REPORT

Isolation identification and control of vancomycin resistant *Staphylococcus aureus*

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Abstract: Vancomycin resistant *Staphylococcus aureus* (VRSA) has been reported from many parts of the world including Asian countries. Hence, main objective of study was to evaluate the possible occurrence of VRSA in hospitals of Lahore city and to ensure the effectiveness of various substitute therapeutic options. A total of 150 samples of pus/wounds were collected from three hospitals of the city and VRSA were isolated and confirmed through recommended method of Clinical and Laboratory Standards Institute. Out of 51 (49.04%) methicillin resistant *S.aureus* (MRSA) isolates, 5 (9.8%) were found resistant to vancomycin. Minimum inhibitory concentration (MIC) of Linezolid (LZD), Moxifloxacin (MFX) and Clindamycin (CD) were calculated against VRSA isolates by broth microdilution test. All 5 (100%) isolates were susceptible to Linezolid and Clindamycin, while 4 (80%) were susceptible to Moxifloxacin. Ethanolic extracts of Turmeric, Mint, Coriander, Garlic, Kalonji, Cinnamon and Cloves illustrate average MIC values of 140.8µg/ml, 563.2µg/ml, 486.4µg/ml, 614.4µg/ml, 409.6µg/ml, 281.6µg/ml and 64µg/ml, respectively against 5 VRSA strains. Concentration dependent increase in growth inhibition zones of ethanolic plant extract was recorded by agar well diffusion test. This study was helpful to find out the effective antibiotic against VRSA. Plant extracts encompass anti-staphylococcal activity and this finding demands necessity of further exploration of potential found in these natural herb.

Keywords: Vancomycin resistant *Staphylococcus aureus,* MIC, broth microdilution test, ethanolic extracts, anti-staphylococcal.

INTRODUCTION

Staphylococcus aureus is an imperative member of the genus *Staphylococcu*s containing pathogenic and non pathogenic organisms. As a Predominantly important fact, this bacteria is overwhelming most of the therapeutic agents which have been built-up against it (Kaleem *et al.,* 2010). *S. aureus* infections may leads to high morbidity and mortality because of its challenging treatment due to the emergence of resistance to many antibiotics including methicillin and vancomycin (Dhanalakshmi *et al.,* 2012). Vancomycin resistant *S. aureus* (VRSA) are those clinical isolate of Methicillin Resistant *Staphylococcus aureus* (MRSA) which shows *in vitro* non susceptibility to vancomycin (Kaleem *et al.,* 2012).

Antimicrobial resistance is continuously developing among *S. aureus* isolates parallel with the drugs developed against it. In 1940's penicillin antibiotic has been launched against gram positive bacteria, and resistance is on track to build up against penicillin in genus *Staphylococcus* (Shahriar *et al.,* 2012). MRSA is a multi-drug resistant isolate, reported soon after methicillin's use in medical practice in 1961 from United Kingdom (Darogha 2009; Khan *et al.,* 2010). In the 1980s, as a result of the increased incidence of MRSA, vancomycin a glycopeptide has been broadly used because it is the solitary predictable active antibiotic against all isolates of *S. aureus* and MRSA. In 1990s arbitrary use of vancomycin increased the selective pressure to facilitate the surfacing of *Staphylococcus* species with lesser vulnerability to glycopeptides. Recently situation is terrified with the discovery of VRSA (Khan *et al.,* 2010; Dhanalakshmi *et al.,* 2012; Shahriar *et al.,* 2012; Zuo *et al.,* 2012). In 1997, vancomycin intermediate resistant *S. aureus* (VISA) were first time reported from Japan. Now the emergence of VRSA from different parts of the world illustrates that it is an international concern. So far VRSA has been reported from Japan, USA, France, Korea, South Africa, Brazil, and Scotland while heteroresistant-VRSA (hVRSA) strains have also been isolated from many countries. Unfortunately after the non-susceptibility to glycopeptides there are no official recommendations about the treatment of infections of *Staphylococci*. Linezolid has been used against MRSA and vancomycin resistant isolates of MRSA, it is quite effective against intricate nosocomial and community acquired illnesses (Watanabe *et al.,* 2008). Similarly Clindamycin have excellent activity against MRSA isolates so it can be a bactericidal alternative for treatment of multidrug resistant isolates (LaPlante *et al.,* 2008). Moxifloxacin is a

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synthetic fluoroquinolone, with broad-spectrum antibiotic activity has documented to comprise killing potential against *S. aureus* and MRSA (Jacobsen *et al.,* 2011).

Modern science is however ardent to ascertain new antimicrobials from various sources such as plant, animal, and microorganisms. Plant has long been known to contain some natural substances which have therapeutic potential and antimicrobial toxic properties against different microorganisms (Khan *et al.,* 2010). World Health Organization (WHO) anticipated that about 80% of the world population uses herbal medicine (Ansari *et al.,* 2011). According to estimation, local communities have used nearly 10% of all the flowering plants present on earth to treat diseases, but only 1% has been renowned by modern science (Khan *et al.,* 2009). The aim of the current study is to isolate and identify VRSA from wounds of clinical patients and to check the invitro effectiveness of antibiotics and plant extracts.

MATERIAL AND METHOD

Collection of samples

A total of 150 samples of surgical and septic wounds exudates/pus were collected from different patients of Myo Hospital, Service Hospital and Sheikh Zayed Hospital Lahore. Samples were taken aseptically by using sterile cotton swabs. Swabs were dipped in transport media and immediately transferred to University Diagnostic Lab, University of Veterinary and Animal Sciences, Lahore.

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 37ºC for 24 hour. Identification was done by colony morphology, gram staining, catalase test, coagulase test and mannitol fermentation (Akhter *et al.,* 2009).

Identification of MRSA

Identification of MRSA was done by Kirby Bauer disc diffusion method on Mueller-Hinton agar using 1µg oxacillin and 30µg cefoxitin discs. Isolates showing no zone of inhibition or zone ≤10mm and ≤21mm for oxacillin and cefoxitin, respectively were taken as MRSA following the criteria of CLSI (CLSI 2011).

Vancomycin agar screening test

Preparation of screening agar

To prepare vancomycin screening agar plates 1000ml of Brain Heart Infusion agar (BHI) was prepared according to manufacturer's instructions (Oxoid). Media was mixed well and dissolved by heating until complete dissolution. Sterilization was done by autoclaving at 121ºC for 15 minutes. Media was cooled to 45-50ºC and 6.0mg of Vancomycin was added aseptically followed by gentle homogenization and pouring into Petri dishes. Prepared medium was stored at 8-15°C (Tiwari and Sen 2006).

Inoculation on BHI Vancomycin Screening Agar

All MRSA isolates were processed for vancomycin agar screening performed on BHI agar supplemented with 6µg/ml vancomycin. One or two pure colonies were directly suspended in sterilized normal saline to obtain the turbidity equal to 0.5 McFarland turbidity standards and 10µl of this suspension was spreader on vancomycin containing BHI agar plates. All the isolates were inoculated in duplicates on vancomycin agar screening pates with positive and negative control strains. Plates were incubated for 24 hours at 37^{o}C in ambient air. Plates containing more than one colony or a thin film of growth or growth similar to positive control strain were taken as positive and isolates showing positive result on both inoculated plates were considered as confirmed VISA. *Enterococcus faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 were used as negative and positive control respectively (Bhateja *et al.,* 2005).

MIC by broth microdilution method

VISA isolates grown on 6µg vancomycin containing agar were further processed to obtain VRSA by performing MIC of vancomycin by broth micro dilution test adapting the CLSI recommended procedure and standards.

Antimicrobial potential of antibiotics against VRSA

MIC and MBC calculation of antibiotic

Linezolid, Clindamycin and Moxifloxacin were tested *in vitro* against VRSA isolates. MIC was performed using standard broth micro dilution test with the final inoculum of 5×10^5colony forming units (CFU)/ml in each well of the 96-well plate according to the CLSI guidelines and incubated at 35°C for 24 hour. MIC is the minimum concentration or highest dilution that prevents visible growth. MBC was determined by culturing 50µl of the suspension from wells where no visible growth was found on MSA plates. MBC will be the minimum concentration or highest dilution of the extract that does not yield any growth on the mannitol salt agar (Zuo *et al.,* 2012).

Antimicrobial potential of herbs against VRSA

Preparation of plant extract

Botanically identified plants including Mint, Coriander, Garlic, Kalonji, Turmeric, Cloves and Cinnamon were collected. The plant material was washed, dried and ground into fine powder. This powder was soaked with ethanol 1:10w/v and placed for extraction for 24 hours with shaking at 150rpm for extraction of active ingredients. Extracts were filtered through Whatman No.1 filter paper and filtrates were dried at 40°C until a solid residue was obtained (Obeidat 2011). Stock solution of each extract was prepared in Dimethyl sulfoxide (DMSO) (Chomnawang *et al.,* 2009).

Table 1: Prevalence of MRSA and VRSA in various hospitals of Lahore city

Name of hospital	Number of sample	*S.aureus.*	MRSA	VISA	VRSA
		No. (%)	No. (%)	No. (%)	No. (%)
Service Hospital	50	31 (62)	13 (42)	6 (46.15)	1 (7.69)
Myo Hospital	50	43 (86)	27 (62.80)	12 (44.44)	4 (14.82)
Sheikh zayed Hospital	50	30 (60)	11(36.70)	4 (36.36)	0 (0)
Total	150	104 (69.33)	51 (49.04)	22 (43.14)	5 (9.8)

Table 2: Minimum inhibitory concentration and minimum bactericidal concentration of various antibiotics against VRSA isolates

Sr. No.	Isolate ID	MIC value in µg/ml			MBC value in µg/ml			Result		
		LZD	MFX	CD	LZD	MFX	CD	LZD	MFX	CD
VRSA1	11	2	0.25	0.5	8	1	2	S	S	S
VRSA2	21	2	0.25	0.25	8	1	0.5	S	S	S
VRSA3	25	4	8	0.5	16	8	2	S	R	S
VRSA4	26	2	0.25	0.25	8	0.5	0.5	S	S	S
VRSA5	45	2	0.5	0.25	8	1	1	S	S	S

Key: LZD: Linezolid, MFX: Moxifloxacin, CD: Clindamycin

Well diffusion test

The antimicrobial activity of ethanolic plant extracts was screened against VRSA isolates by using the agar well diffusion assay (Baeer *et al.,* 1996). Holes of 6mm in diameter were made in the seeded agar using sterile borer. An inoculum suspension containing approximately 1×10^8 CFU/ml was swabbed uniformly on MHA plates and was allowed to dry for 5min. stock solution of plant extract was diluted in DMSO to prepare three concentrations 100 mg, 200mg and 400mg/ml. Aliquot of 50µl from each dilution was added into different wells on the seeded medium which results in three different concentrations of extracts e.g. 5mg, 10mg and 20mg per well. Plates were allowed to stand on the bench for one hour for proper diffusion, and thereafter incubated at 37°C for 24h. Antimicrobial activity was evaluated by measuring the inhibition zone diameter in millimeters (mm) around the wells. Aliquots of 50µl of DMSO were used in the same manner as negative control (Obeidat 2011). Rifampin with a concentration of 5µg per well was used as positive control (Sudhir *et al.,* 2012).

MIC and MBC calculation of plant extracts

Plants with significant antimicrobial activity against VRSA were processed for the MIC and MBC calculation using similar method mentioned above for antibiotics.

RESULTS

Resistance to vancomycin has led to global concerns owing to the fact that vancomycin is well thought-out as the last successful drug to treat the *Staphylococcal* infections. Current study indicated higher rate of VISA occurrence (43.14%) in different hospitals of Lahore (Table 1). and illustrated 9.8% recovery rate of VRSA isolates with a significant variation among three hospitals selected in the study. Occurrence of VRSA was 7.69% in Services Hospital, 14.82% in Mayo Hospital and 0% in Sheikh Zayed Hospital (table 1). These isolates belong to coagulase positive MRSA phenotype as proved by their biochemical and susceptibility testing.

All 5 isolates were susceptible to Linezolid and Clindamycin, while 4 were susceptible to Moxifloxacin. Ethanolic extracts of Turmeric, Mint, Coriander, Garlic, Kalonji, Cinnamon and Cloves illustrate average MIC values of 140.8µg/ml, 563.2µg/ml, 486.4µg/ml, 614.4µg/ml, 409.6µg/ml, 281.6µg/ml and 64µg/ml, respectively against 5 VRSA strains.

DISCUSSION

Reports are present regarding the occurrence vancomycin resistant isolates of *S.aureus* in different parts of the globe. An earlier studies in Pakistan reported the presence of 13% VISA strains (Hakim *et al.,* 2008). Another finding claimed that vancomycin established 38% intermediate resistance among MRSA isolates (Farzana and Hameed, 2006). Up to 6% intermediate resistance to vancomycin was reported by a study done in 2007 (Sonavane and Mathur, 2007) and 7.5% was recorded in 2008 (Mehdinejad *et al.,* 2008). A study done in Bangladesh did not detect any VRSA, but they reported an alarming outcome that only 6.56% *S.aureus* showed sensitivity to vancomycin and 93.44% were intermediate (Shahriar *et al.,* 2012). Our results not only show unique percentages in hospitals but also reveal a distinctive outcome in contrast with literature existed. More recently VRSA strains have been reported from Jordan, Middle East (Bataineh, 2006), and the emergence of

Table 3: Zone of inhibition formed by various concentrations of ethanolic extracts of plants against VRSA isolates as measured through well diffusion method

Ethanolic extracts	Concentration in mg/well	Diameter of zones of inhibition in mm					Average zone diameters in mm
		VRSA1	VRSA2	VRSA3	VRSA4	VRSA5	
Turmeric	5	10	8	7.5	12	12	9.9
	10	13	12	11	14	15	13
	20	15	14	12	17	17	15
Mint	5	16	16	13	19	19	16.6
	10	20	19	17	22	23	20.2
	20	26	26	23	24	26	25
Coriander	5	8	10	7	11	9	9
	10	11	12	10	14	11.5	11.7
	20	13	15	13	16	14	14.2
Garlic	5	8	9	8	8	10	8.6
	10	11	13	10	14	14	12.4
	20	14	17	12	19	19	16.2
Kalonji	5	17	19	16	19	20	18.2
	10	20	23	22	24	22	22.2
	20	25	27	24	27	27	26
Cinnamon	5	15	18	14	19	19	17
	10	19	21	17	21	23	20.2
	20	21	23	20	24	25	22.6
Cloves	5	26	24	20	25	27	24.4
	10	29	26	22	27	29	26.6
	20	31	30	26	31	33	30.2
Rifampin	5µg/well	28	26	26	28	30	27.6
DMSO	Pure	0	0	0	0	0	0

heterogeneous vancomycin resistant *S.aureus* strains have also been reported from several countries of Asia (Song *et al.,* 2004). Some other researchers observed 4 and 1.9% prevalence of VRSA in the clinical samples during recent years while high incidence (79.6%) of MRSA was reported from the same specimens (Bukhari *et al.,* 2011; Thati *et al.,* 2011). A study conducted in India found two MRSA strains which were showing vancomycin resistance with the MIC value of 64µg/ml and 32µg/ml**.** Study further reported the recovery of six VISA strains isolated from the pus specimens of patients admitted in post-operative ward (Tiwari, 2009). A study conducted in Karachi, reported the emergence of VRSA and VISA from Pakistan. VRSA isolate having MIC 32µg/ml was recovered from the pus sample of a 63 year old male and this isolate was found to be resistant to several other antibiotics (Taj *et al.,* 2010).

Development of antibiotics resistance in the organisms is thought to be because of unjustified, irrational and irregular use of antibiotics by the human population, over the counter accessibility without recommendations and unrestricted use of antimicrobials in poultry, farm animals, and fisheries. Most specifically the emergence of VRSA/VISA may be due to building of selective pressure of vancomycin. Uneven frequencies of VRSA among hospitals can be due to variable measures adopted for the control of infection and unsystematic use of antimicrobial agents. High vancomycin prescription rate could also be linked with the development of such antibiotic resistance, however further research is needed to completely explore the phenomenon.

Due to the increasing appearance of VRSA strains, there is a need to find out a good alternative of vancomycin. Linezolid, an oxazolidinones, is being used for hospital acquired and community acquired complicated skin infections, post operative and surgical infections caused by MRSA. Linezolid demonstrated broad activity against all of the Gram-positive bacteria tested, including *Staphylococci, Enterococci and Streptococci* (Wilcox, 2005). Linezolid has been proved to be an ideal alternative of vancomycin and isolates which were found resistant to vancomycin were remained susceptible to linezolid. Superiority of linezolid reported by different studies from USA and Japan as oral administration of linezolid can reduce patient's stay in hospital and better choice than vancomycin for multidrug resistant strains (Weiglt *et al.,* 2004; Khono *et al.,* 2007). In present experiment linezolid was found effective *in vitro* against all five VRSA isolates and it demonstrated 100% susceptibility with the MIC ranges from 2-4µg/ml (Table 2). Similar results have been reported in the past where MIC of linezolid was 2-4µg/ml against *S.aureus* with

Table 4: Minimum inhibitory concentration values of various plant extracts against VRSA isolates

Sample No.	MIC value of Turmeric (µg/ml)	MIC value of Mint (µg/ml)	MIC value of Coriander (µg/ml)	MIC value of Garlic (µg/ml)	MIC value of Kalonji (µg/ml)	MIC value of Cinnamon (µg/ml)	MIC value of Cloves (ug/ml)
VRSA1	128	512	512	512	256	256	64
VRSA2	128	512	128	512	512	256	64
VRSA3	256	1024	256	1024	512	128	64
VRSA4	64	512	1024	512	512	512	64
VRSA5	128	256	512	512	256	256	64
Average	140.8	563.2	486.4	614.4	409.6	281.6	64

Table 5: Minimum bactericidal concentration values of various plant extracts against VRSA isolates

Sample No.	MBC value of Turmeric (µg/ml)	MBC value of Mint (µg/ml)	MBC value of Coriander (µg/ml)	MBC value of Garlic (µg/ml)	MBC value of Kalonji (µg/ml)	MBC value of Cinnamon (µg/ml)	MBC value of Cloves (µg/ml)
VRSA1	256	2048	1024	1024	512	512	128
VRSA2	256	1024	512	2048	1024	512	128
VRSA3	512	2048	512	2048	1024	256	256
VRSA4	256	1024	2048	1024	1024	1024	128
VRSA5	256	1024	2048	2048	512	512	128
Average	307.2	1433.6	1228.8	1638.4	819.2	563.2	153.6

100% susceptibility (Gemmell 2001). Furthermore 100% effectiveness of linezolid has been proved by multiple researchers in the past (Hannan *et al.,* 2002; Fatholahzadeh *et al.,* 2008; Kaleem *et al.,* 2010). However, a finding demonstrated relatively higher MIC values of linezolid (0.4-5µg/ml) in comparison to the present study (Farrell *et al.,* 2011).

Clindamycin a lincosamide antibiotic was also found effective against VRSA isolates with an average MIC value of 0.35µg/ml (Table 2). Similar results were shown in a report with 100% susceptibility of Clindamycin by broth microdilution test with the MIC of ≤ 0.5µg/ml against *S. aureus* (Tang *et al.,* 2002).

Moxifloxacin belongs to 4th generation Quinolones has greater bioactivity against Gram positive bacteria and proved its admirable *invitro* bioactivity against *S.aureus*. Results of the current study revealed an excellent *in vitro* bioactivity of Moxifloxacin against VRSA with MIC values between 0.25-8µg/ml (table 2). Analogous results were depicted by a study where multidrug resistant isolates were tested against Moxifloxacin and found susceptible with the MIC value up to 0.12µg/ml (Entenza *et al.,* 2001). In a study conducted in New Jersey, Moxifloxacin was considered effective antibiotics against most of the examined bacterial strains with MIC values ranging between 0.5-2µg/ml (Morrow *et al.,* 2011). Reliable bioactivity of Moxifloxacin was reported by Farrell *et al.* (2011).

Ethanolic extracts of different plants has shown remarkable antibacterial potential against VRSA. Black seed commonly known as Kalonji (*Nigella sativa*) have been extensively studied for its antimicrobial potential against a wide range of microorganisms. No published data is available pertaining to the activity of black seed against VRSA; however a study conducted in Pakistan proved its effectiveness against MRSA and can be correlated with our results. That study reports a complete inhibition of MRSA growth at a concentration of 4mg/disc, but a concentration of 0.5mg per disc failed to inhibit any of the strains. Literature documented that zone size >12 mm was considered to be significant in case of plant extracts (Hannan *et al.,* 2008). In another study the researchers tested methanolic extract of black seed against *S.aureus*. MIC for methanolic extract was 0.125g/100 ml (1250µg/ml) whereas our study proves MIC to be 409.6µg/ml on an average. (Mashhadian and Rakhshandeh 2005).

Clove (*Syzhygium aromaticum*) and **Cinnamon (*Cinnamomum zeylanicum*)** were also found effective according to the present outcomes. Cinnamon's average zone diameters against VRSA isolates were recorded as 17mm, 20.2mm and 22.6mm in 5mg/well, 10mg/well and 20mg/well concentrations, respectively. While inhibition zone diameters caused by cloves were 24.4mm, 26.6mm and 30.2mm at 5mg/well, 10mg/well and 20mg/well, respectively (table 3). A study conducted in India documented zone diameters of 22-27mm and 19-23mm against ethanolic extract of cinnamon and cloves, respectively. Further that study reported MIC ranges from 64-256µg/ml for cinnamon and 64-512µg/ml for clove extracts (Mandal *et al.,* 2011) which are comparable to the present findings.

Antibacterial activity of Garlic (*Allium sativum*) is well proven against *S.aureus* and many other bacterial pathogens. In the current study, garlic showed 8.6, 12.4 and 16.2mm of the inhibitions zones of by well diffusion approach at 5, 10 and 20mg concentration per well, respectively (table 3). Average MIC value of ethanolic garlic extract is 614.4µg/ml which is a bit higher than the MIC values of the extracts of other plants (table 4). Another work also reported the similar efficacy of garlic extracts showing the zone of inhibition of 20.33 ± 0.47 mm (Sudhir *et al.,* 2012). Ethanolic extract of Turmeric (*Curcuma longa*) exhibited good bioactivity against VRSA with an average zone diameter of 9.9mm, 13mm and 15mm by well diffusion test, and a low MIC value (140.8µg/ml) was observed. An earlier study depicted the antimicrobial activity of methanolic extracts of turmeric with the zone ranges between 10-15mm at a concentration of 1mg/disc and MIC value of 8mg/ml. (You *et al.,* 2003).

Mint (*Mentha piperita*) and **Coriander (*Coriandrum sativum*)** showed average MIC values of 563.2µg/ml and 486.4µg/ml (table 4). At 5, 10 and 20mg concentration, mint formed average inhibition zones of 16.6, 20.2, and 25mm, while coriander formed 9, 11.7 and 14.2mm average zones of inhibitions. Antistaphylococcal activity of coriander was proved by a study where ethanolic extracts showed 1.3mg/ml (1300µg/ml) MIC value (Zardini *et al.,* 2012). Mint leaves were found having strong antibacterial activity against a range of pathogenic bacteria as revealed by *in vitro* agar well diffusion method (Bupesh *et al.,* 2007). MBC values of ethanolic extracts revealed that these plants extracts not only hold growth inhibitory potential but also can act as bactericidal agents. Statistical analysis of the data by Fisher Exact test showed that a significant relation exists between prevalence of MRSA and VRSA. Statistical analysis by ANOVA revealed that there exists a significant difference in zone of inhibition among herbal types, ($p<0.05$) but no significant difference in antibacterial activity of extracts of mint and cinnamon was observed ($p>0.05$).

CONCLUSION

This study has revealed the presence of VRSA in different hospitals. Consequently, there should be an immediate response from the concerned authorities to check further emergence and spread of these notorious VRSA strains. A strict regulatory deterrence on irrational antibiotic usage might be an appropriate and effective approach in this direction. Rational use of vancomycin and the approval of adequate control measures of infection are necessary to avoid surfacing of glycopeptide resistant microorganisms and their spread within hospitals.

The degree of antibacterial activity of plant extracts tested can be graded in the following order: Cloves> Turmeric>Cinnamon>Kalonji>Coriander>Mint>Garlic. It is anticipated that using natural products as therapeutic agents will perhaps not bring forth resistance in microorganisms. It is essential that research should continue to extract and purify the active components of these drugs and their bioactivity, to determine the toxicity of active ingredients, their side effects, pharmacokinetic assets, pharmacological standardization and clinical assessments.

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