ANTI-BACTERIAL EFFECT OF EXTRACTS OF OCIMUM ON IMIPENEM RESISTANT GRAM NEGATIVE BACTERIA ISOLATED FROM BURN WOUND INFECTIONS

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ABSTRACTS

Background & Objectives: Emergence of imipenem resistance in gram negative bacteria (GNB) is threatening to become a devastating condition especially in hospital critical care areas like burn units. In the grim scenario of non-availability of newer effective broad-spectrum antibiotic, we decided to determine the antibacterial activity of Ocimum (Tulsi) against imipenem resistant (IR) bacteria isolated from burn wound infections (BWI).

Methods: 108 GNB isolated from burn wounds were included in this study. Screening of imipenem resistance was done by disk diffusion (Imipenem disk =10μg) method & confirmed by microbroth dilution method (0.25-128μg/ml). Imipenem-EDTA disk diffusion method was used for detection of metallo-β-lactamase (MBL) production & confirmed by PCR detection of the gene. Ethanolic soxhlet extract & essential oil (EO) of Ocimum was tested against imipenem resistant gram negative bacteria (IRGNB) by agar disk diffusion method. Tests for synergism between Ocimum extract & imipenem was also done by disk potentiation test. Chemical fingerprint of essential oil was obtained by GCMS.

Results: 32 (29.62%) of the GNB isolated were identified as IRGNB having MIC in the range of 16 to ≥128 μg/ml. 37.5% IR isolates were MBL producers. 6 (23.07%) of 26 isolates tested gave significant inhibition zones & corresponding MIC values ranging 24-26mm & 4-32μg/ml respectively with EO of Ocimum. Synergistic interaction was observed in enterobacteriaceae isolates when EO of Ocimum was tested along with imipenem (10μg) disk.

Interpretation & Conclusions: The incidence of 29.62% IR in this study is quite alarming as retrospective analysis of institutional data shows increasing trend. Crude ethanolic soxhlet extract of Ocimum was seen not to be effective against IR strains. However, essential oil of Ocimum may prove to be a panacea in the combat against imipenem resistant infections especially due to enterobacteriaceae.

KEYWORDS: Burn Wound Infections, Gram Negative Bacteria, Imipenem Resistant, Ocimum
INTRODUCTION

Infection is the major cause of death following burn injury [1]. Burn care units can be the site of explosive & prolonged outbreaks caused by resistant organisms. Such outbreaks, especially due to IR organisms are highly worrisome due to non-availability of effective antibiotic panacea [2]. Infection due to IR organisms is a burning problem in the burn units of our hospital as well. In the current scenario of non-availability of newer potent broad spectrum antibiotics against IRGNB, there has been a renewed interest to explore alternative natural sources. The Ocimum (Tulsi) plant, a sacred plant revered by the Hindus, is known for its medicinal properties. Presently, it is recognized that there is some scientific evidence for several of its properties, including its antimicrobial property[3]. The genus Ocimum is reported to have good antibiotic efficacy against pathogenic GNB [4,5]. However, in the best of our knowledge, no literature is available till date, on efficacy of Ocimum against IRGNB. Hence, it seemed prudent to us to test the efficacy of Ocimum against IRGNB isolated from burn wounds.

MATERIALS AND METHODS

The present study was conducted on 108 pure isolates of gram negative bacteria isolated from surface swab cultures of burn wounds of patients admitted to the burn units of J.L.N. hospital, Ajmer over a period of two years from April 2010 to March 2012. Samples were taken from infected burn wound areas mainly on extremities & trunk. The samples obtained were cultured on MacConkey agar (MCA) and Blood agar (BA) and incubated at 37°C for 24 to 48 hrs. All gram negative bacteria grown on culture plates were identified by standard microbiological methods. Antibiotic susceptibility testing by modified Kirby-Bauer disc diffusion method was done as per standards [6]. All isolates showing imipenem resistance on disk diffusion were subjected to imipenem and imipenem-EDTA disc potentiation test for detection of metallo-β-lactamase mediated resistance, ocimun disk diffusion test, MIC determination against imipenem (0.25-128µg/ml) & ocimum (0.5-128µg/ml) by microbroth dilution method as per CLSI guidelines [7]. PCR for MBL gene detection was done at JIPMER, Pondicherry.

Test for efficacy of ethanolic soxhlet extract of Ocimum: Extraction of ethanolic Soxhlet extract of Ocimum

The plants used in this study were collected from local home gardens. 50gm of finely ground powder of dry leaves of Ocimum was placed inside a thimble, and was loaded into the main chamber of the soxhlet extractor. The Soxhlet extractor was placed onto a flask containing 450ml ethanol as extraction solvent. The extraction was continued for 72 hours. After extraction, 5gm of the extracted compound was obtained by evaporation. This was reconstituted to 100% (w/v) and kept in the refrigerator at 2-8°C throughout the study period.

Agar disk diffusion by ethanolic soxhlet extract of Ocimum

Swabbing of MHA plate was done with 0.5 McFarland matched suspension of bacteria. Sterile disk (10mm) was placed on the swabbed MHA plate & 20µl of ethanolic soxhlet extract of Ocimum was dispensed on it. The plate was incubated at 37°C for 24 hrs and inhibition zone sizes were noted.

Test for efficacy of essential oil of Ocimum

Extraction of essential oil (EO): 50gm leaves of Ocimum and 250 ml of water were placed in a Clevenger type apparatus. The EO was isolated by hydrodistillation for 8 h. The obtained EO was separated, dried over anhydrous sodium sulphate and stored at -20°C before usage [8].

Identification of the EO composition: The constituents of essential oil were analyzed by gas chromatography (GC) coupled to mass spectrometry (MS). GC analysis was carried out using a GC chromatograph at National Research Centre for Seed Spices (NRCSS), Tabiji, Ajmer. The compounds were identified by comparing their retention times and mass spectra with those obtained from the authentic samples or the MS library.

Agar disk diffusion by EO of Ocimum: Swabbing of MHA plate was done with 0.5 McFarland matched suspension of bacteria. Sterile disk (10mm) was placed on the swabbed MHA plate & 20µl of EO of Ocimum was dispensed on it. The plate was incubated at 37°C for 24 hrs and inhibition zone sizes were noted.

Agar disk diffusion test for synergism between EO of Ocimum and imipenem: Swabbing of MHA plate was done with 0.5 McFarland matched suspension of bacteria. Three disks namely: imipenem (10mcg), EO of Ocimum (20µl), and imipenem (10mcg) along with the EO of Ocimum (20 µl), were placed on MHA plate at an interdisk distance of 30mm centre to centre. The plate was incubated at 37°C for 24 hrs and zones of inhibition noted.

MIC determination against EO of ocimun : MIC for ocimun was determined by microbroth dilution testing as described by Drummond and Waigh in 2000[9]. 100% v/v EO of Ocimum in 10% DMSO to achieve final concentration in the range 0.5-256 µg/ml was used. Final bacterial inoculum density of 10^8 CFU was achieved in each well. Resazurin was used as the redox dye for observing the results. Results were read after full 24 hours of incubation at 37°C.

E.coli ATCC 25922 , E.coli ATCC 35218, P.aeruginosa ATCC 27853 & A. baumannii ATCC 19606 were used as Quality Control (QC) strains as appropriate for all the tests performed.

RESULTS

Out of 145 bacterial isolates tested, 108 (74.48%) were identified as GNB of which, 32 (29.62%) showed resistance to imipenem (10µg) on the modified Kirby Bauer disk diffusion test [Table 1]. All the 32 IRGB showed MIC in the range of 16 to >128µg/ml [Table 2]. 20 (62.5%) of the IR isolates were non fermenters with
**Pseudomonas spp.** (n=13, 65%) leading the list, followed by **Enterobacteriaceae** (n=12, 37.5%) with **Klebsiella** (n=5, 41.7%) leading the list. 12 (37.5%) of the 32 IR isolates were MBL positive by disk potentiation test & were confirmed by polymerase chain reaction (PCR) for MBL gene at JIPMER, Pondicherry.

Whereas all 32 isolates were tested against ethanolic extract, only 26 could be tested against essential oil of **Ocimum** by disk diffusion method. The results are given in Table 3 & Table 4.

**Table 1: Results of imipenem disk diffusion & MIC testing of imipenem resistant strains**

<table>
<thead>
<tr>
<th>Methods using imipenem</th>
<th>Total tested</th>
<th>Imipenem resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar disk diffusion</td>
<td>108</td>
<td>32 (29.62%)</td>
</tr>
<tr>
<td>MIC by microbroth dilution</td>
<td>32</td>
<td>32</td>
</tr>
</tbody>
</table>

**Table 2: Results of Imipenem & Ocimum MIC for different IRGNB isolates**

<table>
<thead>
<tr>
<th>IRGNB tested</th>
<th>No. of isolates tested</th>
<th>Imipenem MIC (µg/ml)</th>
<th>Ocimum MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterobacteriaceae</strong></td>
<td>N=12</td>
<td>16 to &gt;128</td>
<td>4-128</td>
</tr>
<tr>
<td><strong>Klebsiella pneumonia</strong></td>
<td>N=5</td>
<td>16 to &gt;128</td>
<td>8-32</td>
</tr>
<tr>
<td><strong>Citrobacter diversus</strong></td>
<td>N=4</td>
<td>64 to 128</td>
<td>16-128</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>N=1</td>
<td>128</td>
<td>4</td>
</tr>
<tr>
<td><strong>Enterobacter aerogenes</strong></td>
<td>N=1</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td><strong>Proteus mirabilis</strong></td>
<td>N=1</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>Non Fermenters ( NF )</td>
<td>N=20</td>
<td>16 to &gt;128</td>
<td>32-128</td>
</tr>
<tr>
<td><strong>Pseudomonas spp.</strong></td>
<td>N=13</td>
<td>64 to &gt;128</td>
<td>32-128</td>
</tr>
<tr>
<td><strong>Acinetobacter sp.</strong></td>
<td>N=2</td>
<td>32 to 128</td>
<td>64</td>
</tr>
<tr>
<td><strong>Sphingobacterium sp.</strong></td>
<td>N=1</td>
<td>&gt;128</td>
<td>32</td>
</tr>
<tr>
<td>Unidentified NF*</td>
<td>N=4</td>
<td>16 to 128</td>
<td>NT</td>
</tr>
<tr>
<td>ATCC Q.C strains</td>
<td>N=3</td>
<td>0.25-0.5</td>
<td>4-16</td>
</tr>
<tr>
<td><strong>E. coli 35218</strong></td>
<td>N=1</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td><strong>P. aeruginosa 27853</strong></td>
<td>N=1</td>
<td>0.5</td>
<td>32</td>
</tr>
<tr>
<td><strong>A. baumanii 19606</strong></td>
<td>N=1</td>
<td>0.25</td>
<td>16</td>
</tr>
</tbody>
</table>

* Non fermenters, NT - not tested, Q.C - quality control

**Table 3: Results of efficacy of ethanolic soxhlet extract of Ocimum by disk diffusion method**

<table>
<thead>
<tr>
<th>Total isolates</th>
<th>No zone</th>
<th>Zone sizes (10-14mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>27 (84.3%)</td>
<td>5 (15.6%)</td>
</tr>
</tbody>
</table>

**Table 4: Results of efficacy of EO of Ocimum**

<table>
<thead>
<tr>
<th>IRGNB tested</th>
<th>No. of isolates (N=26)</th>
<th>Inhibition Zones by ocimum disk diffusion (mm)</th>
<th>Inhibition Zones by Synergy (I&amp;O) testing (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Klebsiella pneumonia</strong></td>
<td>N=5</td>
<td>24-26</td>
<td>26-28</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>N=1</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td><strong>Pseudomonas spp.</strong></td>
<td>N=13</td>
<td>No zone</td>
<td>No zone</td>
</tr>
<tr>
<td><strong>Citrobacter diversus &amp; Proteus mirabilis</strong></td>
<td>N=5</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td><strong>Acinetobacter sp.</strong></td>
<td>N=2</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>ATCC Q.C strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC E. coli 25922</td>
<td>N=1</td>
<td>34</td>
<td>NT</td>
</tr>
<tr>
<td>ATCC P. aeruginosa 27853</td>
<td>N=1</td>
<td>11</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Imipenem, O=Ocimum*

**DISCUSSION**

This study was done to determine the magnitude of problem due to IRGNB isolated from burn wounds of patients admitted to the burn critical care units & to study the antibacterial effect of **Ocimum** on these isolates. The estimated annual burn incidence in India is approximately 6-7 million per year & is the second largest group of injuries after road accidents [10]. Annual incidence of burns in our hospital during the study period was 10.25 per
1,00,000 population with mortality rate of 3.2 per 1,00,000 population. Worldwide GNB infections in burn wounds has been reported ranging from 56.0% to 80.71% [11,12,13,14,15]. *P. aeruginosa* was the most frequently (40.62%) isolated organism in this study. Similar incidences have been reported in various studies varying from 26% to 55.3% [13,14,15,16]. The first isolate of an IR strain was *P. aeruginosa* from Japan in the year 1988 [17]. Since then, imipenem resistance has been increasingly reported all over the world. Currently reports of IR up to 61.2% has been documented in BWI [12,13,14,15,16]. In this study, 29.62% burn wound isolates were found to be IR with very high MIC values ranging from 16 to >128µg/ml. Similar results have been reported by various researchers [18,19].

Based on retrospective analysis, the incidence of IR in BWI has risen over the years in our setting from 12% in 2009 (unpublished data from local hospital research study) to 29.62% in 2012, which is food for worry. 5.45% were also found to produce extended spectrum β-lactamases (ESBLs). 39.8% of isolates were found to produce inducible type 1 cephalosporinase. This is probably due to the overuse of cephaplorins to treat BWI as has been evidenced by various researchers [12,14,20]. The only β-lactam antibiotics not adversely affected by induction of these β-lactamases are mecillinam, imipenem & related carbapenem antibiotics. However, due to co-existence of imipenem resistance & MBL production as well, treatment of infection due to these organisms poses a major challenge. In India, MBL producing *P. aeruginosa* was first reported in 2002 [20] & current research reports document upto 39% [12,14,15]. MBL producing strains are associated with a higher case fatality rate & invasive disease. Hence, higher incidence of MBL (37.5%) in this study is a cause of great concern in the therapy of critically ill burn patients. A combination of colistin, cilastatin &/or rifampicin along with imipenem is usually tried in a desperate effort to control such outbreaks & save the life of the patient [22,23,24]. However, many IR bacterial isolates may become resistant to this combination as well during therapy leaving the patient prey to such notorious organisms & this probably is the reason for higher case fatality rate of 32.07% in this study.

Drug discovery is not a fast enough process & with no newer potent antibiotic in the near pipeline, it makes immense sense to explore the empirical wisdom of the ancients with modern technology. Tulsi (*Ocimum sanctum* Linn.) a sacred plant belongs to the family *Lamiaceae*. Different parts of the plant have been reported to be effective in wide spectrum of diseases. Both ethanolic extract & EO of *Ocimum* are found to possess antibacterial properties when tested against various GNB [4, 5, 25, 26]. However, in the best of our knowledge, studies on its efficacy on IRGNB have not been reported in literature till date. Hence it seemed prudent to study its antibacterial effect on IRGNB isolated from burn wounds. *Ocimum sanctum* is composed of aromatic essential oils mainly concentrated in its leaves. This aromatic volatile oil mainly contains phenols, terpenes and aldehydes. The oil extracted from seeds is called fixed oil and mainly composed of fatty acids. Besides oil, the plant also contains alkaloids, glycosides, saponins and tannins. The leaves contain ascorbic acid and carotene as well [27]. The present day information about the chemical properties is based on various studies that have been done in different parts of the world and it is likely that chemical constituents may be varying due to edaphic and geographic factors [28,29].

In this study, ethanolic *Ocimum* extract did not show appreciable inhibition against the various pathogenic bacteria tested. It failed to show good results even with Quality Control (sensitive) strains. However, EO of *Ocimum* showed wide appreciable zones of inhibition against ATCC as well as IR enterobacteriae isolates (Figure 1). *viz. E. coli* & *K. pneumonia* with MIC values ranging 4-32µg/ml (Figure 2). Good inhibitory effect was also seen against *Acinetobacter spp*. Synergistic interaction of imipenem & EO of *Ocimum* was also observed (Figure 1). This shows that EO of *Ocimum* may have a promising role in the treatment of IRGNB infections. EO extracted from the leaves of *Ocimum* has been found to inhibit in-vitro growth of *E. coli* and *P. aeruginosa* in various studies [30]. However, in this study, EO of *Ocimum* showed in-vitro inhibitory effect on IR *P. aeruginosa* strains only at concentrations above 32µg/ml. Nevertheless, MIC of EO of ocimum was lesser in comparison to imipenem MIC for all the isolates tested.

**Figure 1: Agar disk diffusion test showing inhibition zones against imipenem(13mm), Ocimum (24mm) and synergistic interaction of imipenem & Ocimum combined(26mm)**

Chemical characterization of EO of *Ocimum* used in this study showed presence of eugenol (33.56%), caryophyllene (20.68%), & other phenolic compounds as main constituents. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active constituent present in *Ocimum*, has been found to be largely responsible for the therapeutic potentials of Tulsi. Inspite of wide usage of *Ocimum* by practitioners of traditional systems of medicine for curing various ailments, a rational approach to this traditional medical practice with modern system of medicine is, however, not much available [31]. Since, eugenol was the
major active constituent in our preparation, we assume that this was responsible for the antibacterial effect against IR *Enterobacteriaceae* strains when tested in-vitro. Caryophyllene was the second most abundant compound found in our preparation and is also known to have antibacterial effect against some pathogenic GNB\textsuperscript{12,23}. Hence, it would have also contributed to the antibacterial effect in this study to some extent. However, further elaborate studies on a large number of clinical isolates, as well as pharmacological studies after chemical purification, cytotoxicity testing & bioavailability studies are warranted before this can be put to pharmaceutical use & treatment of IRGNB infections.

**Figure 2:** Microtitre plate showing results of Ocimum microbroth dilution MIC test for IRGNB isolates

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