Effect of zinc sulphate on antimicrobial activity of Clitoria ternatea

Kumar Dinesh1,2*, Gajbhiye Archana1, Nagda Vipin1 and Arora Asha2
1. Department of Biotechnology, Sir Padampat Singhania University, Bhatewar, Udaipur-313601, INDIA
2. Department of Biotechnology, B.N. University, Udaipur-313001, INDIA
*dinesh.chhatwani@gmail.com

Abstract
In the present study, the antimicrobial activity in crude extracts of roots of C. ternatea and synergistic effect of zinc sulphate on antimicrobial activity of root extract was evaluated. The root extracts were prepared using water and methanol. The minimum inhibitory concentration (MIC) was measured by broth dilution method. Both crude extracts i.e. aqueous and methanolic showed positive antimicrobial activity at concentration more than 10mg/ml. The combination of methanolic extract (50mg/ml) and zinc sulphate (1% w/v) exerted an elevated effect on antimicrobial activity by approximately 20% as compared to that of only crude methanolic extract against all three pathogenic bacteria.

In a combination of aqueous root extract and of zinc sulphate the antibacterial activity did not show any major change. The MIC of methanolic extract was found to be 0.125±0.03, 2.5±0.05 and 5.00±0.03 mg/ml against E.coli, S.aureus and K. pneumoniae. The significance of results was confirmed from the p-value which was found to be <0.01. The antibacterial activity of methanolic root extract from C. ternatea exhibits maximum bacterial growth inhibition compared to aqueous root extract. Significant synergistic effect between methanol extract and zinc sulphate was obtained to inhibit the growth of all selected bacterial isolates.

Keywords: Escherichia coli, MIC, C. ternatea, K. pneumoniae, S. aureus, ZnSO4.

Introduction
Most of the synthetic drugs cause side effects and also most of the microbes developed resistance against the synthetic drugs1. This situation has led to a re-evaluation of the therapeutic use of ancient remedies such as plants and metals2-4.

Plants produce a diverse range of bioactive molecules, making them rich source of different types of medicines. Thus, it is a logical approach in drug discovery to screen traditional plant based natural products. Wide range of substances present in medicinal plants have been used to treat chronic as well as infectious diseases5. For long, the plant based drugs are less toxic with scanty side effects and comparatively cheaper.

Clitoria ternatea (Papilionaceae) popularly known as Vishnukanta is a perennial twining herb. Its various parts have been reported for its tranquilizing, anti-inflammatory, analgesic, antipyretic and immuno-modulatory properties6,9. The flavonol glycoside present in roots was reported to have antibacterial activity6. There are reports available on the antibacterial activity against the non-pathogenic bacteria more than pathogenic ones.

Apart from natural compounds from plants, metals are also identified and used as antimicrobial agents. The use of metals as medicine was prevalent until the discovery of antibiotics3. Some of the metals reported with the antimicrobial activity are copper, arsenic, platinum, zinc etc3,10. A number of metals including zinc (Zn2+) are among important elements of the biological systems, as they serve essential roles in the maintenance of the 3D structure of many proteins. Zinc is a micronutrient which is considered as an essential element in the growth and development of plants, however, when present in greater amounts, metabolic disorders eventually leading to growth inhibition may follow in both plants and microorganisms.

Considering the vast potentiality of medicinal plants and metals as a source of antimicrobial drugs, the present study was aimed to screen the effect of aqueous and methanolic extracts of C. ternatea root, zinc sulphate and their combination on certain important pathogenic bacteria.

Material and Methods
Chemicals: The chemicals and reagents used were of analytical grade. Zinc sulphate was obtained from Sigma-Aldrich. Methanol, tetracycline, DMSO and 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) were obtained from Fisher Scientific. Biological media like Muller Hinton agar and Muller Hinton broth were obtained from Hi Media lab.

Collection of Experimental Plant Material: The roots of C. ternatea were collected from different localities of Udaipur (Rajasthan) and were identified and authenticated from Botanist, BN University, Udaipur, Rajasthan, India. The herbarium sheets were prepared as per taxonomical guidelines and deposited in department with accession no BIOT/ 2015-17/ 176-R. The root material was washed in running tap water to get rid of soil and other moieties followed by washing through sterile distilled water. Uncontaminated and uninfected washed and neat material was air dried and ground to fine powder using a blender and stored at room temperature in air-tight container for further experimental usage.
Extract Preparation: A fine root powder (10 grams) was added to the solvents (100 ml) methanol and distilled water. The contents were mixed in a rotary shaker at 220 rpm for 48 hours. It was then filtered through Whatmann No.1 filter paper and the filtrate was evaporated to dryness at 80°C.

Test Microorganisms: The test microorganisms used for antimicrobial activities were the pathogenic isolates of Escherichia coli (Gram-negative bacteria), Klebsiella pneumoniae (Gram-negative bacteria) and Staphylococcus aureus (Gram positive bacteria). The cultures were procured from RNT medical college, Udaipur and maintained in the laboratory by serial sub culturing.

Positive and Negative Control: To assess the antibacterial efficiency of selected root extract and zinc sulphate, tetracycline (1 mg/ml) and dimethyl sulphoxide [DMSO (50% v/v)] were used as positive and negative control respectively.

Assay for antibacterial activity: The test samples and the positive and negative control were set to perform the antimicrobial assay against Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus as follows:

<table>
<thead>
<tr>
<th>Set I</th>
<th>Methanolic root extract of C. ternatea (50 mg/ml).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set II</td>
<td>Aqueous root extract of C. ternatea (50 mg/ml).</td>
</tr>
<tr>
<td>Set III</td>
<td>Zinc Sulphate (1% w/v)</td>
</tr>
<tr>
<td>Set IV</td>
<td>Summative methanolic root extract of C. ternatea (50 mg/ml) and Zinc Sulphate (1% w/v).</td>
</tr>
<tr>
<td>Set V</td>
<td>Summative aqueous root extract of C. ternatea (50 mg/ml) and Zinc Sulphate (1% w/v).</td>
</tr>
<tr>
<td>Set VI</td>
<td>Positive control (Tetracycline 1 mg/ml).</td>
</tr>
<tr>
<td>Set VII</td>
<td>Negative control (50% DMSO).</td>
</tr>
</tbody>
</table>

Evaluation of antibacterial activity: The antimicrobial activity of Clitorea ternatea root extracts was analyzed by agar well diffusion method according to Murray et al. The Muller-Hinton agar medium was poured onto the Petri plates with bacterial culture (10⁶ colony forming units (CFU) per ml). The wells (6 mm diameter) were made by using borer. The samples (Set I to Set VII) were dispensed; into the respective wells and were allowed to diffuse for 45 min the plates were incubated at 37°C for 24 h. The tetracycline solution (1 mg/ml) and 50% DMSO were used as positive and negative control respectively. The analysis was carried out in triplicate and the sensitivity of the microbial species to the extract was determined by measuring the diameter of the clear zones.

Statistical analysis: All experiments were carried out in triplicate and are expressed as average of three analyses ± standard deviation. The magnitude of correlation between variables was done using a SPSS statistical software package (SPSS for Windows, ver. XXI, 2018).

Determination of Minimum Inhibitory Concentration (MIC): Minimum inhibitory concentration (MIC) of both methanolic and aqueous extract of Clitoria was determined by broth dilution method. A 96 well micro titre plate was loaded with 100 mg/ml of two-fold serially diluted plant extracts in MH broth. 20 µL of each bacterial suspension in Muller Hinton broth medium was added to the wells. The final volume in each well was made up to 200 µL. Control wells were prepared with culture medium with tetracycline, bacterial suspension only and plant extracts with broth only. The content of each well was mixed in the micro titre plate and incubated for 24 hrs at 37°C.

After incubation, 20 µL of 2, 3, 5- Triphenyl Tetrazolium Chloride (TTC) (20 mg/ml dissolved in autoclaved distilled water) was added to each well and incubated for 30 min in dark. The wells were observed for appearance of red colour which is indicative of respiratory activity of the microorganism. The lowest concentration at which no colour change was observed was used for determining the MIC. The experiment was performed in triplicate.

Results and Discussion

Clitorea ternatea is one of the herbs which is traditionally used as Shankapushpi, an Ayurvedic medicine used to promote neurological health. It has adaptations to a wide range of soil conditions, resistance to drought and several pests and pathogens. These properties of the plants were attributed to the presence of various metabolites produced in the roots. C. ternatea root has been reported with anti-inflammatory, analgesic and anti-pyretic properties. The antibacterial activity of C. ternatea in methanolic extract of roots was studied against E. coli, S. aureus and K. pneumonia, responsible for causing infections of gastrointestinal tract, wound and the respiratory tract respectively.

Antibacterial activity of crude extract of root: In agar well diffusion assay for antimicrobial assay, both the extracts i.e. methanolic (100 mg/ml) and aqueous (100 mg/ml) extracts showed antibacterial activity against Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus (Table 1). The zone of microbial growth inhibition surrounding the extract wells was found to be minimum of 8.00±1.00 mm in aqueous root extract against S. aureus and a maximum of 21.67±02.05 mm in methanolic extract against K. pneumoniae.

The methanolic extract of C. ternatea root was found to be more effective in inhibiting microbial growth of all the three bacterial isolates Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus compared to aqueous extract (Table 1). The inhibitory effect of methanolic root extract on Klebsiella pneumoniae and Staphylococcus aureus was 50% and 25% more pronounced respectively compared to inhibitory effect on Escherichia coli. The antimicrobial properties in root have been mostly reported in ethyl acetate extract against fungi and a few plant pathogens. The
The present study in which zinc sulphate inhibited the growth of all tested bacterial isolates at 10mg/ml was at par with observation of Surjawidjaja et al.\textsuperscript{24} who studied the \textit{invitro} effect of zinc sulphate against enteric bacteria.

Zinc sulphate showed the growth inhibitory effect against all three bacterial cultures at a concentration of 10mg/ml. The growth inhibition zone (clear zone) was either equal or more than 10.00 mm. Compared to the positive control tetracycline at a concentration of 1mg/ml, the inhibitory effect of ZnSO\textsubscript{4} against all the three bacterial isolates was not at par as tetracycline.

\textbf{Antibacterial activity of root extract in combination with zinc sulphate:} The antimicrobial properties of metal salts have been known for long\textsuperscript{3}. It is also important to consider the effect of metals and plant extracts that may have effect on the environment as well as on plant or animal cells. The root extracts were augmented with zinc sulphate and their synergistic effect on antimicrobial activity of root extracts was found to be encouraging. The combination of the two can be of use to lower the effective concentrations of plant extracts and metals than those that are used for each of them individually. Both \textit{Clitoria ternatea} root extract and zinc sulphate in lower concentration have relatively low toxicity to humans, animals and plants.

Methanolic root extract of \textit{C. ternatea} had shown significant antibacterial activity against all \textit{E. coli}, \textit{S. aureus} and \textit{K. pneumoniae} and this activity was found to increase on addition of ZnSO\textsubscript{4} (Table 1). Compared to the individual antibacterial effect of root extract, the combination of methanolic root extract and zinc sulphate showed 11.25\% higher activity against \textit{E. coli} and 11.00\% against \textit{S. aureus} (Figure 2 and Figure 3).

The addition of zinc sulphate enhanced the inhibitory action of methanolic extract by about 12\% against \textit{K. pneumoniae}. This revealed that the enhanced antimicrobial activity was due to the synergistic effect of methanolic root extract and zinc sulphate. In aqueous root extract, the addition of ZnSO\textsubscript{4} enhanced the antibacterial efficacy by 12.22\% against \textit{S. aureus} while it did not show upshot summative effect against \textit{E. coli} and \textit{K. pneumoniae} (Table 1). In both the extracts, ZnSO\textsubscript{4} increased the inhibitory effect against all three pathogenic bacterial isolates. The comparative study of all the extracts, combinations of extract and zinc sulphate and the positive control i.e. tetracycline found that ZnSO\textsubscript{4} synergistically enhanced efficacy of aqueous extract by 11\% to 13\% while that of methanic extract by 20\% to 22\% (Figure 3).

The individual effects of aqueous extract and ZnSO\textsubscript{4} revealed same antibacterial potential against \textit{K. pneumoniae} while for \textit{E. coli}, efficacy of aqueous extract exceeded by nearly 10 percent. An escalated effect of \textit{C. ternatea} was observed in aqueous extracted with ZnSO\textsubscript{4} against \textit{S. aureus} (Table 1). Antimicrobial activity of \textit{C. ternatea} root extracts can be improved by exploiting the synergistic effect of combining simple plant crude extract and metals.

\textbf{Minimum inhibitory concentration assay:} From the broth dilution method, the MIC was determined against the bacterial cultures for both aqueous and methanolic extract. Among the two extracts, methanolic extract exhibited lesser MIC against all tested cultures (Figure 1). The lowest MIC of 0.125 ±0.03 mg/ml was observed in methanolic extract against \textit{E. coli} followed by 2.50±0.05 mg/ml and 5.00 ±0.03 mg/ml against \textit{S. aureus} and \textit{K. pneumoniae} respectively (Table 2). Though aqueous extract exhibited antibacterial activities, the lowest MIC was found to be 25mg/ml against \textit{E. coli}. MIC against \textit{S. aureus} and \textit{K pneumoniae} was found to be 50mg/ml (Table 2).

MIC values observed for methanolic extract were between 1.25 and 2.5mg/ml. In similar studies \textit{C. ternatea} blue flowers have been reported with activities against uropathogenic \textit{E.coli}, Enterotoxigenic \textit{E.coli}, Enteropathogenic \textit{E.coli}, \textit{Klebsiella pneumoniae} and \textit{Pseudomonas aeruginosa}\textsuperscript{20}. The methanolic extract of \textit{Clitoria ternatea} has been reported with high antimicrobial activity compared to that of chloriform and aqueous extracts\textsuperscript{20}. MIC reported in their studies was 2.5mg/ml and 5mg/ml in chloriform extract and in aqueous extract respectively\textsuperscript{20}. Thus methanolic extract of roots of \textit{C. ternatea} can be considered more effective than the aqueous extract.

\textbf{Antibacterial activity of zinc sulphate:} A novel surveillance is on the rise, establishing that zinc also acts as a drug\textsuperscript{21} and it has an \textit{invitro} antibacterial effect on various bacteria. WHO/UNICEF\textsuperscript{22} recommends a dose of 10 mg of zinc daily for children less than 6 months of age and 20 mg zinc per day for children between 6–59 months of age for a period of 10–14 days for management of childhood diarrhoea. Zinc is being efficiently used in various forms like Zinc Sulphate, Zinc Gluconate and Zinc Acetate\textsuperscript{23}.

The present study in which zinc sulphate inhibited the growth of all tested bacterial isolates at 10mg/ml was at par
Figure 1: MIC of methanolic and aqueous root extracts of *C. ternatea* against *E. coli*, *S. aureus* and *K. pneumoniae*.

(a)                                                                                                     (b)

Figure 2: Synergistic activity of *Clitoria ternatea* methanolic root extract and zinc sulphate against
(A) *E. coli* and (B) *S. aureus*.

**Table 1**

<table>
<thead>
<tr>
<th>Pathogenic Bacterial Isolate</th>
<th>ZnSO₄ (10 mg/ml)</th>
<th>Aqueous root extract of <em>C. ternatea</em></th>
<th>Methanolic root extract of <em>C. ternatea</em></th>
<th>ZnSO₄ (10 mg/ml) and Aq. Ext (50mg/ml)</th>
<th>ZnSO₄ (10 mg/ml) and Meth. Ext (50mg/ml)</th>
<th>Positive Control (Tetracycline)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>17.67±0.58</td>
<td>10.33±0.58</td>
<td>15.00 ±1.63</td>
<td>10.00±0.00</td>
<td>17.33±0.58**</td>
<td>18.00±0.25</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>11.33±0.58</td>
<td>8.00±1.00</td>
<td>18.00±2.16**</td>
<td>11.67±0.58**</td>
<td>20.67±1.53**</td>
<td>17.00±0.30</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>10.33±0.58</td>
<td>9.00±1.00</td>
<td>21.67±2.05**</td>
<td>10.00±0.00</td>
<td>23.67±0.58</td>
<td>21.00±0.05</td>
</tr>
</tbody>
</table>

Negative control (50% DMSO) showed no Zone of inhibition in all three bacterial isolates
Values are mean inhibition zone (mm) ± S.D of three replicates; ** Significant at 0.01 %

**Table 2**

<table>
<thead>
<tr>
<th>Pathogenic Bacterial Isolate</th>
<th>Minimum Inhibitory Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanolic Extract</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.125 ±0.03</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>2.50±0.05**</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>5.00 ±0.03</td>
</tr>
</tbody>
</table>

Values are mean inhibition zone (mm) ± S.D of three replicates; ** Significant at 0.01 %
**Conclusion**

The plant *Clitoria ternatea* root extract and zinc sulphate solution combinations synergistically inhibit the growth of a broad range of bacteria including growth of human pathogens. Application of such combinations may provide a simple, cost-effective and efficient alternative to current bactericidal and antibiotic agents.

**Acknowledgement**

The authors express their sincere thanks to Department of Biotechnology, Sir Padampat Singhania University, Bhatewar and Department of Biotechnology, B.N. University, Udaipur, India for providing the research facilities.

**References**


(Received 14th March 2019, accepted 26th May 2019)