Research Article

Determination of Phytochemicals and Minimum Inhibitory Concentrations of Leaf Extracts of *Camellia sinensis* and *Murraya koenigii* on MRSA and MSSA Strains and Virulence Factors of the Strains.

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ABSTRACT

Virulence traits (coagulase, hemolysin, protease, catalase, lipase, slime layer, biofilm and hydrophobicity of 33 MSSA and 11 MRSA strains were studied. All the MRSA and MSSA isolates exhibited lipase, coagulase and catalase activity. The proportion of MRSA isolates producing protease, hemolysin, biofilm and slime layer was higher as compared to MSSA. MRSA appeared more hydrophobic in salt aggregation test and better biofilm producers. The sugar supplemented media (trypticase soya broth and brain heart infusion broth) were better for biofilm production. The susceptibility of all the isolates to aqueous and alcoholic leaf extracts of *C. sinensis* and *M. koenigii* was determined in the order: (aqueous < ethanolic < methanolic). MIC values of both the alcoholic extracts of *M. koenigii* (curry leaves) were lower as compared to that of *C. sinensis*. These values were higher for MRSA. MIC_{50} values of the ethanolic (3.75×10^2 mg/ml) and methanolic extract (6.25×10^2 mg/ml) of *M. koenigii* were lower as compared to the alcoholic extracts of *C. sinensis* for MRSA isolates. In case of MSSA isolates, there was no difference in the MIC_{50} values of ethanolic extracts i.e 3.75×10^2 mg/ml of both the plants but in case of methanolic extracts, the MIC_{50} value of *C. sinensis* was lesser as compared to that of *M. koenigii.* The extracts of both the plants revealed different phytochemicals.

Keywords: MRSA, MSSA, virulence factors, *Camellia sinensis*, *Murraya koenigii*, phytochemicals, Minimum inhibitory concentration (MIC), MIC_{50}.

INTRODUCTION

Staphylococcus aureus is commensal opportunistic bacterium which is highly adaptive and versatile pathogen of man, other mammals and avians. This organism is responsible for a variety of frequent infections ranging from localised to deep invasive systemic infections. Emergence of drug resistant strains in the nosocomial settings has turned hospital into a breeding place for new infections.[1] *S. aureus* is predominant gram positive bacterium associated with variety of such infections.[2] Methicillin sensitive (MSSA) and methicillin resistant *S. aureus* (MRSA) are of much public health concern. Several virulence factors of *S. aureus* contribute to the pathogenesis of the infections due to this organism. These factors involve; surface associated factors which help in the attachment of the bacterium to the host cell surface (MSCRMM), and several invasive factors (lipases, proteases, hemolysins, coagulases, catalases, staphylokinase, hylauronidase). The invasive factors help in invading the host body tissues and immune system. Toxins also play an important role in the spread of infection from one site in the body to the other. Selective virulence traits of these strains have been compared in the present study. The emergence of resistance to multiple antibiotics complicates the treatment of MRSA and MSSA infections. Inadequacy in the availability of novel drugs in the market, side effects or toxic effects of the drugs used for treating *S. aureus* infections are some of other factors that hamper the treatment. Plants and their products can be explored for the invention of new drugs as they are the natural reservoirs of promising phytochemicals.[3] In our laboratory, the inhibitory effects of certain plant extracts have been observed against *S.aureus* and other bacteria under *in vitro* conditions.[4,5] The results of these studies are quite encouraging which prompted us to determine the minimum inhibitory concentrations of alcoholic extracts of leaves of *C. sinensis* and *M. koenigii* against the MRSA and MSSA strains. These strains have been well characterised in our laboratory.[6,7,8]

MATERIALS AND METHODS

Subculturing of Isolates

The *S. aureus* isolates were subcultured on suitable media (nutrient broth, nutrient agar and mannitol salt agar). A total of 44 isolates (33 MSSA and 11 MRSA isolates) were used in the study. These isolates were recovered from blood, pus and urine samples of human patients at Indira Gandhi medical college (IGMC), Shimla, maintained and characterised in our laboratory.

Confirmation of the Isolates

The isolates were confirmed on the basis of their colony morphology on nutrient agar and mannitol salt agar, microscopic examination of Gram's stained preparations and by biochemical tests.

In vitro Studies on Selective Virulence Traits

Lipases

Lipase media was prepared by adding 2% Tributyrin in nutrient agar. Broth cultures of the test isolates were inoculated in the wells containing the lipase media,

incubated at 37° C for 24 – 48 h. The zone of clearance around the wells indicated lipase activity.

Proteases

Gelatin agar was used for detecting the production of protease by MRSA and MSSA strains. Nutrient agar containing 1% gelatine (Himedia) was prepared and the wells cut in the media.

Broth cultures containing the test organisms were inoculated in the wells, incubated at 37° C for 2- 3 days. Turbid zone around the wells indicated the production of proteases. In order to make the zone clearly visible, 0.1% mercuric chloride solution was added to each plate.

Hemolysin production

Blood agar plates were prepared by pouring 5ml blood aseptically in the nutrient agar, inoculated with the test strains, incubated at 37° C for 1-2 days. Zone of clearance or change in the colour of the media around the wells due to hemolysis indicated positive test.

Coagulase

In order to detect the Coagulase production, loopful culture of each test organism was placed on both the ends of the glass slide.

Plasma 1-2 drops were added to the culture. The production of coagulase by *S. aureus* isolates was demonstrated by clumping of the organisms.

Catalase production

Catalase production was determined by adding 1-2 drops of 3% H_2O_2 to each test isolates. The development of effervescences indicated the production of catalase.

Cell surface hydrophobicity

Salt aggregation test (SAT) was conducted for the determining cell surface hydrophobicity. The bacterial suspensions and ammonium sulphate $(NH4)_2SO_4$ solutions used in the test were prepared in phosphate buffered saline (PBS, pH6.8).

The SAT values were recorded as the lowest molarity of ammonium sulphate that demonstrated visible bacterial aggregation. Molarities of ammonium sulphate used in the SAT ranged from 4.0 mol l^{-1} to 0.002 mol l^{-1}. The interpretation of the results was done as follows: strains with SAT values >4.0 mol l^{-1}, 2.0-4.0 mol l^{-1}, 1.0-2.0 mol l^{-1} and 0.0-1.0 mol l^{-1} were designated as no, low, moderate and high hydrophobic strains respectively.[9]

Slime layer formation

For quantitative determination of slime layer, Congo red agar (CRA) method was used. All the strains were inoculated into CRA plates, incubated at 37°C for 24 - 48 h. The slime layer producing isolates developed black colonies, whereas non-slime producers remained non-pigmented (red in colour).[10]

Biofilm formation

Tissue culture plate (TCP) method of Mathur[11] was followed for observing biofilm formation. Precisely, flat bottomed 96 - wells microplates were inoculated with the test MRSA and MSSA strains, incubated for 24 h at 37°C. Optical density of crystal violet stained adherent bacteria was determined with ELISA microplate reader at 595 nm. The experiment was conducted in triplicate and the average of readings and standard deviation were calculated. A three grade scale was employed for evaluating the biofilm producing ability of the strains: OD < 0.120: no or weak; 0.120<OD<0.240: moderate; OD > 0.240: high.

Collection of Plant Materials

Leaves of *Camellia sinensis* and *Murraya koenigii* were collected during the month of October, 2013 from Palampur and Solan regions respectively and authenticated at the Department of Botany Shoolini University, at Solan, Himachal Pradesh (India).

Preparation of Plant Extracts

The method followed for preparation of the extract involved washing of the fresh plant leaves under running tap water, soaking for 5-10 min. in sterile distilled water followed by drying under shade for 5-6 days.

The dried leaves were ground to fine powder using pestle and mortar. The powder was stored in air-tight bottles. Aqueous extract (10% w/v) and alcoholic extracts (methanolic and ethanolic) 20% w/v were prepared for use in the study.

Sterility Testing of Extract

After filtration the extracts were tested for their sterility by inoculating on freshly prepared sterile nutrient agar plates and incubated for 24 h at 37°C. The sterile extracts were used for evaluating their inhibitory activity on test isolates.

Phytochemical Analysis

The plant extracts were screened for their phytochemical constituents such as alkaloids, tannins, saponins, flavonoids, acidic compounds, proteins, glycosides, terpenoids, phenols and steroids etc. according to the procedures as described by[12-14].

Test for flavonoids

Extracts were treated with a few drops of 5% sodium hydroxide solution. Formation of intense yellow colour, which became colourless on addition of dilute sulphuric acid, indicated the presence of flavonoids.

Test for alkaloids

Extracts containing one ml of 2% HCl were heated. A few drops of 5% sodium hydroxide solution were added to the contents. Formation of yellow precipitate or turbidity indicated the presence of alkaloids.

Test for steroids (Salwoski's test)

Dry extracts were dissolved in two ml of choloroform. A few drops of conc. sulphuric acid were added slowly to form a lower layer. A reddish brown ring at the interface indicated the presence of steroids.

Detection of diterpenes (Copper acetate test)

Plant extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicated the presence of diterpenes.

Test for terpenoids

Chloroform in a volume of two ml and conc. sulphuric acid (one ml) were added to the extract. A reddish brown colour indicated the presence of terpenoids.

Test for phenol

Ferric chloride solution (3-4 drops) was added to the leaf extracts of both the plants. Formation of a bluish-green colour indicated the presence of phenols.

Test for tannins

Dry extracts (50 mg) was dissolved in two ml of distilled water. Ferric chloride in a vol. of two ml was added to it. Formation of blue black precipitate indicated the presence of tannins, black colour the catecholic tannins and the blue colour the gallic tannins.

Test for saponins

The leaf extracts were diluted with 10-20 ml of distilled water, agitated for 15 min. The formation of a layer of foam showed the presence of saponins.

Test for glycosides

Each plant extract was dissolved in one ml ethanol. An equal volume of water was added to it. 5% sodium hydroxide (3-4 drops) was added to the contents. The development of yellow colour indicated the presence of glycosides.

Test for acidic compounds

Dry extract (50mg) was dissolved in two ml of ethanol followed by the addition of 5- 6 drops of sodium bicarbonate solution.

The production of effervescence indicated the presence of acidic compounds.

Test for proteins (Xanthoproteic test)

The leaf extracts were treated with few drops of conc. nitric acid. Formation of yellow colour indicated the presence of proteins.

In vitro* Susceptibility of MRSA and MSSA Isolates to the Extracts of *Camellia Sinensis* and *Murraya Koenigii

The leaf extracts of both the plants in different solvents were tested for their inhibitory activity on MRSA and MSSA isolates by disc diffusion method as describe below:

Preparation of inoculum

The inoculums were prepared by suspending 5-8 colonies of each isolate from the fresh culture in 5ml of normal saline. The contents were vortexed. The turbidity of suspension was compared with 0.5 McFarland standard.

Susceptibility of the isolates to plant extracts

The inhibitory activity of the plant extracts to MRSA and MSSA isolates was determined by disc diffusion method using Muller Hinton Agar plates. The medium was sterilized by autoclaving at 121°C for 15 min., poured aseptically in the sterile petri plates and allowed to solidify at room temperature. The MHA plates were incubated overnight at 37°C. The sterilized cotton swabs dipped in bacterial inoculums were swabbed over the surface of agar in order to spread them uniformly. After drying for five min., sterile discs dipped in 10µl of the plant extracts were placed on the surface of the inoculated medium and the extracts were allowed to diffuse for 5 min. The plates were incubated at 37°C for 24-48 h. The diameters of the zone of inhibition around the disc were measured. The zones were measured. Oxacillin (1mcg), vancomycin (10 mcg) and chloramphenicol (10 mcg) were used as positive controls while DMSO was used as solvent control in the susceptibility assays.[1]

Determination of Minimum Inhibitory Concentration (MIC)

The MICs of the leaf extracts of both the plants were determined by transferring aseptically the paper discs in to Muller Hinton agar plates inoculated with the test organisms and impregnating them with 13 µl of extract samples at a concentration ranging from 1g/ml - 62.5mg/ml. The MIC is the lowest concentration that produced a visible zone of inhibition.[15] MIC_{50} of the extracts was calculated using the following formula given by Smith[16] which is as follows:

Minimum Inhibitory Concentration $_{50}$ - MIC $_{50}$

$$MIC = (M < 50) + \frac{(n - x)[(M > 50) - (M < 50)]}{y}$$

M< 50 = MIC of highest cumulative percentage below 50%.

M >50 = MIC of lowest cumulative percentage below 50%.

n = 50% of the number of organisms tested.

x = No. of organism in the group at M < 50.

y = No. of organism in the group at M > 50.

RESULTS

Confirmation of Isolates

All the isolates under study were confirmed as *Staphylococcus aureus*. The identity of each isolate was established on the basis of colony characteristics (on nutrient agar, the colonies were smooth, shiny, entire and opaque. The colonies were yellow coloured on mannitol salt agar), microscopic examination (Gram

positive cocci arranged in grape like clusters) and specific biochemical tests.

In vitro Production of Selective Virulence Traits of MRSA and MSSA Isolates

All the isolates of MRSA and MSSA were positive for lipase (Fig 1 A), coagulase and catalase. All the MRSA isolates tested were also positive for hemolysin (Fig 1 C). All of them exhibited protease activity also while 13/33 (39.4%) of MSSA isolates demonstrated this activity (Fig 1 B). Also 24.24% of MSSA and 69.23% of MRSA isolates respectively were slime layer producers (Fig 1 D).

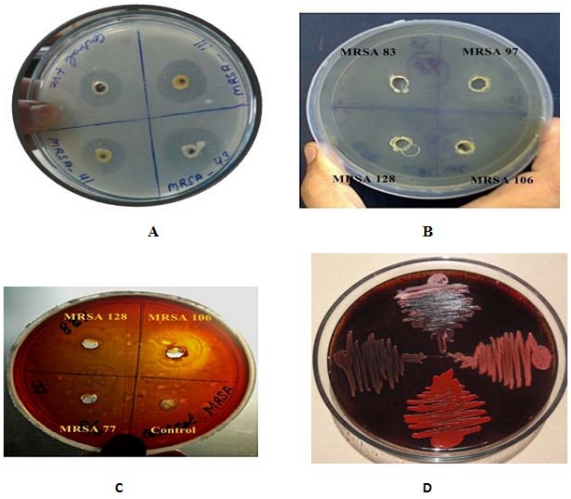

Figure 1: Demonstrating the production of different virulence factors: **A** Lipase activity, **B** Protease activity, **C** Hemolytic activity, **D** Slime layer formation demonstrated by different MRSA isolates.

Hydrophobicity

The SAT values (The lowest ammonium sulphate molarity which gives the visual aggregation was recorded as the cell surface hydrophobicity). The value for MRSA were recorded as 0.002 mol L^{-1} for all MRSA isolates tested whereas these values ranged from 0.01 - 0.002 mol L^{-1} for MSSA.

Biofilm formation

The details of biofilm formation by MRSA and MSSA on different media after 24 h of incubation are given in table 1. All the isolates produced biofilms except MSSA in TSB and TSB supplemented with glucose.

The comparative analysis revealed that the media supplemented with glucose or sucrose was better for biofilm production as compared to TSB alone.

Phytochemical Analysis of Leaf Extracts of *C. Sinensis* and *M. Koenigii*

The aqueous, ethanolic and methanolic extracts of leaves of *C .sinensis* and *M. koenigii* as shown in Table 2 in different solvents had the presence of alkaloids, flavonoids, saponins, tannins, acidic compounds, terpenoids, proteins, steroids, phenols and glycosides as demonstrated by the standard tests of detection.

Inhibitory Activity of Leaf Extracts of *C. Sinensis* and *M. Koenigii* on MRSA and MSSA Isolates

The antibacterial activity of the extracts of both the plants against MRSA and MSSA isolates was determined and found in the following order aqueous < ethanolic < methanolic (Table 3 and 4). Six MRSA isolates were susceptible to the aqueous extract of green tea (Zone size-10 – 11 mm), the zone size with ethanolic extract ranged from 12 – 22 mm in diameter while it was 13 mm – 22 mm for methanolic extract. All the MSSA strains were sensitive to all the three extracts of *C. sinensis,* the zone of inhibition ranged from 10mm – 15mm in case of aqueous extract while it ranged from 12 – 26 mm and 11 – 29 mm respectively for ethanolic and methanolic extracts. In case of *M. koenigii,* four MRSA isolates were sensitive to aqueous extract (zone size 18 -28 mm) while all isolates were sensitive for methanolic extract (zone size 13 - 32 mm and ethanolic extract (11 - 37 mm).

Minimum Inhibitory Concentration (MIC) and MIC_{50} values of the Plant Extracts against MRSA and MSSA strains.

The MIC range of ethanolic and methanolic extracts of *C. sinensis* were recorded as 5 x 10^2 - 10^3 and 2.5x 10^2 - 10^3 mg/ml against MRSA isolates with MIC_{50} values of 6.43 x 10^2 mg/ml and 8.5 x 10^2 mg/ml respectively. The MIC values of both the alcoholic extracts of *M. koenigii* were lower: ethanolic (1.25 x 10^2 mg/ml to 5 x 10^2 mg/ml) and methanolic (0.625 x 10^2 - 10^3 mg/ml) against MRSA as compared to *C. sinensis.* The MIC 50 values of ethanolic extract of *M. koenigii* were recorded as 3.75 x 10^2 mg/ml and methanolic extract as 6.25 x 10^2 mg/ml. For MSSA, the range of MIC for ethanolic extract of *C. sinensis* was 2.5 x 10^2 - 5 x 10^2 and for methanolic extract 1.25 x 10^2 - 10^3 MIC_{50} 3.75 x 10^2 and 2.25 x 10^2 respectively. In case of *M. koenigii*, the MIC ranges for these extracts were 2.5 x 10^2 - 10^3 (ethanolic) and 2.5 x 10^2 - 5 x 10^2 (methanolic) and MIC_{50} value of 3.75 x 10^2 was recorded for both the extracts.

Table 1: Biofilm formation by MRSA and MSSA employing Tissue Culture Plate (TCP) method using different media.

OD Values at 595 nm (Biofilm formation)	TSB		TSB+ glucose		BHIB + sucrose	
	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA
High (>0.240 ± 0.022)	0	2	0	1	2	4
Moderate (0.12-0.240 ± 0.020)	12	8	13	10	8	6
Weak/Non (0.120 ± 0.012)	21	1	20	0	23	1

TSB – Trypticase soya broth BHIB – Brain heart infusion broth; The number indicates the number of isolates positive for biofilm formation.

Table 2: Phytochemical analysis of leaf extracts of *C. sinensis* and *M. Koenigii.*

S. No	Phyto-chemicals analysed	*C. sinensis*			*M. koenigii*		
		Aqueous	Ethanolic	Methanolic	Aqueous	Ethanolic	Methanolic
1.	Alkaloid	+	+	+	+	+	+
2.	Flavonoid	+	-	+	+	-	+
3.	Steroid	-	+	-	-	-	-
4.	Saponins	+	-	+	+	-	+
5.	Tannins	+	-	+	-	-	-
6.	Catecholic tannins	-	+	-	-	+	-
7.	Gallic tannins	-	-	-	+	-	-
8.	Terpenoid	+	-	+	-	-	-
9.	Diterpeins	-	-	-	-	-	-
10.	Phenols	-	-	-	-	-	+
11.	Glycosides	+	-	+	+	-	-
12.	Protein	+	+	+	+	-	-
13.	Acidic compounds	+	-	+	+	-	+

+ represents presence and – absence of the phytochemical

Table 3: Inhibitory activity of leaf extracts of *C. sinensis* and *M. koenigii* against MRSA isolates.

S. No.	Sample no.	Zone of Inhibition (diameter in mm)								
		Extracts						Antibiotics		
		M. koenigii			*C. sinensis*					
		Aqueous	Ethanolic	Methanolic	Aqueous	Ethanolic	Methanolic	Vancomycin (30 mcg)	Oxacillin (1 mcg)	Chloramph (30 mcg)
1.	MRSA- 29	10	14	16	18	21	24	22 (S)	22(S)	37(S)
2.	MRSA- 41	10	19	21	-	12	17	19 (S)	26(S)	33(S)
3.	MRSA- 43	Nil	14	13	24	24	29	21(S)	22(S)	33(S)
4.	MRSA- 64	10	12	21	28	32	37	22(S)	28(S)	13(I)
5.	MRSA- 111	11	14	14	-	13	16	20(S)	22(S)	32(S)
6.	MRSA- 83	Nil	18	19	-	-	11	20(S)	27(S)	31(S)
7.	MRSA- 97	Nil	18	19	21	31	35	22(S)	27(S)	19(S)
8.	MRSA- 102	Nil	22	22	-	17	19	19(S)	25(S)	32(S)
9.	MRSA- 106	10	19	20	-	15	21	19(S)	26(S)	31(S)
10.	MRSA- 128	10	19	20	-	15	16	20(S)	30(S)	32(S)

S- Sensitive, R- Resistant

Table 4: Inhibitory activity of leaf extracts of *C. sinensis* and *M. koenigii* against MSSA isolates.

S. no.	Sample no.	Zone of Inhibition (diameter in mm)								
		Extracts						Antibiotics		
		M. koenigii			*C. sinensis*					
		Aqueous	Ethanol	Methanolic	Aqueous	Ethanol	Methanol	Vancomycin (30 mcg)	Oxacillin (1 mcg)	Chloramph. (30 mcg)
1.	MSSA 02	10	15	20	10	20	21	21 (S)	24 (S)	33 (S)
2.	MSSA 04	29	30	33	10	21	22	24 (S)	10 (R)	34 (S)
3.	MSSA 10	-	-	-	10	21	20	20 (S)	10 (R)	29 (S)
4.	MSSA 55	27	31	35	10	17	16	10 (R)	10 (R)	32 (S)
5.	MSSA 57	28	34	35	13	22	24	22 (S)	25 (S)	36 (S)
6.	MSSA 118	34	32	36	10	21	21	26 (S)	36 (S)	36 (S)
7.	MSSA 78	32	36	40	10	12	11	29 (S)	32 (S)	38 (S)
8.	MSSA 116	17	19	23	12	18	22	10 (R)	0 (R)	10 (R)
9.	MSSA 122	-	19	27	15	26	29	28 (S)	35 (S)	40 (S)
10.	MSSA 132	26	27	28	11	17	20	18 (S)	17 (S)	29 (S)

S- Sensitive, R- Resistant

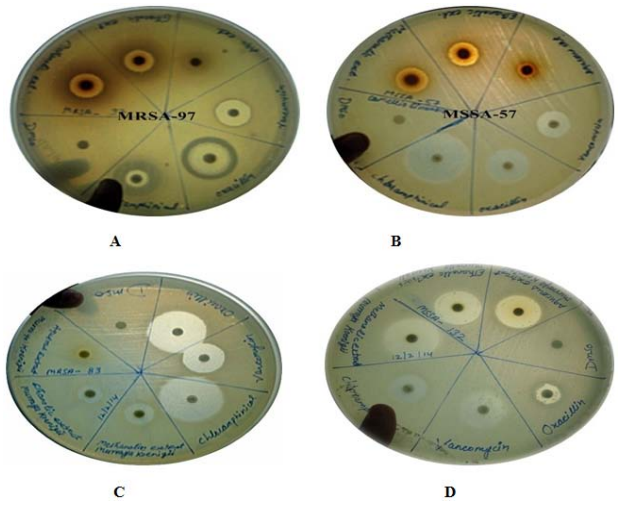

Figure 2: A Inhibitory activity of aqueous and alcoholic extracts of *C. sinensis* on MRSA isolate and **B.** MSSA isolate. Inhibitory activity of extracts of *M.koenigii* on MRSA isolate **(C)** and MSSA isolates **(D).**

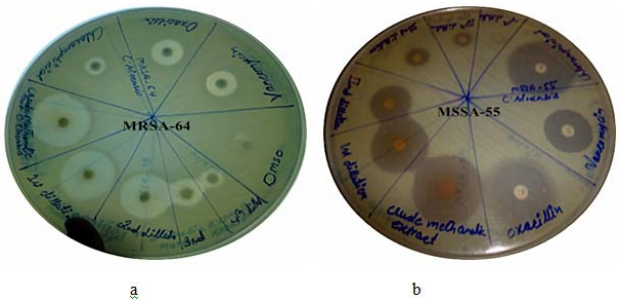

Fig. 3.a Minimal inhibitory concentration **(**MIC) of methanolic extract of *C. sinensis* on MRSA isolates 64. The largest zone is produced by 20 µl of the crude extract containing 1g/ml i.e. 20 µg of the crude extract followed by serial tenfold dilutions upto 4th dilution. Antibiotics vancomycin, chloramphenicol and oxacillin are kept as controls and DMSO as solvent control. **b.** MIC of methanolic extract on MSSA isolate 55.

DISCUSSION

The incredible degree of virulence contributed by various virulence factors and emergence of multi drug resistant strains has made it difficult to treat infections due MRSA and MSSA. Several virulence factors contribute to the pathogenesis of *S. aureus* infections, some of the important ones are: lipase, proteases, hemolysin, coagulase, catalase, slime layer and biofilm formation. The enzyme lipase is one of the important virulence factors which is encoded by certain genes the removal of which also reduces the potential of the *S. aureus* strains to form biofilm.[17] We found all the MRSA and MSSA isolates positive for lipase. The MRSA isolates appeared to be better producers. Coagulase is another virulence factor. All the isolates recovered from the pus and blood samples of the patients in the study produced coagulase. A majority of milk samples (90.91 %) were found positive for coagulase producing *S. aureus*.[18] All the MRSA and MSSA isolates tested produced catalase in the present study (Table 1). Catalase a heme protein enzyme, is yet another important factor which plays an important role in the defence of the host against *S. aureus* infections. All the isolates also produced hemolysins except a single MSSA isolate. Akinkunmi and Lamikanra (2012)[19] observed a lower proportion (56.1%) of strains that expressed hemolytic activity.

In the present study, all the MRSA isolates exhibited protease activity, while only 13/33 (39.4%) MSSA produced protease on gelatine agar. A lower proportion of *S. aureus* isolates (7.3%) was protease positive by gelatin agar method.[19] On the contrary, Saising[20] reported 99% of coagulase positive *S. aureus* expressing protease activity on skimmed milk agar. In the present investigations, 24.24% of MSSA and 69.23% of MRSA isolates respectively were slime layer producers. Other workers have found lesser proportion of *S. aureus* strains that produced slime layer. Bose[21] reported 32.96% of slime producing *S. aureus*.

Hydrophobicity and charge on the cell surface are some of the physicochemical characters which contribute towards the pathogenic nature of *S. aureus*. Ljungh[22] observed that 90.43% *S. aureus* strain recovered from infection due to this organism exhibited hydrophobiciy at 0.002 M solution of phosphate buffer. Also 39% *S. aureus* strains recovered for nasal cavity showed hydrophobicity at this concentration. In the present study, all the MRSA isolates showed hydrophobicity at 0.002 M ammonium sulphate while the concentration for MSSA ranged from 0.1M – 0.002 M for the aggregation of the salt. The MRSA appeared therefore, more hydrophobic than MSSA.

In the present study, the MRSA isolates appeared better biofilm produces as compared to MSSA (Table 2). The media containing sugars were better for biofilm production by both MRSA and MSSA strains. This is in agreement to the observation of Mathur[11] Majority of the MSSA isolates in the present study were either weak or non producers of biofilm. Akinkunmi and Lamikanra (2012)[19] observed (36.6%) *S. aureus* isolates positive for biofilm formation.

The emergence of multidrug resistant strains of *S. aureus* due to indiscriminate use of the antibiotics poses a problem in treating infections due to this organism. Also the unavailability of very effective and useful drugs in the market adds to this problem. Therefore, there is an urgency to explore alternate therapy which is affordable, efficacious and reliable.[23] Scientists worldwide are enthusiastic and expecting good inhibitory activity of compounds from plant sources as they are the natural reservoir of promising phytochemicals and can be explored for the invention of new drugs.[24] In our laboratory, we observed good inhibitory activity of certain extracts of different plants on different microbes.[5,4] Keeping in view the preliminary results of inhibitory activity, we planned to investigate such activity of leaf extracts of *Camellia sinensis* (Green tea) and *Murray koenigii* on MRSA and MSSA isolates which have been well characterized in our laboratory and analysed

their phytochemical constituents also the aqueous extract of *C .sinensis* contained alkaloids, flavonoids, saponins, tannins, acidic compounds, terpenoids, proteins and glycosides. Our observations are in agreement with those reported by Dogra[25] except that we did not detect steroids in our study. Aqueous extract of *M. koenigii* (curry leaf) contained alkaloids, flavonoids, saponins, gallic tannins, acidic compounds, glycosides and proteins. Sarla and Bharathi (2013)[26] however, did not report the presence of alkaloids, flavonoids and steroids in the aqueous extract. Similarly, Baskaran[27] also could not detect terpenoids, tannins, phenols, glycosides and alkaloids. We detected alkaloids, steroids, catecholic tannins and proteins in the ethanolic extract of *C. sinensis*.

The main phytochemicals present in the ethanolic extract of *M. koenigii* were: alkaloids and catecholic tannins only. Argal[28] reported the presence of alkaloids in the ethanolic leaf extract of *M. koenigii* but these workers additionally observed the presence of glycosides, steroids, flavonoids, saponins, proteins and sugars in the extract.

Methanolic extract of *C. sinensis* contained alkaloids, flavonoids, saponins, tannins, acidic compounds, terpenoids, proteins and glycosides in it. Methanolic extract of green tea contained flavonoids, terpenoids and glycosides.[29] Tannins, alkaloids and anthocyans have been reported by Akroum and Lalaoui (2012)[30] in the methanolic extract of tea. This is in accordance with our observations whereas the methanolic extract of *M. koenigii* had alkaloids, flavonoids, saponins, acidic compounds and phenol. Bhaskaran[31] also support our observation in respect of methanolic extracts of *M. koenigii*.

In the present study the order of antimicrobial activity of aqueous, ethanolic and methanolic leaf extracts of both the plants *C .sinensis* and *M .koenigii* using disk diffusion method against both the MRSA and MSSA isolates has been recovered in the order: aqueous < ethanolic < methanolic extract. The diameter of zone of inhibition (ZDI) observed in case of aqueous extract of *C. sinensis* ranged between < 10mm - 11 mm. However, bigger zone of inhibition (19.130 ± 0.250 mm) against MRSA isolates with 20 µl of aqueous extract of this plant containing 0.8mg/ml of this plant have been observed by others by disk diffusion method.[22]

MRSA and MSSA isolates were also sensitive to the alcoholic extracts of both the plants. The methanolic extract had relatively better inhibitory activity as compared to ethanolic extract. However, these isolates exhibited variability in their sensitivity to antibiotics: oxacilin (1 mcg), vancomycin (30 mcg) and chloramphenicol (30 mcg) which were used as controls in the assay. It is interesting to note that intermediate inhibitory activity of chloramphenicol (30 mcg) was observed against one MRSA isolate (no. 64) although the methanolic extract had significant inhibitory activity. The methanolic and ethanolic green tea extract in 15 µl volume that contained 15 µg of crude extract had inhibitory activity comparable to oxacillin and vancomycin. These extracts thus, can offer an alternative rather therapeutic option in treating MRSA and MSSA infections. Also, the active ingredients having inhibitory activity can be further analysed by HPLC and other suitable analytical methods.

Compared to MRSA, MSSA isolates were more sensitive to the green tea extracts. Among the MSSA isolates it is interesting that two isolates (isolates 4 and 10) were resistant to oxacillin and another isolate MSSA 55 to vancomycin as well as oxacillin but all the three strains were sensitive to all the crude extracts of *C. sinensis*.

In case of *M. koenigii,* all the isolates were sensitive to methanolic extract while 9/10 MRSA were sensitive to ethanolic extract and the zone size ranged from 13 - 40 mm and 11 - 32 mm respectively. One isolate (MRSA 64) had intermediate resistance to chloramphenicol. Maheshwari and Cholarani (2013)[32] also observed better inhibitory activity of methanolic extract of *M. koenigii*.

In case of MSSA, 8 isolates were sensitive to aqueous extract while 9 isolates were sensitive to ethanolic and methanolic extracts except one isolate MSSA 116 which was resistant to all the antibiotics included as control but at the same time showed sensitivity toward extracts. Infections due to such isolate might pose challenge as it was resistant to commonly used antibiotics, vancomycin and oxacillin used to treat *S. aureus.*[28] These results reflect that the plant extract offer a therapeutic option.

Our study shows that the aqueous extract of both the plants were not fully effective against MRSA and MSSA isolates, the less effectiveness of aqueous extract is consistent with the study of Elamparithi and Boominathan (2011).[33] Better inhibitory activity of all the extracts of both the plants i.e. *C. sinensis and M. koenigii,* has been previously reported in our laboratory by Katoch.[4] Methanolic extract exhibiting significant antimicrobial activity.

Similarly, Saini[34] have also observed that methanolic extract of *M .koenigii* had better anti bacterial activity than aqueous extract against *S. aureus*. The antimicrobial activity of plant extracts depends on the nature of the plant, mode of extraction and species under testing.[35]

It may be for this reason that some variations have been observed in the present investigations.

The MICs of crude ethanolic and methanolic extracts of *C. sinensis* against MRSA isolates ranged from 5 x 10^2 to 1 x 10^3µg/µl and 2.5x 10^2 to 1 x 10^3µg/µl respectively. MICs of ethanolic and methanolic extract of *M. koenigii,* were in the range of 1.25x 10^2 - 5 x 10^2 and 0.625 x 10^2 - 5 x 10^2 µg/µl respectively.

The MIC values for ethanolic and methanolic extracts *of C. sinensis* ranged from 2.5 x 10^2 - 10^3 µg/µl and 1.25 x 10^2 – 1 x 10^3 µg/µl respectively against MSSA isolates

while in case of *M .koenigii* these values ranged from 2.5 x 10^2 – 1 x 10^3 µg/µl respectively.

These results suggest that MICs of ethanolic extract of *C. sinensis* and *M. koenigii* were same for MSSA isolates (2.5 x 10^2 µg/µl) but MIC of methanolic extract of *C. sinensis* was lesser (1.25 x 10^2 µg/µl) as compared to *M. koenigii* (2.5 x 10^2 µg/µl). Following broth dilution method, Archana and Abraham (2011)[36] reported MIC 40 µl 400 µg of methanolic extract of fresh green tea for *S. aureus.* Hamillton-Miller and Shah (2000)[37] observed the antibacterial activity of green tea with a minimum inhibitory conc. of 0.28 mg/ml. Different workers have found different minimum inhibitory concentration values for different extracts of *M. koenigii*. Mathur[11] found MIC of methanolic extract of *M. koenigii* against *S. aureus* as 12.5 mg/ml while Choudhury (2013)[38] found this value as 64 mg/ml and Saini[34] as 0.312 mg/ml. In the present study, MICs of both ethanolic and methanolic extracts against MRSA of *M. koenigii* were relatively smaller i.e. 1.25x 10^2 and 0.625 x 10^2 µg/µl than the MIC values of *C. sinensis* which were 5 x 10^2 and 2.5 x 10^2 µg/µl. Mehrota[39] reported MIC of ethanolic extract of green tea against *S. aureus* and other pathogens as 0.1µg/µl. Aqil[40] reported that the MIC of ethanolic extract of *C. sinensis* ranged from <1.8 – 7.5 mg/ml for MRSA isolates. All these studies suggest that the leaf extracts of *C. sinensis* possessed significant inhibitory activity against *S. aureus* strains. The MIC values of ethanolic extract of this plant were determined as 6.37 ± 0.18 mg/ml by Handler.[41] Keeping in view the effectiveness of very minute amounts of the plant extracts against MRSA and MSSA isolates, it may be inferred that these extracts have therapeutic potential which needs further exploration. No difference was observed between the MIC_{50} values of ethanolic extract of both the plants against MRSA and MSSA isolates except for *M. Koenigii* against MSSA isolates in which case it was lower. MIC_{50} value of methanolic extract of *C. sinensis* was determined against MRSA as 8.5 x 10^2 and in case of *M. koenigii* this value was 6.25 x 10^2 for MRSA. In case of MSSA, the MIC_{50} of ethanolic extract of *C. sinensis* was recorded as 2.25 x 10^2 mg/ml while for *M. koenigii,* this value was 3.75 x 10^2 mg/ml.

CONCLUSION

It may be concluded from the present study that in general, MRSA in large proportions expressed virulence factors as compared to MSSA strains recovered from human patients.

The leaf extracts of *C. sinensis* and *M. koenigii* had very good inhibitory action on both the type even on those which were resistant to antibiotics which are used to treat *S. aureus* infections routinely. The study can be further extended to active principle based development of newer compounds as an alternative to treat multiple drug resistant MRSA and MSSA isolates.

REFERENCES

1. Inweregbu K, Dave J, Pittard A, Nosocomial infections. Continuing education in Anaesthesia, Critical Care and Pain, 5(1), 2005, 14–17.
2. Cameron DR, Howden BP, Peleg AY, The Interface Between Antibiotic Resistance and Virulence in *Staphylococcus aureus* and Its Impact Upon Clinical Outcomes, Clinical Infectious Diseases, 53(6), 2011, 576–582.
3. Maurice MI, Angela RD, Chris OO, New Antimicrobials of Plant Origin J. Janick (ed.), ASHS Press, Alexandria, VA 1999.
4. Katoch A, Batta B, Kumar A, Sharma PC, screening of *murraya koenigii* (curry) and *camellia sinensis* (tea) leaves for antimicrobial activity against strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida* species and their phytochemical analysis, IJPSR, 4(2), 2013, 862-868.
5. Batta B, Katoch A, Bhatia VK, Patil S, Sharma PC, Screening of leaf extracts of *Azadirachta indica* (neem), *Aegle marmelos* (bael) and *Trigonella foenum graecum* (methi) for their inhibitory activity on the strains of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida spp*, International Journal of Pharmaceutical Sciences Review and Research, 19(2), 2013, 42-46.
6. Patil S, Sharma Dk, Sharma PC, Emergence of Methicillin Resistant *Staphylococcus aureus* Strains (MRSA) and Multi Drug Resistant (MDR) Strains in Himachal Pradesh, Int J Pharm Sci Rev Res, 23(1), 2013, 1-5.
7. Sharma D, Bhatia VK, Patil S, Sharma PC, Antimicrobial activity of some selected Cryptogams from Solan region, International Journal of Biological and Pharmaceutical Research, 4(6), 2013, 448-454.
8. Sharma DK, Sharma PC, Bacteriophage typing of Methicilllin sensitive *Staphylococcus aureus* (MSSA) strains recovered from human clinical cases in Himachal Pradesh and their *in vitro* susceptibility to different antibiotics, Indian Journal of Basic and Applied Medical Research, 3(1), 2013, 296-302.
9. Qiao G, Li H, Xu DH, Park Soo I, Modified a Colony Forming Unit Microbial Adherence to Hydrocarbons Assay and Evaluated Cell Surface Hydrophobicity and Biofilm Production of Vibrio scophthalmi, J Bacteriol Parasitol, 3, 2011, 1.
10. Pourmand BJ, Somogy S, Guardado G, Aguirre C, Chan R, Meyer K, Kuhlman A, Townesmith A, Effio-Carbajal J, Frias-Fernandez F, Benito M, Minimum inhibitory concentrations of medicinal plants used in Northern Peru as antibacterial remedies, J Ethnopharmacol, 132(1), 2010, 101–108.
11. Mathur A, Dua VK, Prasad GBKS, Antimicrobial activity of Leaf Extracts of *Murraya koenigii* against aerobic bacteria associated with bovine mastitis, ijCEPr, 1(1), 2010, 12-16.
12. Tiwari RP, Bharti SK, Kaur HD, Dikshit RP, Hoondal GS, Synergistic antimicrobial activity of tea and antibiotics, J Med. Res, 122, 2005, 80-84.
13. Tariq M, Naveed A, Ali KB, The morphology, characteristics and medicinal properties of '*Camellia sinensis*' tea, J Med Plants Res, 4(19), 2010, 2028-33.
14. Joshua T, Takudzwa M, Antibacterial properties of *Mangifera indica* on *Staphylococcus aureus*, African journal of clinical and experimental microbiology, 14(2), 2013, 62-74.
15. Mwitari PG, Ayeka PA, Ondicho J, Matu EN, Bii CC, Antimicrobial Activity and probable Mechanisms of Action of Medicinal Plants of Kenya: Withania somnifera, Warbugia ugandensis, Prunus africana and Plectrunthus barbatus, PLoS ONE, 8(6), 2013.
16. Smith K, Perez A, Ramage G, Lappin D, Gemmell CG, Lang S, Biofilm formation by Scottish clinical isolates of *Staphylococcus aureus*, Journal of Medical Microbiology, 57, 2008, 1018–1023.

17. Hu C, Xiong N, Zhang Y, Rayner S, Chen S, Functional characterization of lipase in the pathogenesis of *Staphylococcus aureus*, Biochem Biophys Res Commun, 419(4), 2012, 617-20.
18. Friedrich R, Panizzi P, Fuentes-Prior P, Richter K, Verhamme I, Anderson PJ, Kawabata S, Huber R, Bode W, Bock PE, Staphylocoagulase is a prototype for the mechanism of cofactor-induced zymogen activation, Nature, 425, 2003, 535–539.
19. Akinkunmi EO, Lamikanra A, Phenotypic determination of some virulence factors in *staphylococci* isolated from faecal samples of children in Ile-Ife, Nigeria, Afr J Biomed Res, 15, 2012, 123-128.
20. Saising J, Singdam S, Ongsakul M, Piyawan S, Voravuthikunchai SP, Lipase, protease, and biofilm as the major virulence factors in *Staphylococci* isolated from acne lesions, BioScience Trends, 6(4), 2012, 160-164.
21. Bose S, Khodke M, Basak S, Mallick SK, Detection of biofilm producing *Staphylococci*: Need of the hour, Journal of Clinical and Diagnostic Research, 3, 2009, 1915-1920.
22. Ljungh A, Hjerten S, Wadstrom T, High surface hydrophobicity of autoaggregating *Staphylococcus aureus* strains isolated from human infections studied with the salt aggregation test, Infect Immun, 47(2), 1985, 522-526.
23. Radji M, Agustama RA, Elya B, Tjampakasari CR, Antimicrobial activity of green tea extract against isolates of methicillin resistant *Staphylococcus aureus* and multi-drug resistant *Pseudomonas aeruginosa*, Asian Pac J Trop Biomed, 3(8), 2013, 663-667.
24. Maurice MI, Angela RD, Chris OO, New Antimicrobials of Plant Origin, J. Janick (ed.), ASHS Press, Alexandria, VA, 1999.
25. Dogra D, Ahuja S, Krishnan S, Kohli S, Ramteke A, Atale N, Rani V, Phytochemical screening and antioxidative activity of aqueous extract of Indian *Camellia sinensis*, Journal of Pharmacy Research, 4(6), 2011, 1833.
26. Sarala S, Bharathi V, Phytochemical and Antibacterial Activity of *Murraya koenigii* Linn, IJPI's Journal of Pharmacology and Toxicology, 3(13), 2013.
27. Baskaran C, Bai VR, Kanimozhi V, Screening for antimicrobial activity and phytochemical analysis of various leaf extracts of *Murraya koenigii*, IJRAP, 2(6), 2011, 1807-1810.
28. Argal MS, Kumar S, Choudhary HS, Thakkar RM, Verma SK, Seniya C, The efficacy of *Murraya koenigii* leaf extract on some bacterial and a fungal strain by disc diffusion method, J Chem. Pharm. Res, 3(5), 2011, 697-704.
29. Nand P, Drabu S, Gupta RK, Phytochemical and antimicrobial screening of medicinal plants for the treatment of acne, Indian Journal of Natural Products and Resources, 3(1), 2012, 28-32.
30. Akroum S, Lalaoui K, Antimicrobial activity of some alimentary and medicinal plants, African Journal of Microbiology Research, 6(8), 2012, 1860-1864.
31. Baskaran C, Bai VR, Kanimozhi V, Screening for antimicrobial activity and phytochemical analysis of various leaf extracts of *Murraya koenigii*, IJRAP, 2(6), 2011, 1807-1810.
32. Maheswari NU, Cholarani N, Pharmacognostic effect of leaves extract of *Murraya koenigii* Linn, Journal of Chemical and Pharmaceutical Research, 5(4), 2013, 120-123.
33. Elamparithi D, Boominathan M, Comparative evolution of crude extract and fractions of *Camellia sinensis* leaves against human pathogenic bacterial strains *Streptococcus pneumoniae* and *Staphylococcus aureus*, International Journal of Universal Pharmacy and Life Sciences, 1(2), 2011, 147-154.
34. Saini SC, Dr. Reddy GBS, Birari P, Study for anti-infective potential of antibacterial properties of *Murraya Koenigii*, IOSR-JPBS, 7(5), 2013, 26-28.
35. Mbata TI, Debiao LU, Saikia A, Antibacterial activity of the crude extract of Chinese green tea (*Camellia sinensis*) on *Listeria monocytogenes*. African Journal of Biotechnology, 7(10), 2008, 1571-1573.
36. Archana S, Abraham J, Comparative analysis of antimicrobial activity of leaf extracts from fresh green tea, commercial green tea and black tea on pathogens, Journal of Applied Pharmaceutical Science, 1(8), 2011, 149-152.
37. Hamilton- Miller JMT, Shah S, Activity of tea components epicatechingallate and analogueous against methicillin resistance *Staphylococcus aureus*, J Antimicrob Chemotherap, 46, 2000, 852-853.
38. Choudhury S, phytochemical and antimicrobial standardization of the methanolic leaf extracts of *Murraya koenigii* linn, Archives Des Sciences, 66(3), 2013, 67-80.
39. Mehrotra S, Srivastava AK, Shoma PN, Comparative antimicrobial activities of Neem, Amla, Aloe, Assam Tea and Clove extracts against Vibrio cholera, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, Journal of Medicinal Plants Research, 4(18), 2010, 2473-2478.
40. Aqil F, Khan MS, Owais M, Ahmad I, Effect of certain bioactive plant extracts on clinical isolates of beta-lactamase producing methicillin resistant *Staphylococcus aureus*, J Basic Microbiol, 45(2), 2005, 106-14.
41. Handral H, Hoti SL, Shruthi SD, *In vitro* evaluation of antimicrobial activities of crude extracts from *Murraya koenigii* against pathogenic bacteria, International Journal of Pharmacy & Pharmaceutical Sciences, 4(4), 2012, 74.

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