Mode of Action of Killing of Microorganisms with Vasaka Leaf Extract

ABSTRACT: In studying the antimicrobial activity of Vasaka, it has been found that Vasaka leaf extract is a broad spectrum antimicrobial agent and microbiostatic. When plates inoculated with microorganisms were grown in presence of Vasaka, no zone of inhibition was obtained. The organisms have grown resistance against Vasaka. The ethanolic extract of Vasaka have no direct effect on microbes like *S. mutans*, *Pseudomonas sp.*, *Candida albicans* and *Aspergillus sp.*, but should trigger a signal, which would induce some cellular responses. Lastly, performing activity gel of SOD from cell extracts of microorganisms grown in presence of Vasaka extract indicated increased SOD activity. This gave the idea of the mechanism of killing of microbes.

Key words: Vasaka leaf extract, antimicrobial agent, microbiostatic, Superoxide dismutase

Main Text: Ayurvedic system of medicines utilises curative properties of plants for treating diseases. Due to undesirable side effects and long term consequences of antibiotics, search for various naturally derived compounds of herbal origin is on rise that can be less toxic as well as highly effective. During last two decades there has been increasing interest in utilisation of Indian medicinal plants for treating chronic respiratory disorders, as some of these plants are known to be used in traditional medicine for at least alleviating symptoms associated with them1,2.

Phytochemical investigation of herbal drugs or their extracts reveals that possibly due to synergistic effect of the compounds, some ingredients potentiate therapeutic activity of main active ingredient, or even retard the undesirable actions of main compound because individual compound may not be as effective as mixture of compounds present in plant1.

Due to its medicinal properties Vasa or Vasaka or Adhatoda vasica (Linn.) Nees has been recommended by Ayurvedic physicians for management of various types of respiratory disorders. Vasaka belongs to the plant family Acanthaceae, its botanical/taxonomic name is *Justicia adhatoda* L.. Some of its synonyms are *Adhatoda vasica* Nees and *Adhatoda zeylanica* (Medicus). Vasaka appears to be the most common name for this plant.

The crude drug may be derived from powdered dry leaves or from extracts of fresh leaf juice. Other parts of plants that are also used are – roots, bark, flowers1,2,3,4. In our study, only fresh wet leaves and their extract were used to detect antimicrobial activity of Vasaka.

The nature of constituents is quinazoline alkaloid among which vasicine is the chief principle5. Other minor alkaloids are vasicinol, vasicinone, deoxyvasicinone, deoxyvasicine.

Formulation comprises fresh juice, decoction, infusion, powder, alcoholic extract, liquid extract or syrup but are also given along with other expectorants1,2,3,4.

Drug from Vasaka act as a sedative-expectorant, antispasmodic & antihelmenthic. Expectorant activity is due to the essential oils present in leaves5.

Leaf extract has been used for treatment of bronchitis, asthma, fever, jaundice, diarrhoea, dysentery, glandular tumour, cough and breathlessness. Large doses of fresh juices of leaves have been used in tuberculosis. Due to strong coagulation activity it minimizes blood loss. It has uterine stimulatory activity. It acts as a uterotonic & is also useful to control post-partum haemorrhage5. It also acts as antimicrobial & anticancer agent1,3.

Innovation of different methods of preparation of *Adhatoda vasica* leaf juice by quantification of total alkaloids and vasicine have paved the way for giving the best quality juice with highest amount of total alkaloids6.

An efficient regeneration schedule of *Adhatoda vasica* Nees through synthetic seeds provides an efficient plantlet formation of Vasaka in vitro7.

The Vasaka leaf juice being cheaper and having minimum side effects has tremendous applications in various ailments as observed from literature survey. So the aim of this experiment is to perform a preliminary study on leaf extract of Vasaka to determine the mode of killing of microorganisms in presence of Vasaka. The behaviour of microorganisms grown in presence of Vasaka was also studied.

Materials and Methods: Microorganisms used as test organisms: Bacteria - *Escherichia coli*, *Bacillus subtilis*, *Streptococcus mutans* (S. Mutans), *Vibrio cholerae*, *Salmonella sp.*, *Pseudomonas sp.*, *Klebsielle sp.*

Fungi - *Candida albicans*, *Aspergillus sp.*, *Penicilium sp.*
Procedure of collection, identification and preservation of voucher specimen: The aerial portions with maximum leaves along with tender stem were collected from Vasaka plant in Garia area of Kolkata. Identification was done from an Ayurvedic book. It was kept away from direct sunlight and heat in refrigerator for its preservation.

Preparation of leaf extract: 10 g of fresh Vasaka leaves were weighed accurately and several such packets were stored in refrigerator. Leaves from one of the packets were grinded to make the extract with 20 ml 90% ethanol making the final concentration 0.5 g/ml. The extract was then filtered to get a clear solution, devoid of any leaf debris. It was then kept in a sterilized conical flask, corked properly and stored in refrigerator.

Determination of zone of inhibition of Vasaka extract: In 7 autoclaved test tubes, inoculation of 7 different bacterial strains and in another 3 test tubes inoculations of 3 different fungal strains was done separately and aseptically in 5 ml of Nutrient broth and 5 ml Czapek Dox broth respectively. Pure cultures of the 10 different microorganisms were used as inoculums. The test tubes were incubated in the incubator at 37°C for 24 h to allow growth to occur. 0.2 ml of each bacterial and fungal culture were spread in 14 sterile petri plates containing Nutrient agar and 6 other plates containing Czapek Dox agar media. For each organism, 2 plates were assigned. In the hole made at the centre of each plate 200 μl of Vasaka extract was added, in the other 200 μl of 90% ethanol was added. The latter served as the control. The plates were incubated at 37°C in the incubator for 24 h in case of bacterial culture and for 48 h in case of fungal culture.

Determination of Vasaka as microbiostatic or microbiocidal agent: The Vasaka extract used in this experiment was of the concentration 1 g/ml i.e., of higher concentration. Leaves of 2 packets, each containing 10 g of leaves, were used to prepare the extract in 20 ml of 90% ethanol.

Development of resistance property in presence of Vasaka: For every microorganism there were 2 test tubes. At first 5 ml Nutrient broth and Czapek Dox broth was dispensed in each tube and then inoculated with respective microorganisms. In one of the 2 test tubes allotted for each microorganism, 200 μl of Vasaka extract was added. Thus in one set of test tubes the microorganisms were grown in presence of Vasaka and in the other in absence of Vasaka. All the test tubes were incubated at 37°C for 24 h in case of bacteria and for 48 h in case of fungus.

Following the stipulated incubation period, in the petri plates containing solid agar media, 2 wells were made after spreading the plates with 0.2 ml of culture from all the test tubes in respective petri plates. In one of the well 200 μl of Vasaka extract was added and in the other 200 μl of 90% ethanol was added. All the plates were incubated at 37°C for 24 h in case of bacteria and for 48 h in case of fungus.

Enzymatic assay of superoxide dismutase (EC No. 1.15.1.1): All the bacterial strains as mentioned above and among the fungal strains, Candida albicans was used. Two sets of test tubes were prepared. In one set the organisms were grown in absence of Vasaka and in the other set of test tubes the organisms were grown in presence of Vasaka.

To each of 1ml bacterial cultures and fungal culture were treated with lysozyme and sand respectively for disrupting cell walls. The extract was then separated from sand in case of fungal culture. Meanwhile, the mixture of bacterial culture was incubated at 37°C for 15 min. Following incubation the mixture was homogenized properly and then centrifuged for 10 min. The supernatant was collected in separate test tubes for each of the organisms. With the supernatant obtained enzyme assay was performed.

Protein content of each of the sample was determined by Folin-Ciocalteau method.

SOD Activity gel: The supernatant obtained after centrifuging the bacterial and fungal culture prepared following the preceding method were collected in separate

<table>
<thead>
<tr>
<th>Name of organism</th>
<th>Gram character</th>
<th>Zone of inhibition with Vasaka</th>
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<tbody>
<tr>
<td>Escherichia coli</td>
<td>Gram-negative</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>Gram-positive</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>Gram-positive</td>
<td>++</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>Gram-negative</td>
<td>++</td>
</tr>
<tr>
<td>Salmonella sp.</td>
<td>Gram-negative</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>Gram-negative</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella sp.</td>
<td>Gram-negative</td>
<td>-</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>Not applicable</td>
<td>+++</td>
</tr>
<tr>
<td>Aspergillus sp.</td>
<td>Not applicable</td>
<td>+++</td>
</tr>
<tr>
<td>Penicillium sp.</td>
<td>Not applicable</td>
<td>-</td>
</tr>
</tbody>
</table>

Here, '+' sign: presence of zone of inhibition. '-' sign: absence of zone of inhibition. '++' sign: moderate size of zone of inhibition. '+++' sign: large size of zone of inhibition.
test tubes for each of the organisms. Cell-extracts of different microorganisms of 24 h old culture grown in presence (0.5 g/ml) and absence of Vasaka were added from left to right well of the gel. After running the gel was soaked in a solution of NBT for 20 min. The gel was then subjected to incubation in dark for 10 min in a solution of TEMED, riboflavin and phosphate buffer, pH 7.8. After washing excess stain the gel was illuminated for 15 min under artificial white light.

**Results :** Determination of zone of inhibition of Vasaka extract: After 24 hrs the plates were observed and the result was tabulated as follows' (Table 1) (Fig. 1, 2, 3, 4)

**Determination of Vasaka as microbiostatic or microbiocidal agent**: In presence of the Vasaka extract no growth of the sensitive bacterial or fungal cultures were observed. But when the cultures were reinoculated in absence of the extract, turbidity of the growth culture media showed growth of both bacterial and fungal cultures. To confirm the above conclusion, cultures from liquid broth are used to inoculate Nutrient agar and Czapek Dox agar media. Colonies appeared. Gram staining for bacterial culture and fungal staining for fungal culture showed presence of pure culture of desired organisms.
Considering the microorganisms insensitive to Vasaka, in both types of plates – containing microorganisms grown in presence of Vasaka as well as those grown in absence of Vasaka – no zone of inhibition was obtained in either of the cases.

**Enzymatic assay of superoxide dismutase (EC No. 1.15.1.1) :** Graphically the specific activity of SOD in different microorganisms is shown in the Fig. 5.

**SOD Activity gel :** In black background white band of SOD are seen in each of the lanes. Band intensity is higher in those lanes containing cell extract of microorganisms grown in presence of Vasaka (data not given).

**Discussions : Determination of zone of inhibition of Vasaka extract :** Sensitive strains were Streptococcus mutans. (Gram + ve Bacteria), Pseudomonas sp. (Gram - ve Bacteria), Candida albicans. (Fungi) and Aspergillus sp. (Fungi) because in case of Streptococcus mutans, Pseudomonas sp. Candida albicans, Aspergillus sp. zone of inhibition for Vasaka (Fig 1, 2, 3, 4 and Table 1) was much larger than that for 90% ethanol. Escherichia coli and Bacillus subtilis were found to be insensitive to both Vasaka extract and absolute alcohol. Salmonella sp, Vibrio cholerae, Klebsiella sp., Penicillium sp. showed sensitivity to alcohol, but not to Vasaka extract. Therefore, these organisms were not sensitive to Vasaka.

Since the sensitive bacteria may be gram positive and gram negative and the sensitive fungi may be mold and yeast so in broader sense Vasaka is a broad spectrum antimicrobial agent. Thus Vasaka can be used for treatment of various infections caused by different microorganisms.

**Determination of Vasaka as microbiostatic or microbiocidal agent :** The observation revealed that Vasaka leaf extract is microbiostatic in its mode of action.

**Development of resistance property in presence of Vasaka :** From above observations we can hypothesize that if the organisms are allowed to grow in presence of Vasaka, then probably some of the derivatives are produced from Vasaka in the organism that may activate some enzyme system in due time so that the resistance may appear. The concept of herbal resistance may be taken into consideration during treatment of a disease while more and more herbal drugs are being prescribed.
are coming into consideration and becoming more popular.

**Enzymatic assay of superoxide dismutase (EC No. 1.15.1.1)**: In *Streptococcus mutans* SOD was absent because it is an aerobic organism. As oxygen free radical was generated, spontaneous death occurred. Some operon may have been induced, not definitely SOD, which can antagonize oxygen free radicals. In *Pseudomonas sp.* and *Candida albicans*, increased SOD activity was observed in presence of Vasaka. Hence, Vasaka extract would protect them from further Vasaka supplemented medium.

The mechanism of killing was concluded from oxygen free radical formation. As SOD is absent in *S. mutans*, resistance against Vasaka in *S. mutans* may be due to some other mechanism whereas *Pseudomonas sp.* or *Candida albicans* which can produce excess SOD in presence of Vasaka, resistance is due to increased SOD activity.

In other microorganisms tested, this SOD enzyme activity was shown.

**SOD Activity gel**: In black background white band of SOD were seen in each of the lanes. Band intensity (SOD native gel) was higher in those lanes containing cell extract of microorganisms grown in presence of Vasaka which indicated increased SOD activity (Data not given).

The mode of killing of microorganisms by Vasaka leaf extract was found to be increased SOD activity due to generation of oxygen free radicals except in *S. mutans*.

**Acknowledgements**

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**Appendix**: NBT, Nitro Blue Tetrazolium, TEMED, Tetramethylethylenediamine, EDTA, Ethylenediaminetetraacetic acid, SOD, Superoxide dismutase.