MATERIAL AND METHODS

Identification:
(a.) Study area, sampling, and sample processing
The study will be carried out in the Microbiology Laboratory of the Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur. Clinical specimens [urine, pus, blood, sputum and throat swab etc] will be collected aseptically with a sterile cotton swab. The samples will be inoculated on the Mannitol salt agar (MSA) (HiMedia, India) plates immediately after sample collection for isolation of S. aureus and transported to the laboratory as early as possible.

(b.) Isolation and identification of Staphylococcus aureus
All MSA plates will be incubated for 24 hours at 37 °C. After incubation, isolated colonies suspected to be Staphylococcus will be allowed to grow on nutrient agar plates (HiMedia, India) and then identified microscopically, biochemically, and serologically. For microscopic observation, a pure colony will be selected and subjected to Gram staining. Then the shape, arrangement, and Gram reactions of the isolates were observed under a light microscope. Required confirmatory biochemical tests including catalase and triple sugar iron agar tests will be performed to identify suspected S. aureus following standard protocols.

Biochemical Identification of S. aureus
a.) By Gram Staining: 1.) Crystal violet-1min, 2.) Grams iodine-1min, 3.) acetone/alcohol-30sec, 4.) Saffarin-30sec
b.) Catalase Test: using 3 % H₂O₂
c.) Coagulase test: Can be done by using two methods:
(a.) Tube Coagulase Test: About 0.1ml of a young broth culture or agar suspension of the isolate is added to 0.5 ml of human or rabbit plasm in a narrow test tube. EDTA, oxylate or heparin may be used as the anticoagulant for preparing plasma. Positive and Negative controls are also set up. The tube are incubated in water bath at 37 degree C for 3-6 hours. If positive, the plasma clots and does not flow when the tube is tilted.
(b.) Slide Test: The isolate is emulsified in a drop of saline on a slide. After checking for absence of autoagglutination a drop of human or rabbit plasma is added to the emulsion and
mixed Prompt clumping of the cocci indicated a positive test. Positive and Negative controls are also set up.

d) **Hemolytic activity:** The hemolytic activity of *S. aureus* isolates will be tested using blood agar plates containing 5% defibrinated sheep blood. An isolated colony from a nutrient agar (NA) plate will be inoculated on blood agar and incubated at 37°C for 24 hours. The hemolytic zones will be characterized as alpha (partial hemolysis), beta (complete hemolysis), and gamma (no hemolysis) depending on the extent of each colony.

**Assay of antibacterial susceptibility**

A standard agar-disc diffusion (Kirby-Bauer) assay using Mueller-Hinton agar (MHA) (HiMedia, India) plates will be conducted to determine the susceptibility of the isolated *S. aureus* to different antibiotics. A suspension of the test organism will be prepared by adjusting the turbidity of the broth in a phosphate buffer saline by comparing it with that of the McFarland standard solution of 0.5. By means of a sterile cotton swab, a uniform lawn of bacterial growth will be prepared on the MHA plates. Antibiotic disc including Ciprofloxacin (CIP) (30µg), Tobramycin (TOB) (10µg), Cotrimoxazole (COT) (25µg), Penicillin (P) (10µg), Imipenem (IPM) (10µg), Cefoxitin (CX) (30µg), Amikacin (AK) (30µg), Netilmicin sulphate (NET) (30µg), Oxacillin (OX) (1µg), Erythromycin (E) (15µg), Clindamycin (CD) (2µg), Azithromycin (AZM) (15µg), Gentamycin (GEN) (10µg), Tecoplanin (TEI) (30µg), Linezoid (LZ) (30µg), Tetracycline (TE) (30µg), Vancomycin (VA) (30µg), Norfloxacin (NX) (10µg), Nitrofurantoin (NIT) (300µg), Ofloxacin (OF) (5µg), Chloramphenicol (C) (30µg) will be applied aseptically on the surface of the inoculated plates in an appropriate spatial arrangement using a sterile needle. The plates will be incubated at 37°C for 24 hours and examined for zones of inhibition (mm).

Optimum temperature required for the Antibiotic Cefoxitin is at 37°C and Oxacillin at 35°C. If large doses of vancomycin might still be able to kill the *S. aureus*, it is called Vancomycin-intermediate *S. aureus* (VISA). If no amount of vancomycin will kill the *S. aureus*, it is called Resistance to Vancomycin (VRSA).

- Cefoxitin and Oxacillin if not resistance: MSSA
- Cefoxitin and Oxacillin if resistance: MRSA
- Vancomycin if resistance: VRSA
Vancomycin large doses if able to kill: VISA

Identification of MRSA
For the detection of MRSA, oxacillin (1 µg) and cefoxitin (30 µg) will be introduced on the MHA plates against the growth of S. aureus. For this purpose, a bacterial suspension will be prepared in sterile saline by selecting colonies produced by the overnight incubation on Nutrient agar plates. After 5-7 hours of incubation, the cell turbidity will be adjusted to 0.5 McFarland standards [98,99]. Subsequently, the suspensions will be inoculated onto MHA plates and the antibiotic discs will then be placed onto the plates [95,101]. All plates will be incubated for 24 hours at 35°C and 37°C to observe for oxacillin and cefoxitin resistant S. aureus.

Identification of VRSA through disc diffusion methods
MHA plates will be inoculated with the bacterial suspension which was previously adjusted to 0.5 McFarland standards. Afterward, a 30 µg vancomycin disc and a blank disc as a control will be aseptically placed over the surface of the MHA plates at a distance of 5 mm to observe the range of the zone diameter for the detection of strains of VRSA [95,101].

Determination of vancomycin resistance by minimum inhibitory concentration test
The minimum inhibitory concentration (MIC) of vancomycin will be determined by the tube dilution method [102-105]. Muller-Hinton Broth will be prepared with 2-128 µg/mL of vancomycin. By using a direct colony suspension method, 0.5 McFarland equivalent bacterial inoculums will be prepared in normal saline after culturing for 24 hours on an agar plate. The suspension was further diluted to achieve the desired inoculum concentration. If it is <=2 µg/mL then it is Sensitive, if it is >=4-8 µg/mL then it is Intermediate and if it is >=16 µg/mL then it is Resistance, according to the CLSI guidelines 2016.

All strains will be spotted onto Muller-Hinton plates containing different concentrations of vancomycin. The plates will be incubated for 24 hours at 37°C and checked for any visible growth [97].
MRSA isolated from Rama Medical college

MSSA Isolated from Rama Medical College
VISA Isolated from Rama Medical College

VRSA isolated                     Subculture of MRSA, VRSA and VRSA and MSSA
Collection of plant material
The leaves of the Cannabis sativa and Allium sativum examined in this study will be collected from the Rama Medical College Hospital and Research Centre, Mandhana, Kanpur. The taxonomic identity of the plants was authenticated at the Central National Herbarium, Botanical survey of India-Howrah, West Bengal, India. The plant leaves of cannabis sativa and peeled garlic bulbs will be washed thoroughly and then shade dried in room temperature for 4 days, finely powdered and stored in a sterile airtight container for further use.

Preparation of the plant extract
Extraction procedure
The plant leaves of cannabis sativa and peeled garlic bulbs will be washed thoroughly and then shade dried in room temperature for 4 days, finely powdered and stored in a sterile airtight container for further use.
Now 10 g of powder will be dissolved in 100 ml of the different solvents including polar solvents as methanol, ethanol, water and diethyl ether as a non polar solvent in a conical
flask. The flask will be Plugged with cotton and will be kept on a rotary shaker at 250 rpm for three days. After three days filtered with the help of Whatmann No.1 filter paper (Harbone et. al. 1973) \(^{106}\) and the solvent was evaporated carefully by using a rota vapour until reach its maximum concentration supposed final concentration as 100% and it diluted in respected solution to form different concentration as 50%, 25%, and 12.5% then store at 40ºc for phytochemical analysis and testing their antimicrobial properties.

**Antimicrobial agents Susceptibility test**

The antimicrobial activities will be determine by the disc diffusion method (Bauer et.al.1996, Andrews’s et.al.2001)\(^{107,108}\) on Mueller Hinton Agar. All plates will be inoculated with the test bacterium which will be previously adjusted to the 0.5 McFarland standard solution to comprise approximately 1.5x10^8 CFU/ml. A sterile cotton swab will be dipped into the suspension, rotated several times. The surface of the agar plate will be streaked over the entire sterile agar surface rotating the plate to ensure an even distribution of inoculum with the final swab around the rim in the laminar air flow. The plates will be allowed to dry for 3 to 5 min before placing the sterilized discs. Mark the swabbed plate with marker as 100%, 50%, 25% and 12.5%.

Sterile discs (Hi media) (6 mm in diameter) will be impregnated with 25µl in respective percentage solution and then placed on the swabbed MHA plates. Meanwhile, 25µl of the different solvents will also be pipetted onto the sterile discs (Hi media) as a control. The plates will be incubated at 37ºC for 24 hours and a ruler scale will be used to measure the inhibition zones in millimetres (mm). The antimicrobial activity was then compared with standard antibiotics.

**Purified compound**

Purification is a process to get purified compound. Number of different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography, Silica gel, Centrifugal Thin Layer Chromatography (CTLC) and HPLC, should be used to obtain pure compounds. The pure compounds are then used for the determination of structure and biological activity.

Extraction is the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. The basic operation included steps, such as pre-washing, drying of the plant materials or
freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available for the extraction of the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate. For the extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some instances, extraction with hexane is used to remove chlorophyll (Cos et al., 2006).[109]

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Cos et al., 2006).[109].

**Column Chromatography** is a preparative technique which is used to purify compounds depending on their polarity or hydrophobicity. In column chromatography, a mixture of molecules is separated based on their differentials partitioning between a mobile phase and a stationary phase.

**Thin Layer Chromotography (TLC)** is a simple, quick, and inexpensive procedure that gives the researcher a quick answer as to how many components are there in a mixture. TLC is also used to support the identity of a compound in a mixture when the $R_f$ of a compound is compared with the $R_f$ of the known compound. Additional tests involves the spraying of phytochemical screening reagents, which cause color changes according to the phytochemicals existing in a plants extract, or by viewing the plate under the UV light. This has also been used for the confirmation of purity and identity of isolated compounds.

High performance liquid chromatography (HPLC) is a versatile, robust, and is a widely used technique for the isolation of natural products. Natural products are frequently isolated following the evaluation of a relatively crude extract in a biological assay in order so that to fully characterize the active entity. Purification of the compound of interest using HPLC is the process of separating or extracting the targetted compound from the other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions.

We will perform Column Chromotography to purify the compound, as there are various compounds present in Cannabis Sativa L. and Allium Sativum. Garlic has definite antibiotic
properties and is effective against a wide spectrum of bacteri, fungi and viruses \cite{53,54}. The antimicrobial activities of garlic are linked to the presence of some bioactive compounds \cite{55}. Ingredients in fresh garlic, other than illicit, have strong natural antibiotic effects \cite{56}. Garlic extract, DAS and DADS provide powerful protective activity against MRSA. Garlic includes to >200 components such as volatile oils (allicin, allin and ajoene) consisting of sulphur, enzymes (allinase, peroxidase and miracyanase), carbohydrates (sucrose, glucose), minerals (germanium, selenium, zinc), aminoacids like cysteine, glutamine, isoleucine and methionine, bioflavonoids like quercetin and cyaniding and allistatin I and allistatin II, C, E and A vitamins and niacin, B1, B2 vitamins and beta carotene (Goncagul and Ayaz, 2010) \cite{110} Organosulfur compounds are also present in Garlic. Allicin is a very small molecule that is easily able to penetrate bacterial cell walls and is reported to have strong SH-modifying and antioxidant properties.

Cannabis is a complex plant which contains 421 known compounds from 18 different chemical classes. Sixty one Cannabinoids and their homologs \cite{111} are known to occur in plant \cite{112}. Cannabidiol (CBD) is one of at least 113 active cannabinoids identified in cannabis \cite{113,114}. CBD has a wide range of medical benefits. Although CBD and THC act on a different pathways of the body, they seem to have many of the same medical benefits. According to a 2013 review published in the British Journal of Clinical Pharmacology, studies have found CBD to possess the following medical properties \cite{115}.

<table>
<thead>
<tr>
<th>Medical Properties of CBD</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiemetic</td>
<td>Reduces nausea and vomiting</td>
</tr>
<tr>
<td>Anticonvulsant</td>
<td>Suppresses seizure activity</td>
</tr>
<tr>
<td>Antipsychotic</td>
<td>Combats psychosis disorders</td>
</tr>
<tr>
<td>Anti-inflammatory</td>
<td>Combats inflammatory disorders</td>
</tr>
<tr>
<td>Anti-oxidant</td>
<td>Combats neurodegenerative disorders</td>
</tr>
<tr>
<td>Anti-tumoral/Anti-cancer</td>
<td>Combats tumor and cancer cells</td>
</tr>
<tr>
<td>Anxiolytic/Anti-depressant</td>
<td>Combats anxiety and depression disorders</td>
</tr>
</tbody>
</table>

We will perform column chromatography to purify the compounds. Solvents such as Hexane, Ethyl acetate will be used to run the column. Purity of the compound could be check with HPLC and TLC and also by silica plate.
PHYTOCHEMICAL ANALYSIS METHOD FOR PLANT EXTRACTS-

i. **Alkaloids**- In 300 microlit. extract dissolve in 1 ml distill water and add 2 molar HCl with extract and mix 500 microlit. Dragendorff reagent result (A: bismuth nitrate 1.7gm+ glacial acetic acid +80ml D/w. B: 5gm potassium acetate +10ml D/W (A+B) red or orange ppt. confirmation alkaloid.

ii. **Flavanoids**- In 300 microlit. extract add 5-10 drop of conc. HCl followed by Zn powder. Pink or brown colour indicate present of Flavanoids.

iii. **Glycosides**- In 300 micro extract dissolve 2 ml distill water and add 1 ml of 1% NaOH solution (1 gram in 100ml). Yellow colour shows the present of Glycoside.

iv. **Phenol**- To 300 microlit. extract dissolve in 2 ml of distill. H2O add few drop of 10% ferric chloride solution blue green color shows the presence of Phenol.

v. **Tannins**- To 300µl of leaf extract add few drops of 5% aqueous ferric chloride. Blue black colour appears. Then add dil H2SO4 drop wise. Green brown colour will appear if tannins are present. This colour disappears when excess of dil H2SO4 is added.

**DNA Extraction:**

DNA will be extracted using the DNA extraction kit as per manufactures guidelines. Extracted DNA will be stored at 20°C till amplification. PCR will be done for the detection of Van A gene.

**Amplification of extracted DNA**

Primers and cycling conditions used for amplification of Van A genes.

**Van A gene Primer:**

**Primers** The primers will be for (van A) gene detection “vancomycin resistance gene”

(forward primer EA1, 5’-GGGAAAAACGACGACAATTTGC-3’)

(reverse primer EA2, 5’-GTACAATGCGGCGGTGTTA-3’)

**Primers** The primers will be for (van B) gene detection “vancomycin intermediate gene”

Van B Forward primer 5’-GTG ACA AAC CGG AGG CGA GGA-3’

Van B Reverse primer 5’-CCGCCATCCTCCTTCG AAA AAA-3’
Primers will be obtained from Quiagen, Germany and will be reconstituted with sterile double distilled water based on the manufacturer’s instruction.

**PCR reaction mix (working concentration)**

The working conditions for PCR reaction mixture will be consumed from Bangalore Genei, India:

- 1. 10 x PCR Buffer: 1X
- 2. dNTPs
- 3. Forward primer
- 4. Reverse primer
- 5. Taq polymerase
- 6. Template DNA
- 7. Distilled water: to make up the volume

**PCR conditions:**

- 1) Initial denaturation: 94 °C - 5 mins
- 2) Denaturation: 94 °C - 30 secs
- 3) Annealing: 58 °C - 45 secs
- 4) Extension: 72 °C - 45 secs
- 5) Cycling condition: 30 cycles
- 6) Final extension: 72 °C - 7 mins
- 7) Hold at 4 °C

Data will be collected and entered in MS excel worksheets and results will be analysed with appropriate statistical tools like, tests of significance, logistic regression analysis etc using SPSS software.
MATERIALS AND METHODS

Study Design

It is a Cross Sectional Case Control Study.

Study Place

Rama Medical College Hospital & Research centre, Kanpur, Uttar Pradesh.

Study Period

The study will be conducted within a period of duration 12-18 months.

Study Population

The clinical samples like blood, pus, wounds, nasal swab, urine, plural fluid will be collected from different wards patients and send to the microbiology laboratory for the culture identification and antimicrobial sensitivity testing will also be included in the study. A detailed clinical history of the patients will be collected.

Sample Size

The Sample size was calculated so that the test of hypothesis is able to detect the difference of 0.6 cm in zone size between the two groups with 80% power and 95% Confidence.

The formula used is:

\[ Z_{\text{power}} = \frac{\text{difference}}{\text{SE of difference}} \times Z_{\alpha/2} \]

For 80% power, \( Z_{\text{power}} = 0.84 \)

For 95% Confidence \( Z_{\alpha/2} = 1.96 \)

difference = 0.6
\[ S_e = \sqrt{\frac{s_1^2 + s_2^2}{n}} \]

Using the data collected on 30 patients and considering 10 patients in each group as a pilot study, S.E. has been calculated as 4.22.

Putting the values and solving for \( n \)

\[ n = 139.6 \]

So, we decided to take a sample of size 140 for each group.

**Inclusion criteria**

Any of the specimen from patients collected from different wards and confirmed for GPC after proceeding will be included in the study.

**Exclusion criteria**

Patients specimen confirmed with GNB will be excluded from the study.

**Specimen transport**

Specimens will be transported to the laboratory as soon as possible after collection.

**Ethical consideration**

Ethical clearance will be taken from ethical committee.