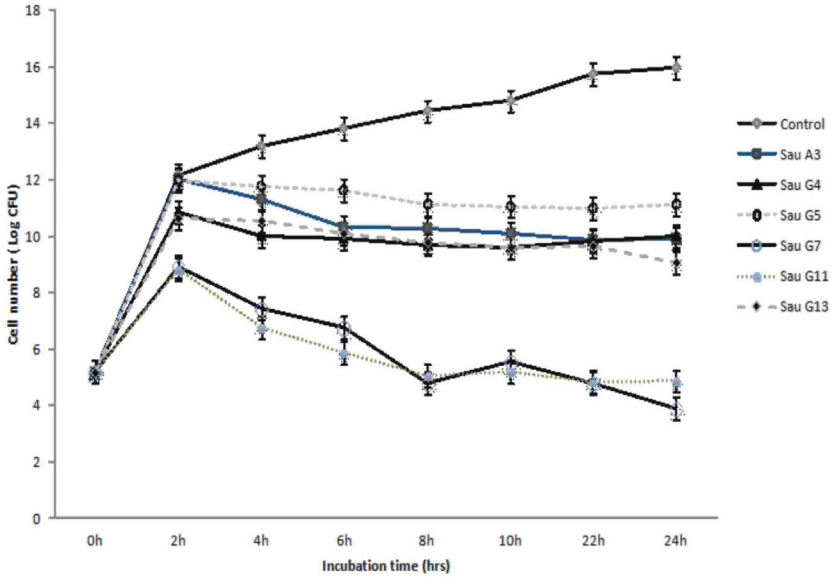

Figure 4: Kill pattern of urine and vaginal isolates by bioactive alkaloid fraction at MIC

74.5 µg/ml. Sau G4 exhibited the least IC_{50} value among the Urine and vaginal isolates (Table 3).

A non-significant difference was found in the average IC_{50} values of pus-wound, burn and urine-vaginal isolates by one way ANOVA at $p<0.05$. Similarly Bonferroni's multiple comparison tests also indicated a non-significant difference in the three groups of test isolates.

4 DISCUSSION

Plant alkaloids are known to possess anti-cancer and anti-bacterial activities. Alkaloid rich fractions from leaves of *Prosopis julifora* exhibited a MIC of 50 µg/ml against *Staphylococcus aureus* (Singh *et al*., 2011). Alkaloid extract from stem bark of *Mahonia manipurensis* exhibited an antibacterial activity against *Bacillus cereus* and *Enterococcus faecalis* however *Staphylococcus aureus* was not tested by them (Pfoze *et al*., 2011). Garba and Okeniyi (2012) have evaluated the total alkaloid extracts from *Jatropha curcas, Calotropis procera, Mangifera indica, Carica papaya* and *Psidium guajava* and found that the possessed a broad spectrum antibacterial activity. All the alkaloids from leaves of *Elaeagnus mollis* had obvious anti-microbial effect on *Bacillus*

Table 3: IC_{50} values of ABF against test panel staphylococcal groups.

Test Isolate Groups	Mean* IC_{50} Values (µg/ml)	Culture Id	**IC_{50} ± SD of ABF (µg/ml)
Pus and Wound Isolates	IC_{50} = 27.22 ± 2.86	Sau A1	7.24 ± 2.5
		Sau A2	3.82 ± 1.9
		Sau A4	3.42 ± 1.71
		Sau G1	189.9 ± 4.46
		Sau G2	20.49 ± 1.94
		Sau G3	46.7 ± 6.6
		Sau G10	96.8 ± 2.63
		Sau G14	12 ± 1.6
		Sau G23	42.7 ± 1.7
		Sau G24	122 ± 5.24
		Sau G25	41.68 ± 1.41
		Sau G27	6.89 ± 4.67
		Sau G28	151.1 ± 3.55
Burn Isolates	IC_{50} = 13.57 ± 2.48	Sau G9	68.9 ± 2.14
		Sau G15	12.03 ± 2
		Sau G16	6.4 ± 3.4
		Sau G17	20.4 ± 1.4
		Sau G18	10.71 ± 7
		Sau G19	9.1 ± 2.3
		Sau G26	80.9 ± 1.8
Urine and Vaginal Isolates	IC_{50} = 13.12 ± 2.45	Sau A3	20.3 ± 145
		Sau G4	4.21 ± 1.86
		Sau G5	74.5 ± 6.92
		Sau G7	10.3 ± 3.72
		Sau G11	4.25 ± 2.14
		Sau G13	18.3 ± 1.48
Standard Culture	IC_{50} = 30.9 ± 1.3	Sau NCTC	30.9 ± 1.3

∗ *Geometric means*
∗∗ *All values are means of triplicate readings*

subtilis, *Staphylococcus aureus*, *V. ceratosperma* and *Verticillium dahliae*. The MIC of these alkaloids against *Bacillus subtilis* and *Staphylococcus aureus* was 40 µg/ml and 80 µg/ml respectively (JiaMing *et al.*, 2010). Total alkaloid fraction of *Halorrhena floribunda* stem bark extracts by *in vitro* microbroth dilution assay was 96.25 ± 36.25 µg/ml. The IC_{50} value was 46.3 ± 14.7 µg/ml using test panel of *Bacillus* sp (Patrice *et al.*, 2007). Alkaloid from *Sida acuta* leaves exhibit potential antibacterial activity in the range of 16 - 400 µg/ml (Karou *et al*., 2005). Our results also exhibit a potential activity of the alkaloid bioactive fraction from *Callistemon rigidus* with MIC_{50} ranging between 80-320 µg/ml and IC_{50} values ranging between 3.422-189.9 µg/ml. The presence of alkaloids possessing anti-bacterial activity in *Callistemon rigidus* is being reported for the very first time. Further separation of individual alkaloids from the alkaloid bioactive fraction and their assessment against the same test panel of clinical, multidrug resistant Staphylococci would help in finding out relevant alkaloids which could be developed into drugs. Further synergistic activities of these alkaloids would also be helpful in developing formulations in development personal care and hygiene products to prevent transmission of Staphylococcus in the community and hospital settings.

5 CONCLUSION

The alkaloid bioactive fraction from *Callistemon rigidus* was as efficient as evident from its MIC_{50} and IC_{50} values against the test panel isolates. The ABF was having potential anti-Staphylococcal activity and was efficacious as Cefixime which is a pure antimicrobial. Hence further separation of individual alkaloids from the ABF would reduce the MIC_{50} and IC_{50} value than currently observed to overcome the refractory strains of *Staphylococcus aureus*.

ACKNOWLEDGMENT

The authors are grateful to Dr. Aarti Kapil, Department of Microbiology, All India Institute of Medical Sciences (AIIMS) and Dr. Amarjit Kaur Gill, ex-Head, Department of Microbiology, Government Medical College, Patiala for providing us multidrug resistant cultures of *S. aureus*. Dr. S. Natesh and Dr. M. Aslam are gratefully acknowledged for sponsoring the project BT/ PR/ 3982/ PBD/ 17/257/2003. Charu Gomber acknowledges DBT, Government of India for providing fellowship under the project number BT/PR/3982/ PBD/17/257/2003.

REFERENCES

Garba, S., Okeniyi, S.O. (2012) 'Antimicrobial activities of total alkaloid extracts from some Nigerian Medicinal plants'. *J. Microb and Antimicrobials,* Vol 4, 3, pp. 60-63. http://dx.doi.org/10.5897/JMA11.081

Gomber, C., Saxena, S. (2007) 'Anti-staphylococcal potential of *Callistemon rigidus'*. *Central European J Medicine,* Vol 2, 1, pp. 79-88 http://dx.doi.org/10.2478/s11536-007-0004-8

Hadi, S., Bremner, J.B. (2001) 'Initial studies of alkaloids from Lombok Medicinal Plants.' *Molecules* Vol 6, pp. 117-119 http://dx.doi.org/10.3390/60100117

JiaMing, Z., Nan Mu, YuXiang, S., Xiaochun, S., GuiQin, Y. (2010) 'Study on anti-microbial activities of total alkaloids in *Elaeagnus mollis' Med Plant,* Vol 1, 8, pp. 62-64

Jirovetz, L., Fleischhaker, W., Buchbauer, G. and Ngassoum, M.B. (1997) 'Analysis of essential oils from *Callistemon rigidus* (Myrtaceae) from Cameroun by GC/FID and GC/MS'. *Sci Pharm,* Vol 65, pp. 315-319

Karou, D., Savadogo, A., Canini, A., Yameogo, S., Montesano, C., Simpore, J., Vittorio Colizzi, V., Traore, A.S. (2005) 'Antibacterial activity of the alkaloids from *Sida acuta'*. *African J Biotech,* Vol 4, 12, pp. 1452-1457

Miles, A.A., Mishra, S.S. (1938) 'The estimation of bactericidal power of the blood'. *J Hygiene (Camb.),* Vol 38, pp. 732-749 http://dx.doi.org/10.1017/S002217240001158X

Patrice, B.K., Veronique, P.B., David, L., Xavier, E.F. (2007) 'Antibacterial activities of extracts and conissine from *Halorrhena floribunda* G. Don (Apocyanceae)' *Afr J Trad CAM*, Vol 4, 3, pp. 352-356.

Pfoze, N.L., Kumar, Y., Myrboh, B., Bhagobaty, R.K. and Joshi, S.R. (2011) 'In *vitro* antibacterial activity of Alkaloid extract from Stem bark of *Mahonia manipurensis* Takeda'. *J Med Plant Res*., Vol 5, 5, pp. 859-861

Saxena S., Gomber, C. (2006) 'Antimicrobial potential of *Callistemon rigidus* R.Br.' *Pharm Biol* Vol 44, 3, pp. 194-201 http://dx.doi.org/10.1080/13880200600685899

Saxena, S., Gomber, C. (2010) 'Surmounting antimicrobial resistance in the Millennium Superbug: *Staphylococcus aureus' Central European J Medicine,* Vol 5, 1, pp**.** 12-29 http://dx.doi.org/10.2478/s11536-009-0079-5

Saxena, S., Kumar, D. (2000) 'Tailoring biodiversity in development of new therapeutics' *Nat Prod Radiance*, Vol 1, pp.18-25

Shea O', S., Lucey, B., Cotter, L. (2009) '*In vitro* activity of Inula helenium against clinical *Staphylococcus aureus* strains including MRSA'. *British J Biomed Sci* 66, 4, pp.186-189.

Singh, Sachi, Swapnil, Verma, S.K. (2011) 'Antibacterial properties of alkaloid rich fractions from various parts of *Prosopis julifora'*. *International J Pharm Sci and Res,* Vol 2, 3, pp. 114-120

Slack, M.P.E., Wheldon, D.B. (1978) 'A simple and safe volumetric alternative of the Miles, Mishra and Irwin for counting viable bacteria'. *J Med Microbiol,* Vol 11, pp. 541-545. http://dx.doi.org/10.1099/00222615-11-4-541