EFFECT OF POLY HERBAL FORMULATION AGAINST KLEBSIELLA PNEUMONIA CAUSING PNEUMONIA IN CHILDREN’S

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ABSTRACT

Pneumonia is an inflammatory condition of the lung, especially of the alveoli (microscopic air sacs in the lungs) associated with fever, chest symptoms, and consolidation on a chest radiograph. While typically caused by an infection there are a number of non-infectious causes. Infectious agents include: bacteria, viruses, fungi, and parasites. Typical symptoms include cough, chest pain, fever, and difficulty breathing. With increasing use of antibiotics these bacteria have evolved itself into the way that it is becoming more and more antibiotic resistant and case of ESBL producing bacteria has been reported in genus Klebsiella. The aim of this work is based on assessing the prevalence of the bacteria Klebsiella pneumonia in the patients of pneumonia, and to check the activity of medicinal plants and their effect in synergism with antibiotics. Fruit and aerial parts of five medicinal plants of Dasmodium Gangeticum, Nelumbo Nucifera, Canabis, Sesame White, and Sesame Black have been used in the form of three different extract i.e. Methanolic, Ethanolic and Aqueous and their activity was assessed.

Key words: Klebsiella pneumonia, Cefazidime, Ofloxacin, Gentamicin, Ciprofloxacin.

INTRODUCTION

K. pneumoniae is a gram negative bacterium. It is facultative anaerobic. It is rod-shaped and measures 2 µm by 0.5 µm. In 1882, Friedlander C. Ulber first discovered Klebsiella to be a pathogen that caused pneumonia (Friedlander C, 1882). Many hospital cases around the world have been linked to K. pneumoniae. Therefore, more studies of the strains were important and performed. The bacterium was isolated and sequenced from a patient in 2004. K. pneumoniae is commonly found in the gastrointestinal tract and hands of hospital personnel (Podschun, R 1998). The reason for its pathogenicity is the thick capsule layer surrounding the bacterium. It is 160 nm thick of fine fibers that protrude out from the outer membrane at right angles (Lawlor, M., 2005). Another site on the human body that this bacterium can be found is the nasopharynx. Its habitat is not limited to humans but is ubiquitous to the ecological environment. This includes surface water, sewage, and soil (Brisse, S., 2001). It is clinically the most important member of the Klebsiella genus of Enterobacteriaceae (Postgate J., 1998). The detailed mechanisms of this action are not known yet, though much information about the capsules is accumulating from chemical, physiological, and immunological studies. Many types of bacteria produce extracellular polysaccharides (EPSs). Klebsiella are non-motile, rod-shaped, aerobic bacteria that possess a prominent polysaccharide capsule. The diseases caused by K. pneumoniae can result in death for patients who are immunodeficient. Differences in the diseases are determined by the different virulence factors. For example, mucoid phenotype varies as the strains for mucoid bacteria has been reported in genus Klebsiella. Therefore, differences in pathogenicity and virulence factors are non-motile, rod-shaped, aerobic bacteria that possess a prominent polysaccharide capsule. The diseases caused by K. pneumoniae can result in death for patients who are immunodeficient. Differences in the diseases are determined by the different virulence factors. For example, mucoid phenotype varies as the strains for mucoid bacteria have evolved itself into the way that it is becoming more and more antibiotic resistant and case of ESBL producing bacteria has been reported in genus Klebsiella. The aim of this work is based on assessing the prevalence of the bacteria Klebsiella pneumonia in the patients of pneumonia, and to check the activity of medicinal plants and their effect in synergism with antibiotics. Fruit and aerial parts of five medicinal plants of Dasmodium Gangeticum, Nelumbo Nucifera, Canabis, Sesame White, and Sesame Black have been used in the form of three different extract i.e. Methanolic, Ethanolic and Aqueous and their activity was assessed.

MATERIALS AND METHODS

Collection of sample

In the present investigation, the total 130 sputum samples of children (age 3-5) and swabbed sample of ventilator were collected from Civil Hospital and Puran Hospital of Paonta Valley. The sample were collected aseptically in sterile 50 ml Oakridge tubes and inoculated in nutrient broth for 24 hrs at 37°C. The isolates were isolated from patients who had a minimum of 5 days stay in the hospital prior to sample collection and the patient must be free of infection at the time of hospital admission.

Isolation and Identification of Bacterial strain

Recovered isolates were identified with Colony Characteristics, Gram Staining and Biochemical Rxn. (Holt et al, 1994. Cappuccino and Sherman Microbiology (A Laboratory Manual 7th edition.).

ANTIBIOTIC SENSITIVITY TESTS

Antibiogram study of the predominant bacterial isolates against commercial antibiotics like Cefazidime, Ceftriaxone, Amikacin, Cefotaxime, Ofloxacin, Gentamicin, Amoxicillin, Ciprofloxacin, Cephalexin, Chloramphenicol, Tetracycline were used against the pneumonia causing bacteria Klebsiella was made by Kirby-Bauer disc diffusion method (Cappuccino 1999).

COLLECTION OF PLANT MATERIAL

Fresh fruits of Nuficer Indicum, Desmodium Gangeticum, Sesame Indicum White seeds, Sesame Indicum Black seeds and Canabis were collected from surrounding areas of Paonta Sahib (H.P.) and identified by Botanical survey of India, Dehradun. For the preparation of plant extract the fruits were dried under shade and stored into fine powder using electric blender. 50 mg of dried powder sample was taken and extracted by Soxlet apparatus using distilled water, methanol and ethanol separately. The solvents were removed under reduced pressure in a rotary evaporator until they become completely dry. Filtrates were preserved at 4°C.

DETERMINATION OF THE ANTIMICROBIAL ACTIVITY

From the dry filtrate material, the 500mg/ml dilutions of plant paste were prepared for antibacterial assay. The modified agar well diffusion method was employed to determine the antimicrobial activity of plant extracts. Three different extractions (Aqueous, methanol and ethanol) were taken. In agar well diffusion method, 100µl of the extracts (500mg/ml) were poured in to the wells. All the agar plates incubated at 37°C. If antimicrobial activity was
present on plates, it was indicated by an inhibition zone. The
diameters of the inhibition zone were measured in millilitre after
24 hrs. The experiments were conducted in triplicate for each test.
The mean of triplicate result were taken.

DETERMINATION OF SYNERGESTIC EFFECT OF PLANT EXTRACT
AND ANTIBIOTIC DISCS

Antibacterial activity was measured using a well diffusion method
according to the clinical and laboratory standard institute
(formerly, the NCLLS). Briefly petri plates containing approximately
25-30ml of Mueller Hinton Agar medium were inoculated using a
cotton swab in with a 4-6 hour old culture of bacterial strain. Wells
(6mm diameter) were punched in the agar and filled with 30μl of
plant extract or antibiotics and in case of synergistic effect 30μl of
plant extract in the well alongside with the disc were placed on the plate.
Replicate of each plate was done. The plates were incubated at 37°C
for 18-24 hrs. The antibacterial activity was assessed by measuring
the inhibition zone diameter (mm) around the well and the
antibiotic disc. The average of total three replicates of the plates of
each plate with antibiotic disc, plant extract and the synergistic effect
of both the plant extract has been calculated. Synergism effect was
considered when combinations exhibited with enlargement of
combined inhibition zone size by 5mm. (National Committee for
Clinical Laboratory Standards, 1993).

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION
(MIC)

MIC is defined as the lowest concentration of extracts that
completely inhibits the growth of the microorganism in 24 hrs
(Thongson C, 2004). The MIC for the extracts was determined by
broth dilution method. The highest dilution of the plant extract that
retained its inhibitory effect resulting in no growth (absence of
turbidity) of a microorganism is recorded as the MIC value of the
extract. A control experiment was run in parallel to study the impact
of the solvent alone (without plant extracts) on growth of the five
test organisms. Methanol, Ethanol and water were diluted in a
similar pattern with sterile nutrient broth followed by inoculation
and incubation.

RESULTS AND DISCUSSION

Prevalence of Klebsiella pneumonia the bacterium in total
samples:

Total recovered isolates were 105 out of which 70 Klebsiella
Pneumonia were isolated during the study period in 130 sputum
and ventilator samples. So the prevalence of recovered isolates was
54%. The incidence of Klebsiella Pneumonia isolates varies according
to countries, regions or even hospitals. In 50-60 years age group, the
most prevalent organism was also Klebsiella pneumoniae; 26 out of
80 (32.5%) followed by 07 out of 38 (19%) for the age group of 30-
50 years. The count of recovered isolate was negligible as the
ventilator masks applied to the patient were constantly sterilized by
disinfectants; hence the recovered isolates in case of ventilator were
3 out of 10 (3%).

<table>
<thead>
<tr>
<th>Total Samples</th>
<th>Recovered isolates</th>
<th>Klebsiella Pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>85</td>
<td>50</td>
</tr>
</tbody>
</table>

Antibiotic Sensitivity Test

The antibacterial activity of 12 commercial drugs was assayed by
Kirby-Bauer disc diffusion method. Approximately 80% of the
recovered isolates showed the sensitive activity against Ceftazidime,
Ciprofloxacin, Gentamycin, Ciprofloxacin, and resistant activity against
Carbenicillin, Ceftizoxime, Amikacin, Cefotaxime, Amoxicillin,
Cephalixin, Chloramphenicol and Tetracycline. The diameter of
inhibition zones observed in 4 drugs namely Ceftazidime (16mm) Cefloxaclin (20mm), Gentamycin (22mm), Ciprofloxacin (20mm).
Whereas Carbenicillin, Ceftizoxime, Amikacin, Cefotaxime, Amoxicillin,
Cephalixin, Chloramphenicol and Tetracyline showed very small zone of inhibition against the growth of Klebsiella pneumonia, hence considered to be resistant against these drugs. The etiological picture of lactation mastitis and
sensitivity of its causative agent to some antibiotics were studied.

Out of 12 antibiotics used the pictures having the activity if 4 antibiotics have been shown along with the diameter of the antibiotic showing the sensitivity of bacteria against the antibiotics. The activity was assessed by applying the plot of activity of the 12 antibiotics against the number of isolates.
Antibiotic sensitivity of *Klebsiella pneumonia* against the disc used (Bars with red colour shows the resistance of organism towards the antibiotics and Blue colour bars shows the sensitivity of organism against the antibiotics used).

**ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANT**

The results of antimicrobial activity of ethanol, methanol and aqueous extracts of *Dasmodium Gangeticum*, *Nelumbo Nucifera*, *Canabis*, *Sesame White* and *Sesame Black* by agar well diffusion method. Our study showed that aqueous extract gave maximum zone of inhibition against *Klebsiella pneumoniae* i.e., *Nelumbo Nucifera* (16mm), *Sesame White* (09mm), *Sesame Black* (12mm), *Dasmodium Gangeticum* (12mm), *Canabis* (23mm). Followed by methanolic extract *Nelumbo Nucifera* (24mm), *Sesame White* (00mm), *Sesame Black* (09mm), *Dasmodium Gangeticum* (07mm), *Canabis* (00mm). Ethanollic extract gave following zone of inhibition i.e., *Nelumbo Nucifera* (06mm), *Sesame White* (00mm), *Sesame Black* (09mm), *Dasmodium Gangeticum* (06mm), and *Canabis* (00mm). Highest mean diameter of inhibition zone was produced by the methanolic extract of *Nelumbo Nucifera* (24mm), against *Klebsiella pneumoniae* and followed by aqueous extract of *Sesame White* (09mm), *Sesame Black* (12mm), *Dasmodium Gangeticum* (12mm) and *Canabis* (23mm) extracts.

**Antibacterial activity of *D. gangeticum* (Aq) against *Klebsiella pneumoniae* (12mm)**

**Antibacterial activity of *Sesame Black* aq. against *Klebsiella pneumonia* (12mm)**

**Antibacterial activity of *N. Nucifera* methanolic extract against *Klebsiella* (24mm)**

**Antibacterial activity of *Sesame White* aq. against *Klebsiella pneumonia* (9mm)**
Antibacterial activity of Cannabis Aq. against Klebsiella pneumonia (23mm)

Table 1: The antibacterial activity of plant extracts.

<table>
<thead>
<tr>
<th>Plants</th>
<th>Aqueous extract</th>
<th>Methanolic extract</th>
<th>Ethanollic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. Nucifera</td>
<td>16 mm</td>
<td>24 mm</td>
<td>00 mm</td>
</tr>
<tr>
<td>Sesame White</td>
<td>09 mm</td>
<td>00 mm</td>
<td>00 mm</td>
</tr>
<tr>
<td>Sesame Black</td>
<td>12 mm</td>
<td>09 mm</td>
<td>09 mm</td>
</tr>
<tr>
<td>D. Gangeticum</td>
<td>12 mm</td>
<td>07 mm</td>
<td>06 mm</td>
</tr>
<tr>
<td>Canabis</td>
<td>23 mm</td>
<td>00 mm</td>
<td>00 mm</td>
</tr>
</tbody>
</table>

Chart 1: Antibacterial activity of plant extracts

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC is defined as the lowest concentration of extracts that completely inhibits the growth of the microorganism in 24hrs. MIC of five medicinal plants i.e. N. Nucifera, Canabis, Sesame White, Sesame Black and D. Gangeticum were observed with different extracts, namely aqueous, ethanolic and methanolic which were showing maximum activity in against the organism. The dilutions were made by taking 800µl of the plant extract with concentration of 512mg/ml, 100µl of distilled water and 100µl of broth culture thus making the total concentration of 1ml solution. The spreading of the culture on MHA plates was done after 8 hr incubation and later the plates were kept for an incubation period of 24 hrs. The MIC of the extract was determined after the colony counting on the plate was done and the count was less than 10.
MIC of aqueous extract of Sesame Black at 512 mg/ml

<table>
<thead>
<tr>
<th>Plants</th>
<th>Extract</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nelumbo Nucifera</td>
<td>Methanolic</td>
<td>256 mg/ml</td>
</tr>
<tr>
<td>Sesame White</td>
<td>Aqueous</td>
<td>512 mg/ml</td>
</tr>
<tr>
<td>Desmodium Gangeticum</td>
<td>Aqueous</td>
<td>512 mg/ml</td>
</tr>
<tr>
<td>Cannabis</td>
<td>Aqueous</td>
<td>256 mg/ml</td>
</tr>
<tr>
<td>Sesame Black</td>
<td>Aqueous</td>
<td>512 mg/ml</td>
</tr>
</tbody>
</table>

By combining the above MIC of five medicinal plants we formulated the drug against the recovered isolates causing pneumonia disease

256 mg + 512 mg + 512 mg + 256 mg + 512 mg = 2048 mg

At the concentration of 2048 mg/ml the dilution were made in the same manner as the above method and final MIC was determined.

**SYNERGIC EFFECT OF PLANT EXTRACT AND ANTIBIOTICS:**

Ceftazidime (CAC) [25 mm], Ofloxacin (OF) [26 mm], with CANNABIS aq.

Gentamicin (G) [27 mm], Ciprofloxacin (CIP) [27 mm] with Cannabis aq.

**Synergic activity of plant extract and the antibiotics used.**
CONCLUSION

Pneumonia is the most prevalent disease and one of the reasons for high mortality rates among the infants and the people of 40-50 years of age group. The incidence of *Klebsiella pneumonia* isolates varies according to countries, regions or even hospitals. The antibacterial activity of 12 commercial drugs was assayed by Kirby-Bauer disc diffusion method. Approximately 80% of the recovered isolates showed the sensitive activity against Ceftazidime, ofloxacin, gentamycin, ciprofloxacin, and resistant activity against Carbencillin, Ceftizoxime, Amikacin, Cefotaxime, Amoxicillin, Cephalexin, Chloramphenicol and Tetracycline. The diameter of inhibition zones observed in 4 drugs namely Ceftazidime (16mm), ofloxacin (20mm), gentamycin (22mm), ciprofloxacin (20mm). The results of antimicrobial activity of ethanol, methanol and aqueous extracts of *Dasmodium Gangeticum*, *Nelumbo Nucifera*, *Canabis*, *Sesame White* and *Sesame Black* by agar well diffusion method. Our study showed that aqueous extract gave maximum zone of inhibition against *Klebsiella pneumonia* i.e., *Nelumbo Nucifera* (24mm), *Sesame White* (20mm), *Sesame Black* (12mm), *Dasmodium Gangeticum* (12mm), *Canabis* (23mm) against *Klebsiella pneumonia*. MIC is defined as the lowest concentration of a extracts that completely inhibits the growth of the microorganism in 24hrs. MIC of five medicinal plants i.e *N. Nucifera*, *Canabis*, *Sesame White*, *Sesame Black* and *D. Gangeticum* were observed with different extracts, namely aqueous, ethanolic and methanolic which came out to be 512mg/ml for the concentration of 2048mg/ml. Study of the synergic effect helped to understand the combined activity of the plant and antibiotics. Applying the plant extract and antibiotic discs collectively showed enhanced activity in case of four out of five plants. In case of *sesame black* the zone of inhibition was shown only by antibiotic discs. In our study the synergic effect was applied with sole purpose enhancing the antibacterial activity of antibiotics with help of plant extract. The results were comparable to standards fixed by National Committee for Clinical Laboratory Standards, 1993. As they were 4-5 mm more as compared to the individual activity of plants and antibiotics. *N. Nucifera*, *Canabis*, *Sesame White*, *Sesame Black* and *D.Gangeticum* showed maximum of 22 mm, 23 mm, 23 mm and 27 mm zone with antibiotics against organism. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries. The medicinal value of these plants lies in some chemical active substances that produce a definite physiological action on the human body. Natural products of higher plants possess a new source of antimicrobial agents with possibly novel mechanisms of action. Systematic screening of them may result in the discovery of novel active compounds.
REFERENCES


