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ANTIBACTERIAL ACTIVITY OF SOME PLANTS AGAINST DRUG RESISTANT NEONATAL SEPSIS CAUSING BACTERIA

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ABSTRACT

The study was undertaken to investigate the organisms responsible for sepsis in the Neonatal Unit at S.V.B.P. Hospital, Meerut Uttar Pradesh, to determine their resistance pattern to antibiotics. A total of 120 neonates having one or more signs of sepsis as respiratory distress, jaundice, cyanosis and lethargy and aged from 0 to 28 days were included in this study. A blood sample was taken each subject, cultured and then antibacterial susceptibility test was performed for isolates. 80 (66.66%) cases yield positive cultures. The gram negative bacteria constituted 57.5% of the total isolates, from which *E. coli* was the predominant pathogen (27.5%) followed by *Klebsiella pneumoniae* (17.5%) and *Pseudomonas aeruginosa* (12.5%). The gram positive bacteria constituted 42.5% of the total isolates, from which *S. aureus* was the prominent pathogen (21.25%) followed by *Enterococcus spp.* (13.75%) and Coagulase Negative *Staphylococci* (7.5%).

KEYWORDS: *Enterococcus sp, E. coli, Klebsiella pneumonia, Pseudomonas aeruginos Staphylococcus,* Medicinal plants and Antibacterial Activity.

1. INTRODUCTION

Neonatal sepsis is a clinical syndrome characterized by systemic signs of infection and accompanied by bacteremia in the first month of life. Neonatal sepsis is among the three most common illnesses among newborns, and is second most common cause of neonatal mortality especilaay among preterms and low birth weight babies. Neonatal infections are estimated to cause 1.6 million deaths annually in developing countries. Sepsis and meningitis are the most commonly implicated factor for most of these deaths (Stoll, 1997).

The continuous spread of multidrug resistance pathogens has become a serious threat to public health and a major concern for infection control practitioner's worldwide. In addition to increasing the cost of drug regimens, this scenario has paved way for the re-emergence of previously controlled disease and has contributed substantially to the high frequency of opportunistic and chronic infection cases in neonates. This situation has forced scientists to search for new antimicrobial substances from various sources as novel antimicrobial chemotherapeutic agents.

As such, there is an urgent need to develop new, effective, biodegradable agent, which could be free from side ill effect. According to Brandes, (1958) " Much of our efforts are being wasted in routine testing of the standard bactericides, often there is a pressing need to investigate new sources of effective bactericide. Green plants appear to be reservoir of effective chemo-therapeutants and can provide renewable sources of useful bactericide and thereby reducing pressure on foreign exchange (Swaminathan, 1978).

Plants are used medicinally in different countries and are a source of many potent and powerful drugs. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases.

Ten plants *Foeniculum vulgare* (Mill), *Syzygium aromaticum* (L.) Merrill & Perry, *Citrus sinensis* (Risso), *Psidium guajava* (L.), *Murraya koenigii* (L.) Bergera Koenigii, Chalcas Koenigii, *Curcuma longa* (L.), *Punica granatum* (L.), *Hibiscus rosa-sinensis* (L.), *Eucalyptus tereticornis* (L'Hér.), *Piper nigrum* (L.) were identified and used in the research work. These plants are well known as medicinal plants because of their biological and pharmacological properties.

2. MATERIALS AND METHODS

The study was conducted in Neonatal Intensive Care Unit, Department of pediatrics, "Sardar Vallabh Bhai Patel Hospital" Meerut on 120 neonates admitted with features of suggestive of sepsis from February 2013 to June 2013. The isolation and identification of the microorganisms from the collected sample were carried out in the Department of Microbiology, "Lala Lajpat Rai Memorial Medical College" Meerut.

2.1. Detection of microorganisms by Versa TREK ™

The blood samples of neonates suffering from sepsis were collected under sterile condition from Sardar Vallabh Bhai Patel Hospital in Versa TREK vial. Blood sample of 0.5-1.0 mL was injected in each bottle. Once collected, the samples were transported with in 5-6 hours to the Department of Microbiology, L.L.R.M. Medical College Meerut. The Versa TREK vial with blood sample was placed in Versa TREK Automated Microbial Detection System and incubated for 5 days.

2.2. Isolation of microorganism

Positive blood culture vials were cultured onto blood agar and then the media were inspected after 24-48 hours at 37°C for the presence of bacterial growth. The isolated bacteria were defined by colonial morphology, Gram staining and biochemical tests.

2.2 Antibiotic resistance profile of bacterial isolates by Kirby Baurer disc diffusion method

The Kirby Baurer disc diffusion assay on Mueller-Hinton Agar plates was used for antimicrobial susceptibility testing against the microorganisms. The following antibiotics were used (µg/disc): Meropenem (ME) - µg , Imipenem (IM) – 10µg; Colistin (CL) – 10 µg; Polymixin-B (PB) – 300 units, Pipracillin/Tazobactum (Pt) – 100/10 µg; Amikacin (AK) – 30 µg; Netilmicin (Nt) – 30 µg; Aztreonam (AT) – 30 µg; Gentamycin (G) – 10 µg; Vancomycin (VA) - 30 µg, Teicoplanin (TEI) – 30 µg; Linezolid (LZ) – 30 µg; Cefoxitin (CX) – 30 µg; Erythromycin (ERT) - µg.

2.3 Selection of plants

Ten plants were collected from local areas of Meerut and all were selected from various sites of Meerut. Out of ten plants, five were used as leaf extract, two were used as fruit extract and one was used as flower bud extract while the remaining two was used as peel extract.

2.4 Preparation of plant extracts

The crude plant extracts were prepared by shade drying the leaves for few days. When the moisture content of the leaves was totally evaporated, they were crushed to a very fine powder with the help of a mixture grinder. 0.5gm leaf, fruit, bud and peel powder of different plants was then extracted in 2ml each of six different solvents i.e. Acetone, Benzene, Chloroform, Ethanol, Ethyl acetate, Methanol separately. The overnight extracts were filtered with a Whatman's No. 1 filter paper and stored at 4^{o}C in refrigerator.

2.5. Antimicrobial screening of plant extracts against isolated bacteria

Agar well diffusion method

The plant extracts were tested by the traditional agar well diffusion method. 400 µL of overnight inoculum of the test microorganism was spread on the surface of a Mueller-Hinton Agar plate with the help of a glass spreader. Then, with the help of a sterilized borer, 3 equidistant wells were made on the two different plates. The care was taken that wells were made without damaging the whole agar plate. 100 µL of each solvent extract of a plate was pipette in those wells and the plates were incubated at 37^{o}C. In control 100 µL DMSO was used in place of plant extract. Plates were examined for inhibition zones after incubation for 24 hours. A clear zone around the well was measured by Hi-antibiotic zone scale (Hi-media).

2.6. Determination of Minimum Inhibitory Concentration (MIC) by microtiter plate assay

MIC is expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube. A broth microdilution assay was adopted using 96 well micro titer plates with resazurin. It was carried out to assess the microbial growth and determine the Minimal Inhibitory Concentration Sarker *et al*. (2007).

2.7. Preparation of microtitre plates

A sterile 96 well microtiter plate was labelled. A volume of test extract (50 µL/mL acetone) was pipetted into the first row of the plate, from A1 to H1. Wells from A2 to H2 till A10 to H10 were dispensed with 50 µL of nutrient broth. 50 µL of test extract was transferred from test solution (A1-H1) to next wells (A2-H2) and so on to create serial dilutions. Tips were discarded after use such that each well had 50µL of the test material in serially descending concentrations. To each well, from A2 to H2 till A10 to H10, 20 µL of resazurin indicator solution was added. Finally, 30 µL of bacterial suspension (5×10^{6} cfu/ml) was added to each well ensuring a final volume of 100 µL in them. A9 and A10 served as controls having 50 µL of solvents of analytical grade (acetone) in place of oil.

The plates were prepared in replicates, sealed with cling film and placed in incubator set at 130 rpm at 30^{0}C for 24 hours. The colour change was then assessed visually. Any colour changes from purple to pink or colourless were recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value. The average of two values was calculated and that was the MIC for the test material and test organism strain.

2.8. Determination of minimum bactericidal concentration (MBC) values

To determine the MBC for each set of wells in the MIC determination, a loopful of broth was collected from those wells that did not show any growth or color change and inoculated on sterile agar plate by streaking. Nutrient agar plates were streaked with loopful of culture only to serve as control. The plates were incubated at 30°C for 24h. After incubation the concentration at which no visible growth was observed as noted as the MBC.

3. RESULTS AND DISCUSSION

In an attempt to observe the microbial infection in neonates, for the study of neonatal septicemia, taken from the NICU of Sardar Vallabh Bhai Patel Hospital Meerut. Antibiotic sensitivity tests for the important isolates were also conducted in the Department of Microbiology L.L.R.M. Medical College Meerut on 120 samples. Out of 80 blood samples, 27.50% *E.coli* were isolated, 17.50% *Klebsiella* were isolated, 21.25% *Staphylococcus aureus* were isolated, 7.50% CoN*S* were isolated, 13.75% *Enterococci* were isolated while 12.50% *Pseudomonas* were isolated.

E.coli was 100% resistant to Aztrenam and Gentamycin, *Klebsiella* was 100% resistant to Imipenem, Aztreonam and Gentamycin and *Pseudomonas aeruginosa* was 100% resistant to Imipenem, Polymixin-B, Pipracillin/tazobactum, Aztrenam and Gentamycin.

S.aureus was 100% resistant to Amikacin, Netilmicin, Cefoxitin, Erythromycin, Gentamycin and Polymixin-B, CoN*S* was 100% resistant to Amikacin, Netilmicin, Cefoxitin, Erythromycin, Gentamycin, *Enterococci* was 100% resistant to Amikacin, Netilmicin, Cefoxitin, Erythromycin, Gentamycin and Polymixin-B.

The present investigation has led to the conclusion that the phytochemicals (extracts) produced by *Syzygium aromaticum* possess the antimicrobial properties against the sepsis causing bacteria in neonates. The lowest MIC value of Acetone extract was 1.56×10^{-6} µL, Ethanol extract was 1.56×10^{-6} and Methanol extract was 0.78×10^{-7}.

Neonatl septicemia is one of the major causes of mortality and morbidity. Septicemia usually consists of bacteremia plus a constellation of signs and symptoms caused by the microorganisms or their toxic products in the circulation. The usual source of infection include incubators (especially humidity tank), resuscitators, ventilators, solutions for cold

sterilization, feeding bottles, catheters, face masks and infusion sets and sites *etc* Cassone *et al.* (2003).

Sepsis is a significant cause of death in the newborn, particularly among those of very low birth weight and premature infants. The World Health Organization (WHO) estimates that worldwide 1.6 million newboen baby die every year from neonatal infections. Despite recent advances in neonatal intensive care and current strategies to treat neonatal sepsis, mortality rates have not fallen for over three decades except in babies born to mothers who have received intrapatum prophylaxis (IAP) for Group B *Streptococcus* Al-Shamahy *et al.* (2012).

The MIC values of acetone, ethanol and methanol extracts of *Syzygium aromaticum* for *E.coli* were 3.12×10^{-5}(µg/mL), 3.12×10^{-5}(µg/mL) and 3.12×10^{-5}(µg/mL) respectively. The MIC values of acetone, ethanol and methanol extracts against *Klebsiella* were 1.56×10^{-6}(µg/mL), 1.56×10^{-6}(µg/mL) and 0.78×10^{-7}(µg/mL). The MIC values of acetone, ethanol and methanol for *Staphylococcus* were 1.56×10^{-6}(µg/mL), 1.56×10^{-6}(µg/mL) and 0.78×10^{-7}(µg/mL). The MIC values of acetone, ethanol and methanol extracts against CoNS were 3.12×10^{-5}(µg/mL), 3.12×10^{-5}(µg/mL) and 1.56×10^{-6}(µg/mL). The MIC values of acetone, ethanol and methanol against *Enterococcus* were 1.56×10^{-6}(µg/mL), 3.12×10^{-5}(µg/mL) and 0.78×10^{-7}(µg/mL). The MIC values of acetone, ethanol and methanol extracts against *Pseudomonas* were 3.12×10^{-5}(µg/mL), 3.12×10^{-5}(µg/mL) and 1.56×10^{-6}(µg/mL).

Table-1: Isolated bacterial species and their percentage from blood sample.

S.NO.	Isolated Bacteria	No. of cases	Percentage
1.	*E.coli*	22	27.50%
2.	*Klebsiella*	14	17.50%
3.	*Pseudomonas aeruginosa*	10	12.50%
4.	*Staphylococcus aureus*	17	21.25%
5.	CoN*S*	6	7.50%
6.	*Enterococcus*	11	13.75%
Total		80	100%

Table-2 Antibiotic resistance pattern shown by Gram negative bacterial isolates from blood sample

Gram Negative Organisms Antibiotics	**Number and % of resistance cases**		
	***E.coli* [n=22]**	***Klebsiella* [n=14]**	***Pseudomonas* [n=11]**
Meropenem (ME)	16 (72.7)	8 (57.1)	7 (63.6)
Imipenem (IM)	16 (72.7)	14 (100)	11 (100)
Colistin (CL)	11 (50)	9 (64.2)	2 (18.1)

Polymixin-B (PB)	18 (81.8)	10 (71.4)	11 (100)
Piperacillin/tazobactum (Pt)	19 (86.3)	13 (92.8)	11 (100)
Amikacin (AK)	20 (90.9)	12 (85.7)	1 (9.09)
Netilmicin (Nt)	20 (90.9)	13 (92.8)	3 (27.2)
Aztreonam (AT)	22 (100)	14 (100)	11 (100)
Gentamycin (G)	22 (100)	14 (100)	11 (100)

Table-3: Antibiotic resistance pattern shown by Gram positive bacterial isolates from blood sample

Gram Positive Organisms Antibiotics	**Number and % of resistance cases**		
	***S. aureus* [n=17]**	**CONS [n=6]**	***Enterococci* [n=11]**
Vancomycin (VA)	10 (58.8)	3 (50)	8 (72.7)
Teicoplanin (TEI)	9 (52.9)	3 (50)	8 (72.7)
Linezolid (LZ)	9 (52.9)	3 (50)	5 (45.4)
Amikacin (AK)	17 (100)	6 (100)	11 (100)
Netilmicin (Nt)	17 (100)	6 (100)	11 (100)
Cefoxitin (CX)	17 (100)	6 (100)	11 (100)
Erythromycin (ERT)	17 (100)	6 (100)	11 (100)
Gentamycin (G)	17 (100)	6 (100)	11 (100)
Polymyxin-B (PB)	17 (100)	1 (16.6)	11 (100)

Table-5: Minimum inhibitory concentration of bioactive plant extracts (*Syzygium aromaticum*) against test organisms.

Microorganism	**Minimum inhibitory concentration (µg/mL)**		
	Acetone extract	**Ethanol extract**	**Methanol extract**
E.coli	3.12	3.12	3.12
Klebsiella spp.	1.56	1.56	0.78
Pseudomonas spp.	3.12	3.12	1.56
Staphylococcus aureus	1.56	1.56	0.78
CoNS	3.12	3.12	1.56
Enterococcus spp.	1.56	3.12	0.78

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