ANTI TUBERCULOSIS ACTIVITY OF PIMENTA OFFICINALIS EXTRACT USING THE MICRO PLATE ALAMAR BLUE ASSAY

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ABSTRACT

The present study describes the anti-tuberculosis (anti-TB) activity of oil obtained by hydro-distillation from the leaves of *Pimenta officinalis* by Micro-plate Alamar blue assay (MABA). The hydro-distillation of leaves is done by Clevenger apparatus. The emergence of multi-drug resistant and extensively-drug resistant strains of *Mycobacterium tuberculosis* has created the problem in treatment. In present study a hope for developing alternate medicine for the treatment of TB. *Pimenta officinalis* shows good anti-tuberculosis activity at different concentration. In this study, standard drugs selected were Pyrazinamide, Streptomycine and Ciprofloxacine. Traditionally the plant was used for relief of painful menstruation and stomach-ache and from present study it can be concluded that it can be used for treatment of tubercular infection.

Key words: *Pimenta officinalis*, *Mycobacterium tuberculosis*, Micro-plate, Almar blue assay, oil, Anti-tuberculosis Activity.

INTRODUCTION

According to world health organization (WHO) reports, more than 80 per cent of the world population relies on traditional medicine for their primary healthcare needs[1]. Synonyms of *Pimenta officinalis* are Caryophyllus pimento, Eugenia micrantha, Eugenia pimenta etc. Family-Myrtaceae. *Pimenta officinalis* commonly known as clove pepper, Jamaica pepper, pimento, Allspice. Allspice is an evergreen tree and native to Middle America and the West Indies.
It has many small fragrant, greenish-white flowers. The trees can remain productive for about 100 years. The leaves can be used either fresh or dried. The handling of allspice can cause dermatitis\(^2\). This species is well known spice, which is used in Jamaica in the preparation of liqueuer pimento dram. It is used for relief of painful menstruation and stomach-ache. The unripe berries contain 3-4.5 per cent oil, tannin, resin, sugar, fat and conine like alkaloid. It also contains traces of aldehyde and ketone\(^3\). The parts of plant used are leaves, flowers, berries and bark. The odour of *Pimenta officinalis* resembles that of mixture of cinnamon, cloves and nutmeg, hence it is called as allspice. The main use of *Pimenta officinalis* is as spice and condiment and also the berries are added to curry powder and to mulled wine. The oil occasionally used in medicine and resembles with clove oil \(^4\). The therapeutic properties of the essential oil of *Pimenta officinalis* are analgesic, antimicrobial, antioxidant, antiseptic, carminative, muscle relaxant, rubefacient, stimulant and tonic. The essential oil can also help in case of depression, nervous exhaustion, tension, neuralgia and natural repellant, stress. In case of cramp, digestive system, nausea, flatulence the oil is used\(^5\) and is also used in fungal infection \(^6\). Recently the natural and synthetic eugenol and essential oils from *Pimenta officinalis* is used for prevention and treatment of animal diseases caused by bacteria, fungi and parasites have been patented. In addition to these, eugenol has also some activities like nematicide, allelopathic effects \(^7\). The powdered air-dried leaves of *Pimenta officinalis* extracted with 80 per cent Methanol also shows lymphoproliferative effect towards T-lymphocytes \(^8\). The pimento oil may causes irritation \(^9\).

According to World Health Organization (WHO), tuberculosis remains the second leading cause of death worldwide, killing nearly 2 million people each year (WHO, 2005) \(^10\) and also remains among the top ten killers of children worldwide \(^11\). The tuberculosis is caused by inhaling a few rod shaped bacteria, *Mycobacterium tuberculosis* (Figure 1) \(^12\). Tuberculosis is a chronic granulomatous disease and a major problem in developing countries. For tuberculosis treatment the first and second line drugs are given according with its efficacy and toxicity \(^13\). The availability of first line anti-TB drug has significantly improved but the persistent gaps in access to first line treatments for drug susceptible TB reduce the efficiency to control disease \(^14\). The disease is passed when an infected person coughs, sneezes or spits and another person inhales the infected air \(^15\). Tuberculosis not only infects human body but also infect animals like cattle by a causative agent *Mycobacterium bovis* called as bovine tuberculosis \(^16\). The tuberculosis may be pulmonary tuberculosis or extra-pulmonary...
tuberculosis. In pulmonary tuberculosis the infection caused to lung parenchyma or the trachea-bronchial tree, while the extra-pulmonary tuberculosis refers to any bacteriologically confirmed diagnosed case of TB involving organs other than lungs, e.g. pleura, lymph nodes, abdomen, genitourinary tract and meninges [17]. Often the individual who’s infected with Mycobacterium tuberculosis required high standard of care to restore the health of individual and prevent the disease in their families and communities. Sub-standard care will result in poor patient outcomes with transmission of tuberculosis to family and generation of drug resistance [18]. In such patient DOT (Directly Observed Treatment) should be considered the standard treatment. The use of DOT has been shown to reduce the rate of drug resistance and relapse when compared with self-administered treatment [19].

Figure 1 Mycobacterium tuberculosis

Figure 2 Pimenta officinalis Leaves

In India two deaths occur every three minute due to tuberculosis [21]. The only way to cure tuberculosis is with a combination (H, R, Z, E, and S) of drugs [22]. But the drugs in the therapy in addition to their role of killing mycobacterium effectively could cause different kinds of adverse reactions (ADRs) such as drug allergy, arthralgia [23], skin reaction, hepatotoxicity (in children also) [24], hyperuricemia [25] and gastrointestinal reactions [26]. The Anti-tuberculosis drug induced liver injury (ATDILI) ranks the first in all kinds of drug-induced liver injuries [27]. Although it is well known that, those ADRs will do great harm to patients and are regarded as one of the major causes of incompliance of anti-TB treatment [23] more than 1 million tuberculosis (TB) patients are receiving the standard anti-TB treatment. Tuberculosis also affects diabetes by causing hyperglycemia and causing impaired glucose tolerance. The drugs used to treat tuberculosis (R, H) interact with oral anti-diabetic drugs and may lead to suboptimal glycemic control [28]. In case of children infected with TB immunization (BCG) is one of the method for preventing serious forms of TB but in such condition there is risk of
redeveloping TB which depends on two sets of factors - the risk of becoming infected and the risk of developing disease after infection\(^{[29]}\).

**MATERIAL AND METHOD**

**The plant material**
The leaves of *Pimenta officinalis* were collected from Sangli district in morning hours. They were stored in sterile polythene bags and transferred to the laboratory and stored at 4\(^\circ\)C till the time of use.

**Micro-organisms and culture**
The microorganism *Mycobacterium tuberculosis* and the culture as *Middlebrook 7H9 broth* were used in the present study. The only media that allow abundant growth of *Mycobacterium tuberculosis* are egg-enriched media with glycerol and asparagine (Lowenstein-Jensen) or agar based media supplemented with bovine albumin. In order to distinguish between different *Mycobacterial* species as well to perform drug susceptibility, culture examination becomes necessity \(^{[30]}\) and mostly Lowenstein-Jensen medium are used for culture of *Mycobacterium tuberculosis*\(^{[31]}\).

**Separation of oil**
The volatile oils of *Pimenta officinalis* leaves were obtained by hydrodistillation in Clevenger apparatus. In this method, the sample is completely immersed in water and the still is brought to the boil. After 3hrs of continuous extraction oil was separated and collected. This method yield about 1.5 ml of oil. The collected oil was stored in air tight umber glass bottle protected from light.

**Determination of anti-tuberculosis activity**
A variety of methods have been developed to measure the sensitivity of *Mycobacterium tuberculosis*\(^{[32]}\). In the present study we used Micro-plate Alamar blue assay (MABA) in which Alamar blue was used as the dye. It is a rapid and low-cost method for the sensitivity study of *Mycobacterium tuberculosis*. This bioassay may also be used to establish relative cytotoxicity of agents within various chemical classes \(^{[33]}\).

Over the years, a number of improved and high throughput techniques towards screening of anti-Mycobacterial agents have been developed. The Micro-plate Alamar Blue Assay is a colorimetric oxidation-reduction based assay. It is a non-radiometric, rapid, comparatively
low cost assay producing results with a high degree of confidence. Moreover, this technique has been used by a number of researchers for testing anti-Mycobacterium activity of several plants.

**Procedure**

1) The anti-Mycobacterial activity of compounds was assessed against *M. tuberculosis* using micro-plate Alamar Blue assay (MABA).
2) This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method.
3) Briefly, 200µl of sterile deionised water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during inhibition.
4) The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilutions of compound were made directly on plate.
5) The final drug concentrations tested were 100 to 0.2 µg/ml.
6) Plates were covered and sealed with parafilm and incubated at 37°c for five days.
7) After this time, 25 µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs.
8) A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth.
9) The MIC was defined as lowest drug concentration which prevented the color change from blue to pink[^34].

**RESULT AND DISCUSSION**

The anti-TB sensitivity of essential oil from leaves against *Mycobacterium tuberculosis* was observed by using Micro-plate Alamar Blue Assay by observing intensity of color. This method uses thermally stable reagent (Almar Blue reagent) and is non-toxic. The results are shown in the Table no.1. The *Mycobacterium tuberculosis* sensitive at a concentration of 100, 50, 25 and 12.5µg/ml. The bacteria exhibited resistance at a concentration of 6.25, 3.125, 1.6 and 0.8µg/ml. Anti-TB activity found from 12.5 to 100µg/ml.
The intensity of color decreases as the concentration decreases. Here the standard drug was taken as Pyrazinamide, Streptomycin and Ciprofloxacin (Figure 3) which were compared with result (Figure 4).

### Table No:-1 Standard Drug Concentration

<table>
<thead>
<tr>
<th>Standard drug</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>P Pyrazinamed</td>
<td>3.1258µg/ml</td>
</tr>
<tr>
<td>S Streptomycin</td>
<td>6.258µg/ml</td>
</tr>
<tr>
<td>C Ciprofloxacin</td>
<td>3.3258µg/ml</td>
</tr>
</tbody>
</table>

The standard values for the Anti-TB test which was performed here are

### Table No:-2. Result of Anti-Tuberculosis Activity Of Test Oil

<table>
<thead>
<tr>
<th>Sr.</th>
<th>Compound</th>
<th>100</th>
<th>50</th>
<th>25</th>
<th>12.5</th>
<th>6.25</th>
<th>3.125</th>
<th>1.6</th>
<th>0.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Po-1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

S- Sensitive
R- Resistance

Po-1 –: Pimenta officinalis oil
CONCLUSION
An exclusive literature survey did not afford any information regarding anti-tuberculosis activity of this plant. Thus in the present study an attempt is made to explore anti-tuberculosis potential of oil from the leaves of *pimento officinalis*. It gives good result at four different concentration and it may used as an alternative drug in case of XDR and MDR tuberculosis.

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