European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 7, Issue 09 , 2020

ANTIBACTERIAL ACTIVITY OF SELECTED NATURAL EXTRACT AGAINST METHICILLIN SENSITIVE STAPHYLOCOCCUS AUREUS

Ms. Asalatha.R[1], Dr. Anand Thirupathi[2]

[1]Research Scholar, SRK University, Bhopal, India.

[2]Research Supervisor, SRK University, Bhopal, India.

ABSTRACT

Antibiotic toxicity and multi drug resistant pathogens are the two greatest challenges being faced by today's medical world. In the present study, the antimicrobial activity of spices has been investigated as an alternative to antibiotics in order to tackle these dangers. In search of bioactive compound, methanol ethanol and aqueous extract of 3 three Indian spices were screened for antibacterial property. The antibacterial activity of three common Indian spices namely clove, cinnamon and turmeric against bacteria Staphylococcus aureus. The results revealed that the methanol extracts of spices (MIC values of 20- 100 µl/ml) have high antimicrobial activities on all test organisms (range of inhibition, 6- 16 mm) as compare to ethanol and aqueous extracts of spices in same concentration. Results concluded that these spices contain high amount of secondary metabolites due to these metabolites they have high antimicrobial activity and it can be used as good bio- preservater and it can also use for medicinal purpose.

Key words: Staphylococcus aureus, MSSA, ethanol, cinnamon.

INTRODUCTION

Plants have been a valuable source of natural products for a long period of time to maintain human health, especially with more intensive studies in the last decade for natural therapies (Gislene et al., 2000). Spices have been used for not only flavor and aroma of the foods but also to provide antimicrobial properties (Nanasombat et al., 2002). Spices may contribute piquancy of

foods and beverages (Praveen et al., 2006). In addition to these spices are some of the most commonly used natural antimicrobial agents in foods. Some of the natural compounds found in various spices possess antimicrobial. (Hatha et al., 2006). Keeping in view this fact the present study was conducted to find out the antimicrobial activity of three spices including Clove (Eugenia caryophyllus, family Mytraceae), Cinnamon (Cinnamomum zylancium, family Lauraceae), and Turmeric **(**Curcuma longa family Lauraceae,).

In order to fight bacterial infections, medicine has largely relied on antibiotics, which are naturally occurring or artificially created chemical substances capable of killing or controlling the growth of bacteria. In present times, however, bacteria have developed resistance to many of the antibiotics commonly used to treat infections (Kaneria et al.,2009). Methicillin Sensitive Staphylococcus aureus (MSSA) causes a type of staph infection that is moderately sensitive to these antibiotics(Lissner et al., 2001).

MATERIAL AND METHOD

Preparation of Spice extracts

The plant materials used in this study consisted of as turmeric, clove and cinnamon and the plants material were collected, authenticated and processed before carrying out the bioactivity studies. Extracts were made by taking 25g of powdered samples separately in a thimble and extracted successively with ethanol, methanol and water using a soxhelt for 48 hrs. The turmeric and cinnamon and clove were then filtered and centrifuged to remove the ground spice particles from the mixtures. All the three spice extracts were concentrated using rotary flash evaporator and stored at 5°C in air tight bottle until further use.

Collection of samples

The samples were collected from 25 patients of wound pathogens from the Royal Care Hospital, Madurai, Tamilnadu, India. Samples were taken aseptically by using sterile cotton swabs. Swabs were dipped in transport media and immediately transferred.

Isolation and identification of S. aureus

Samples were directly inoculated on Mannitol Salt Agar MSA (Oxoid), a selective and differential media of S. aureus and incubated at 37**o**C for 24 hour. Identification was done by colony morphology, gram staining, catalase test, coagulase test and mannitol fermentation (Alabri et al., 2014.

Identification of MRSA

Sampling was done as.10^{-1} dilution was inoculated on Baird Parker Agar plate. Black colonies surrounded by clear zone were the characteristic of staphylococcus colonies on this agar. Coagulase test was performed to confirm Staphylococcus aureus.

Antibiotics and Non-Antibiotics used

Vancomycin, Cefotaxime, Ofloxacin, Ceftriaxone, Ceftazidime, Tetracyclines, Amikacin**,** Chloramphenicol**,** Gentamicin**,** Ampicillin**,** Neomycin, Cefazolin, Cefalexin, Nalidixic acid, Co-trimoxazole, Erythromycin, Pencillin G and Rifampicin and the non-antiiotics like Loperamide, Paracetamol and Vitamin C were purchased from Kavitha pharmacies in Madurai city, Tamilnadu.

Antibiotics activity assay

Antibiotic discs were placed on the surface of a Mueller-Hinton agar that has been inoculated with test microorganisms. During incubation, the antibiotics diffuse outward from the discs creating a concentration gradient. After 18-24 hours, the zone diameter of inhibition is measured and reference tables are used to determine if the bacteria are Sensitive (S), Intermediate (I) or Resistant (R) to the antimicrobial drugs.

Paper Disk Diffusion Assay

Suspensions of testing microorganisms were spread on Muller Hinton Agar (MHA) medium. The filter paper discs (5mm in diameter) was placed on the agar plates which was inoculated with the tested microorganisms and then impregnating with 20µl of plant extract (concentration 200 mg/ml). The plates were subsequently incubated at 37° C for 24 Hrs. After incubation the growth inhibition zone were quantified by measuring the diameter of the zone of inhibition in mm.

Well diffusion method assay

An inoculum suspension was swabbed uniformly to solidified 20 mL Mueller-Hinton Agar (MHA) for bacteria, and the inoculum was allowed to dry for 5 min. Holes of 6 mm in diameter were made in the seeded agar using glass Pasteur pipettes. Aliquot of 20 µl from each plant crude extract (200 mg/ml) was added into each well on the seeded medium and allowed to stand on the bench for 1 h for proper diffusion and thereafter incubated at 37°C for 24 h. The resulting inhibition zones were measured in millimeters (mm).

Determination of MIC of plant extract by Microdilution Method

The isolates grown on 6µg methicillin containing agar were further processed to obtain MSSA by performing MIC of methicillin by broth micro dilution test adapting the CLSI recommended procedure and standards.MIC was defined as the lowest sample concentration showing no color change (clear) and exhibited complete the inhibition of growth.

Synergism activity between plant extract, antibiotics and Non-antibiotics

The bacterial cultures were grown in BHI broth at 37º C. After 4 h of growth, each bacterium was inoculated on the surface of Mueller-Hinton agar plates. Subsequently, the antibiotic disk (diameter-5mm) was placed on the surface of each inoculated plate and then added 20 µl of plant extract (at a concentration of 200mg/ml), to identify synergies effect between the plant extract and antibiotics, and in the same way 20 µl was taken from each dilution of the Non-antibiotic drugs and put on antibiotic disk, to identify synergies between the Non-antibiotics and antibiotic. While to identify synergies between the plant extract & Non-antibiotics, 20 µl of Non- antibiotics and 20 µl of plant extracts were mixed and put together on a filter paper disk which was left for one hour to dry. The plates were incubated at 37º C for 24 h. The diameters of clearing zones were measured.

Antibiotic discs were placed on the surface of a Mueller-Hinton agar that has been inoculated with test microorganisms. During incubation, the antibiotics diffuse outward from the discs creating a concentration gradient. After 18-24 hours, the zone diameter of inhibition is

measured and reference tables are used to determine if the bacteria are Sensitive (S), Intermediate (I) or Resistant (R) to the antimicrobial drugs.

RESULTS AND DISCUSSION

Spices have been used for not only flavor and aroma of the foods but also to provide antimicrobial properties. Spices may contribute piquancy of foods and beverages. In addition to these spices are some of the most commonly used natural antimicrobial agents in foods. Some of the natural compounds found in various spices possess antimicrobial. Therefore, actions must be taken to control this problem by using the plant extracts containing phytochemical having antimicrobial properties (Yamaguchi et al., 2009).

The quantitative phytochemical analysis of ethanolic, methanolic and aqueous extract of turmeric contains presence of alkaloids, tannins, flavonoids, saponins, proteins and terpenoids. The clove yielded the presence of alkaloids, carbohydrates, phenolic compounds, tannins, saponins, flavonids and terpenoids. Cinnamon extracts observed the phytochemical compounds like contains Tannin, Terpenoids, Alkaloids, Terpenoids, amino acids and Polyphenol.

Resistance to methicillin has led to global concerns owing to the fact that methicillin is well thought-out as the last successful drug to treat the Staphylococcal infections. Based on the distribution of characteristic colonies on the culture plate and the identification of the representative colony, the relative distribution of the various bacterial groups obtained. Each wound samples showed methicillin resistant Staphylococcus aureus (MSSA) were predominant isolates (60%).

Previous studies selected locally available plants like C.infortunatum, H. indicus, H. anti dysenterica, M. indica and P. granatum, were selected and their antimicrobial activity was evaluated using Disc diffusion method. Most tested plant extracts showed antibacterial activity against E.coli, S.aureus and P.aurgenosa which may reflect the antibacterial activity of plant active ingredients that inhibit bacterial growth (Voravuthikunchai et al., 2004).

The evolutionary origin of methicillin-sensitive strains of S. aureus is of immense importance to the medical community. Researchers have used a DNA microarray representing >90% of the S. aureus genome to characterize genomic diversity, evolutionary relationships, and virulence gene distribution among 36 strains of organisms causing toxic shock syndrome (Eze et al., 2013). It has been found that genetic variation in S. aureus is very extensive, with ≈22% of the genome comprised of dispensable genetic material (Gitig, 2013). Some of these genes represent nonessential genetic information that is strain-specific. Some of these genes encode factors that facilitate colonization of specialized host or environmental niches.

Antibiotics activity against methicillin sensitive staphylococcus aureus (MSSA)

By disc plate method, the effectiveness of a range of antibiotics was determined against methicillin sensitive staphylococcus aureus. Chloramphenicol was showed the highest inhibition zone against MSSA (24 mm). While it was resistance to tetracyclines, ofloxacin, ampicillin, cefazolin, nalidixic acid and co-trimoxazole (Fig- 1).

Evaluation of plant extracts bioactivity

Well Diffusion Method for MSSA

The results of the effects of methanolic, ethanolic and aqueous extracts of the plants natural extracts using 20µl from the extracts (200 mg/ml crude extract) against the tested methicillin sensitive S.aureus (MSSA). It is shown that methanolic and ethanolic extract of turmeric have the highest effect on MSSA, with a zone of inhibition (19 mm) and (20 mm) respectively (Table-1).

In aqueous extracts, clove was showed the highest effect against MSSA. In which clove extract had a zone of inhibition (14 mm) which was more effective. Turmeric was less effective against MSSA, and each of cinnamon does not have any effect against MSSA.

Disc Diffusion Method for MSSA

The methanol and ethanol extracts of cinnamon showed the highest effect towards MRSA (with a 15 mm zone of inhibition) followed by clove (with a 14 mm zone of inhibition) (by methanol extract). Turmeric (extracted by ethanol) with a zone of inhibition (13mm). The aqueous extracts for 2 h of clove showed the highest activity against MSSA with 15 mm and turmeric showed 12 mm zone of inhibition, respectively (Table-1). Cinnamon showed little activity against MRSA (with 8mm inhibition zone).

MIC of methicillin sensitive S.aureus (MSSA)

The MIC of the methanol extracts against MSSA for each of turmeric, 12.5 mg/ml and clove and cinnamon were 6.25 and 50 mg/ml respectively. The MIC of the ethanolic extract for each of turmeric and clove against MSSA were 25 and 6.25 mg/ml, respectively. But for cinnamon was from 6.25 to 12.5 mg/ml. The MIC of the aquatic extracts of turmeric against MSSA was 50 mg/ml and of clove was 25mg/ml; cinnamon MIC was from 3.125 to 6.25 mg/ml (Table-1).

The MIC of ampicillin against MSSA ATCC 11632strain was 6.25 µg/ml, which is within the acceptable range of susceptibility. A betalactam antibiotic inhibits the PBPs involved in late stage of peptidoglycan biosynthesis. Interference with peptidoglycan biosynthesis causes deformities in the bacterial cell wall and eventually leads to cell death due to high internal osmotic pressure (Mosby, 2009).

Evaluation of Non-Antibiotics activity

Loperamide HCl (with all concentrations) was exhibited distinct antibacterial activity against MSSA, but Paracetamol and Vitamin C did not show this antibacterial activity. Vitamin C did not show any antibacterial activity at any of the concentrations used against MSSA (Table-2).

Evaluation of the Synergistic effect against MSSA

Concept of synergism is applied to treat infections caused by deleterious pathogens to tackle the complex multi-drug resistance issues (Zibbu and Batra, 2010). A review by Gibbons, compiled antibacterial and modifying resistance properties of compounds from plant origin against Staphylococcal species showing use of plant products as anti staphylococcal agents

.Several compounds, such as epicatechin gallate, totarol and corilagin reversed methicillin-resistance in MRSA by reducing MIC of betalactams when combined (Bhunia, and Ray,2008).

Methanolic Extraction and Antibiotics

Turmeric extract has the best synergistic effect on MSSA when added on amikacin disk (19mm) followed by neomycin (17mm) and chloramphenicol (26mm). As for tetracycline and ampicillin, their influences (on MSSA) with the turmeric extract indifference. With the rest antibiotics there was either no effect or there was antagonism.

The clove extract showed the best synergism with amikacin (19mm) followed by ceftriaxone (13mm), and as in influence the turmeric extract with Tetracycline and Ampicillin, It was their influence with clove extract indifference. The cefotaxime, ceftazidime, cefazolin and co- trimoxazole have antagonism effect.

Finally the synergistic effect of cinnamon extract with amikacin (19mm) was the highest synergistic effect on this bacteria followed by chloramphenicol (28mm). It had the same synergistic effect with both of tetracycline and nalidixic acid (9mm) against MSSA. With regard to ofloxacin, ceftriaxone and gentamicin there was no any synergistic effect with cinnamon extract. On the other hand, there was antagonism with ceftazidime, neomycin, cefazolin and ceflexin (Table-3).

Ethanolic Extraction and Antibiotics

Turmeric extract had the highest synergistic effect with amikacin (19 mm) followed by ceftriaxone (14mm). ampicillin, ofloxacin, nalidixic acid and cefazolin had weak or negligible synergistic effect (8, 7, 7 and 7mm, Respectively) and tetracycline with ethanolic extract of turmeric was indifference. And in clove extract showed it with both of amikacin and ceftriaxone has the best synergy (17 and 16mm, respectively). And each of ampicillin, ceftazidime, Cefotaxime and nalidixic acid are shown with it the same effect on MSSA (Inhibition zone 9mm). As for effect each of tetracycline, cefazolin and co- trimoxazole was indifference. The reason is that effect of ethanolic extract of clove alone is (7mm). And these antibiotics have no effect on MSSA, whether alone or with this extract. As for the ethanolic extract each of cinnamon had the highest synergistic effect with amikacin (20 and 21mm, respectively). And their influence with ceftriaxone, ceftazidime, cefazolin and cephalexin are antagonistic (Table-4).

Aquatic Extraction and Antibiotics

The best synergistic effect was with cefotaxime with aqueous extracts of the clove (inhibitory zone 14 mm). As well ofloxacin had better synergistic effect with clove extract (19 and 17 mm, respectively) and with the rest of the extracts, there was no any effect or influence of the antagonistic. The ceftriaxone has increased effectiveness with presence an extract of the turmeric (16 mm) and followed by cinnamon (15 mm) and clove extract (14mm). As for amikacin has increased its effectiveness with all three natural extracts. But the best effect with both of the extract of clove and cinnamon (Inhibition zone for each them 18 mm).

As for gentamicin was his best effect with aqueous extract of turmeric (10mm). While each of tetracycline and co- trimoxazole there has not been any synergistic effect to them except with clove though it's little effect (8mm).As for ampicillin there has not been any effect but only with both extract of clove and cinnamon. It was their best with extract of the turmeric. While chloramphenicol has had a synergistic effect with both extracts turmeric (22mm) and clove (24mm). It was his best effect with extract of the cinnamon (28mm). And while neomycin has a synergistic effect with all extracts except extract of the turmeric (13mm) (Table-5).

The Synergistic Effect of Non-Antibiotics with Antibiotics

Ivanescu et al, 2015, reviewed synergistic effects of Chinese herbal medicine with conventional antimicrobials and discussed various models to demonstrate the same. They described Disc diffusion assay as a simple, visual and qualitative microbiology assay to represent synergistic or antagonist effect of individual components used together. It was further stated that, it is critical to distinguish between synergistic effect and simple additive effect of individual herbs or active ingredients in a complex herbal formulations.

The combinations of antibiotics and Non-antibiotics was showed a weak synergistic activity. As Non-antibiotics drugs showed antagonistic effect with most antibiotics, the best synergistic activity was between Ampicillin and Paracetamol (at concentration of 100 μM), and between Pencillin G and also Paracetamol(at concentration of 10 μM). Loperamide Hcl had the best synergistic activity wih Ceftazidime and Ampicillin (at concentration of 100 μM), and with Vancomycin (at concentration of 50 μM), and with Ampicillin and Pencillin G (at concentration of 50 and 10 μM). While the combination of antibiotics with Vitamin C has shown antagonistic effect with all antibiotics, except Ceftazidime (Fig-2).

Besides that, previous studies show plant metabolites have tendency to bind protein including PBPs or cell-wall building blocks which explains their antibacterial activity against MSSA (Nadkarni, 1954.).

COCLUSION

In present studies antimicrobial activity of three spices clove, cinnamon and turmeric were done. The data supports the hypothesis that some common Indian spices have an inhibitory effect on the growth of certain food borne pathogens in tissue culture. The results suggest that turmeric, clove and cinnamon powder, they produced significant antimicrobial effects.

This study concludes that the plant extracts like turmeric, clove and cinnamon may be further tested to understand their clinical application as potential dermally applicable ointments to alleviate MSSA wound infections. It could also be particularly beneficial towards treating hospital patients with MRSA or MSSA infections, as well as healing people in underdeveloped countries where antibiotics are not readily available.

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Plant extract	**Well diffusion method**				**Disc diffusion method**				**MIC**		
	M	**E**	**W**	**C**	**M**	**E**	**W**	**C**	**M**	**E**	**W**
turmeric	19	20	-	-	09	13	12	-	50	12.5	6.25

cinnamon	-	-	-	-	15	14	8	-	6.25-12.5	25	6.25
clove	-	13	14	-	14	09	15	-	3.125	50	6.25

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Table-1:Antimicrobial Activity of Plant extracts on MSSA by well diffusion method and disc diffusion method

***** Antimicrobial Activity Assays.
Control= DMSO
Method of extraction: M= methanol, E= ethanol, W= water
(-) No inhibition zone

Table-2:Non-antibiotic activity assay

Non-Antibiotics	**Paracetamol**				**Loperamide Hcl**				**Vitamin C**			
Microorganism	100 µM	50 µM	10 µM	C*	100 µM	50 µM	10 µM	C*	100 µM	50 µM	10 µM	C**
Staphylococcus aureus MSSA	0	0	0	0	11	11	12	9	0	0	0	0

* Control = methanol
** Control = Distilled water

Table-3: Synergism Between Antibiotics and Methanolic Extracts of Plant against MSSA

Antibiotic	Antibiotic alone	Turmeric		Clove		Cinnamon	
		Ex.	Ex+ Anti	Ex.	Ex+ Anti	Ex.	Ex+ Anti
CTX	8	7	-	9	8	7	10
OF	0		-		8		7
CTR	9		-		13		9
CTZ	11		-		-		7
AK	10		19		18		19
GN	6		-		9		7
TE	0		7		9		9
AMP	0		7		9		8
CL	24		26		25		28
N	14		7		15		13
N.A.	0		-		7		9
KZ	0		-		-		-
CN	7		7		7		-
STX	0		-		-		8

(-) No synergism; CTX: Cefotaxime; OF: Ofloxacin; CTR: Ceftriaxone;
CTZ: Ceftazidime**;** AK: Amikacin; GN: Gentamicin; TE: Tetracycline; AMP: Ampicillin; CL: Chloramphenicol; N: Neomycin; N.A: Nalidixic acid; STX: Co- trimoxazole; KZ: Cefazolin; CN: Ceflexin (Cephalexin).

Table-4: Synergism Between Antibiotics and Ethanolic Extracts of Plant against MSSA

s	Antibiotic alone	Turmeric		Clove		Cinnamon	
		Ex.	Ex+ Anti	Ex.	Ex+ Anti	Ex.	Ex+ Anti

CTX	8	6	10	7	9	7	10
OF	0		7		8		8
CTR	9		14		16		7
CTZ	11		10		9		7
AK	10		19		17		21
GN	6		8		8		9
TE	0		6		7		9
AMP	0		8		9		9
CL	24		26		25		28
N	14		16		15		14
N.A.	0		7		9		7
KZ	0		7		7		-
CN	7		8		10		-
STX	0		8		7		8

(-) No synergism; CTX: Cefotaxime; OF: Ofloxacin; CTR: Ceftriaxone;
CTZ: Ceftazidime**;** AK: Amikacin; GN: Gentamicin; TE: Tetracycline; AMP: Ampicillin; CL: Chloramphenicol; N: Neomycin; N.A: Nalidixic acid; STX: Co- trimoxazole; KZ: Cefazolin; CN: Ceflexin (Cephalexin).

Table-5: Synergism between Antibiotics and Aquatic Extracts of Plant against MSSA

Antibiotic	**Antibiotic alone**	**Turmeric**		**Clove**		**Cinnamon**	
		Ex.	**Ex+ Anti**	**Ex.**	**Ex+ Anti**	**Ex.**	**Ex+ Anti**
CTX	8		19		14		-
OF	0		-		17		-
CTR	9		13		-		-
CTZ	11		-		7		-

AK	10	8	17	7	18	7	17
GN	6		-		-		-
TE	0		-		-		-
AMP	0		7		-		-
CL	24		28		26		-
N	14		15		15		13
N.A.	0		-		-		-
KZ	0		-		-		-
CN	7		7		-		-
STX	0		-		-		-

(-) No synergism; CTX: Cefotaxime; OF: Ofloxacin; CTR: Ceftriaxone;
CTZ: Ceftazidime**;** AK: Amikacin; GN: Gentamicin; TE: Tetracycline; AMP: Ampicillin; CL: Chloramphenicol; N: Neomycin; N.A: Nalidixic acid; STX: Co- trimoxazole; KZ: Cefazolin; CN: Ceflexin (Cephalexin).

Fig-1: Evaluation of antibiotics activity S.aureus

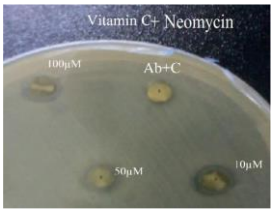
Inhibition zone (mm) of some antibiotics against MSSA

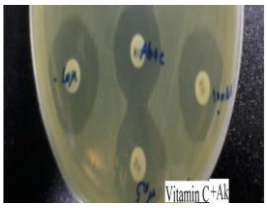
Fig-2: Synergistic effects between Antibiotics and Non-Antibiotics

Cinnamon extract

The combination effect of Ceftriaxone with

Paracetamol and Loperamide Hcl on MSSA





The combination effect of Neomycin and

Amikacin with Vitamin C on MSSA