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# Antitubercular activity of plumbagozeyalanica leaves methanolic extract linn using Microplate Alamar Blue Assay

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**ABSTRACT:**

Increasing resistance to drugs for Tuberculosis (tb) is a major health problem worldwide. The most commonly used agents isoniazid and rifampicin are no longer effective.however natural compounds always served as lead molecules in various diseases treatment *P.zeylanica*is a widely spread shrub throughout the tropical and subtropical climate is known for its use in treatment of skin problems and intestinal worms. The main active compound in the plant is plumbagin which has been shown to possess antimicrobial, antiplasmodial, anticancer and antifertility actions. The present study aims at evaluating the anti tb potential of methanolic extract *P.zeylanica*leaves both (dried and fresh) against M.tuberculosis H37RV strain using microplatealamar blue assay. The methanolic extract showed a significant antimycobacterial activity at 50 and 100 $\mu g/ml$.

**KEYWORDS**: Tuberculosis, *P.zeylanica,* M.tuberculosis H37RV strain, microplatealamar blue assay

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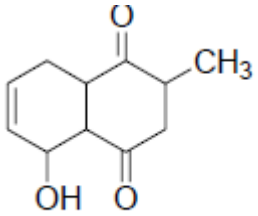
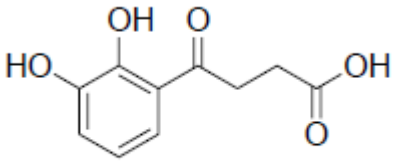
**INTRODUCTION:**

*Plumbago zeylanica* is an ayurvedic plant, popularly known as leadwort [latin] Vernacular Names: Chitraka, Doctorbush, Ceylon leadwort. Bogaagechita(Assamese), Safidsitarak(Bengali), Chitrakamula(Gujarati), Chitra(Hindi), Chitramulika (Kannada), Chitramulam(Telugu). The genus plumbago exist in three species are *P.indicalinn, P. capenisislinn, P. zeylanicaLinn.*among all the three species *Plumbago zeylanica*is widely cultivated because it has more therapeutic usesIt is native to south east asia and distributed in tropical and sub tropical region. In India *Plumbago zeylanica*grows in all districts of plains from central India to West Bengal, Maharastra, Karnataka, Tamilnadu, Andhrapradeshetc[1]

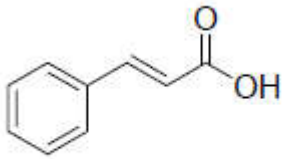
Plumbagin, plumbagic acid, vanillic acid, trans cinnammic acid, 2,5- dimethyl hydroxyl chromone, isoshinanolone, indole-3-carboxylaldehyde,plumbginol, sitosterol,stigmasterol, campesterol are the phytochemicals isolated from the *P. zeylanica*[2,3,4]



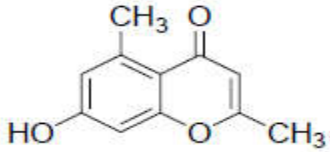
PLUMBAGIN



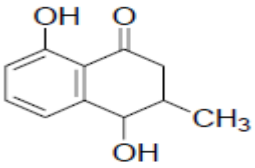
PLUMBAGIC ACID



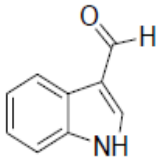
TRANS CINNAMIC ACID



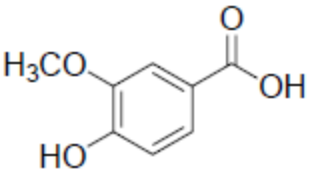
2,5-DIMETHYL-HYDROXY CHROMONE



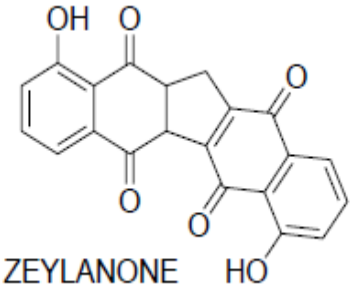
ISOSHINANOLONE



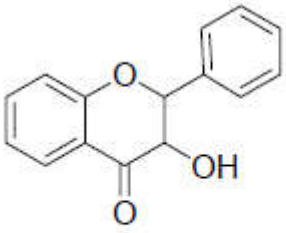
INDOLE-3-CARBOXALDEHYDE



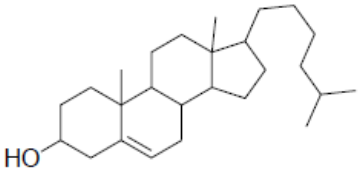
VANILLIC ACID



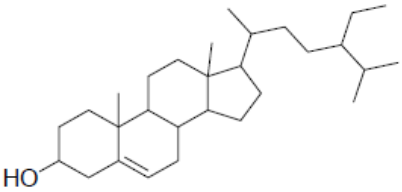
ZEYLANONE



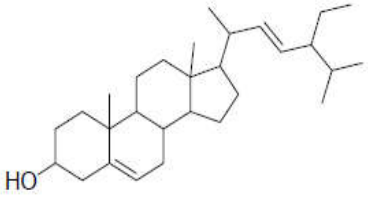
DIHYDROFLAVINOL-PLUMBAGINOL



CAMPESTEROL



SITOSTEROL[S]



STIGMASTEROL

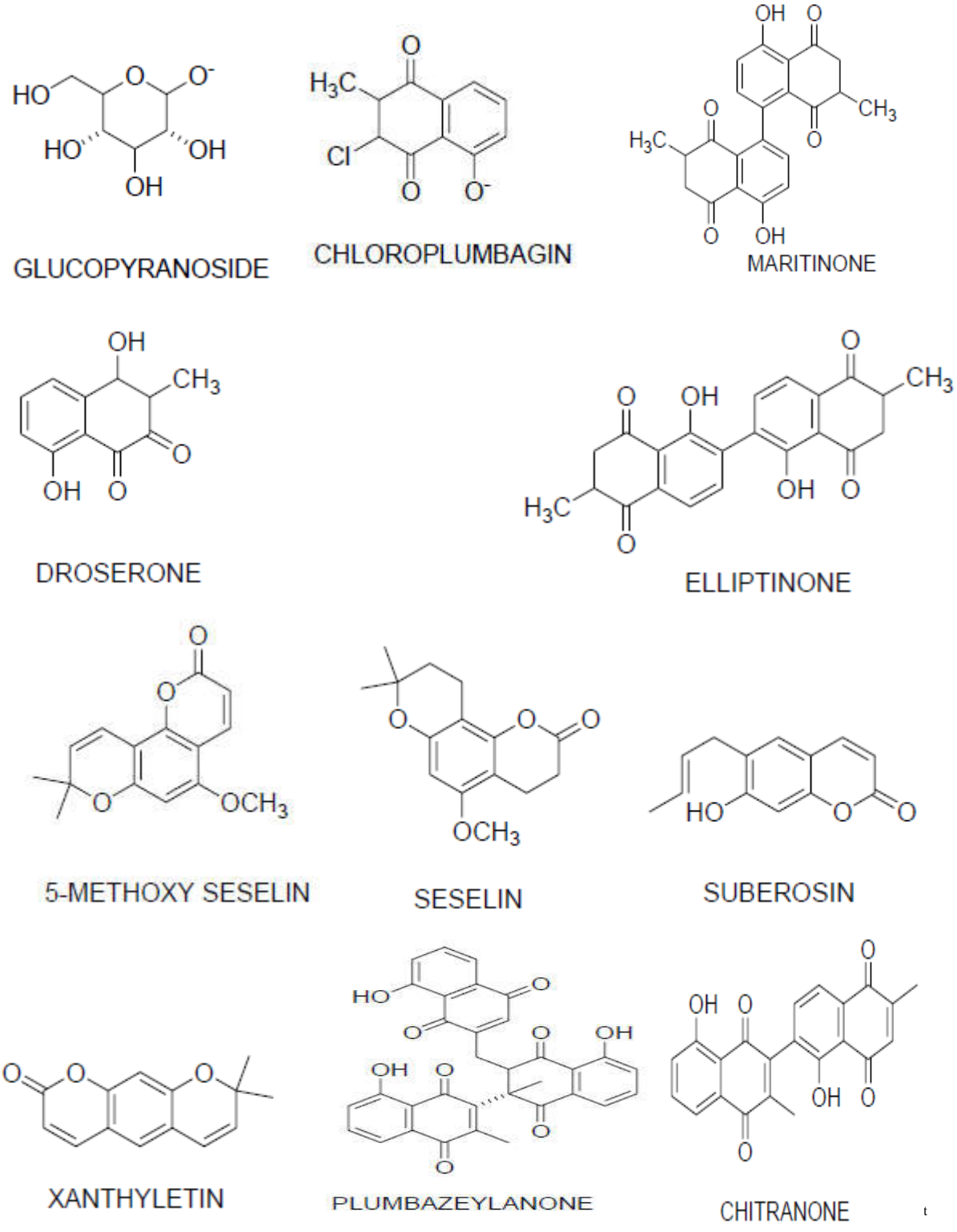



**Fig 1: Structure of Chemical constituents of *Plumbago zeylanica***

**Pharmacological Uses:**

Traditionally *Plumbago zeylanica* is used as a stimulant, digestant, expectorant, laxative, in treatment of muscular pain and rheumatic diseases. In india it is usually used to treat fever and malaria. Pharmacological studies have indicated that Plumbago zeylanica extract has anti plasmodial, antimicrobial, antifungal, anti-inflammatory, antihyperglycemic, hypolipidemic, and anti-atherosclerotic activities. It has other activities like anti fertility, anticancer, antioxidant and cardio protectant activities.[5,6,7]

Tuberculosis (TB) is caused by a bacterium called *Mycobacterium tuberculosis*. The bacteria usually attack the lungs, but TB bacteria can attack any part of the body such as the kidney, spine, and brain[8]

The bacteria that cause tuberculosis (TB) can develop resistance to the antimicrobial drugs used to cure the disease. Multidrug-resistant TB (MDR-TB) is TB that does not respond to at least isoniazid and rifampicin, the 2 most powerful anti-TB drugs.

In some countries, it is becoming increasingly difficult to treat MDR-TB. Treatment options are limited and expensive, recommended medicines are not always available, and patients experience many adverse effects from the drugs. In some cases even more severe drug-resistant TB may develop. Extensively drug-resistant TB, XDR-TB, is a form of multidrug-resistant TB with additional resistance to more anti-TB drugs that therefore responds to even fewer available medicines. It has been reported in 117 countries worldwide[9]

Natural products are proven templates for the development of new scaffolds of drugs .They have received considerable attention as potential anti-TB agents. Natural products have been used worldwide in traditional medicine for the treatment of various diseases. An approach to the search for new drugs is to look in nature, mainly for the extremely rich and varied flora of the tropical areas. In this search various reasons have been put forward to explain the success of natural products in drug discovery; their high chemical diversity, the effects of evolutionary pressure to create biologically active molecules, the structural similarity of protein targets across many species, and so on . In our present study we have phytochemical screening and anti –TB activity using MABA assay which is a preliminary screening test used for testing anti –mycobacterial potential of compounds.

**MATERIALS AND METHODOLOGIES:**

**Plant material:**

The leaves of *Plumbago zeylanica*was collected in month of November 2018 from medicinal garden of Koringa College of Pharmacy, Korangi, A.P and authenticated by the botanist Dr S. B. PadalDept. of. Botany, Andhra University and a voucher specimen no.23307 is deposited in the herbarium (AUV).

**PREPARATION OF PLANT EXTRACT :**
**PZME**

The collected plants were shade dried in a room at normal room temperature ,the leaves were separated from the remaining aerial parts and roots, and they were powdered by using blender.. The obtained powder (100g) was macerated successively two times using methanol for about one month to fully extract the secondary metabolites present in the stem, solvent obtained was collected in a round bottomed flask concentrated using simple distillation. A crude extract of 3g was obtained and it is stored in a dessicator containing fused calcium chloride for 24hr to get semisolid mass(PZME).[10]

**PZE**

10 g of fresh leaves of P.zeylanica were grinded and the obtained paste is extracted with 50% ethyl acetate and hexane(1:1) the obtained liquid is repeatedly extracted with chloroform using separating funnel. The obtained chloroform layer is evaporated to dryness leaving light green coloured liquid (PZE).[11]

**PHYTOCHEMICAL EVALUATION:**

The presence of various phyto constituents like alkaloids, carbohydrates, glycosides, flavonoids, saponins, steroids, tannins, terpenoids, amino acids in the methanol extract (ieaves) of the plants under study were tested as per available standard

procedures for the test of phytochemicals .The test methods are described below[12]

**PROCEDURE:**

**Test for carbohydrates:**

a. **Molisch's test:** To small amount of extract add α napthol solution and concentrated sulphuric acid was added slowly along side of test tube. The mixture was observed for formation of any violet ring at junction of two liquids indicates the presence of carbohydrates.

b. **Fehling's test:** To small amount of extract add equal amounts of fehling's A and B. The mixture was observed for formation of any precipitate. Formation of red precipitate indicates presence of reducing sugars.

c. **Iodine test:** To the extract add iodine solution and observe for colour change. Blue colour indicates presence of starch.

**Test for alkaloid:**

**a. Dragendroff's test:** To the extract add few drops of dragendroffs reagent. The mixture was observed for formation of any precipitate. Formation of red precipitate is the indication of presence of alkaloids.

**b. Mayer's test:** To the extract add little amount of diluted sulphuric acid and mayer's reagent. Then the mixture was observed for the formation of any precipitate. Formtaion of white or creamy precipitate is indication of presence of alkaloids.

**Test for flavanoids:**

**a. Lead acetate test :**To the extract add lead acetate solution. The mixture was observed for color change. Formation of yellow colour indicates presence of flavanoids.

**b. Ferric chloride test :**To the extract add freshly prepared ferric chloride. Then the mixture was observed for color change. Formation of blue colour indicates presence of flavanoids.

**Test for saponins:**

**Foam test :**To the extract add 20 ml of distelled water and shaken in a graduating cylinder for 15 minutes vigorously. The mixture was observed for foam formation and thickness. Formation of two centimeter layer of foam indicates presence of saponins.

**Test for tannins:**

**Lead acetate test :**To the extract add few ml of lead acetate and observed for formation of any precipitate. Formation of white precipitate indicates presence of tannins.

**Test for steroids and terpenoids:**

**Libermann-buchard test:** extract (4mg) was treated with 0.5ml of acetic anhydride and 0.5ml of acetic acid. Then concentrated $H_2SO_4$ was added slowly and blue green colour was observed for terpenoids and reddish brown colour for steroids.

**Test for Cardiac glycosides:**

Approximately 0.5 g of extract diluted in 5 ml in distilled water was added with 2 ml of glacial acetic acid containing one of 1% $FeCl_3$. This was underplayed with 1 ml concentrated sulphuric acid. A brown ring at the interface indicated in the presence of deoxysugar characteristic of cardinolides. A Violet ring appeared below the brown ring while in the acidic layer; a greenish ring formed just above the brown ring and gradually spread throughout this layer. Glycosides consists of a sugar covalently bound to a different structure called the glycine. Glacial acetic acid and$FeCl_3$indicates the presence of glycine sugars thus forming the brown ring.

**IN VITRO ANTI TB USING ALAMAR BLUE ASSAY**

MicrotitreAlamar Blue Assay: it is a rapid and inexpensive colorimetric method based on the oxidation-reduction indicators Alamar blue. The Alamar Blue Assay is designed to measure quantitatively the proliferation of various human and animal cell lines, bacteria and fungiAlamarbluedye (Resazurin)

**Resazurin**(7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) is a blue dye, itself weakly fluorescent until it is irreversibly reduced to the pink colored and highly red fluorescent resorufin

Alamarbluedye (Resazurin) As cells being tested grow, innate metabolic activity results in a chemical reduction of alamarBlue®. Continued growth maintains a reduced environment while inhibition of growth maintains an oxidized environment. Reduction related to growth causes the REDOX indicator to change from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form.

**Procedure:**

Standard Strain used: *Mycobacteria tuberculosis* (*Vaccine strain, H37 RVstrain): ATCC No- 27294*.

1. The antimycobacterial activity of compounds were assessed against M. tuberculosis using microplateAlamar 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 deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation.

4. The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds 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 37ºC for five days.

7. After this time, 25µl of freshly prepared 1:1 mixture of Almar 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 colour change from blue to pink.

**RESULTS AND DISCUSSION:**

The preliminary phytochemical screening revealed the presence of flavanoids, cardiac glycosides, phenolic compounds, saponins.

**Table no 1: Phyto chemical screening of PZME and PZE**

| Sl.no | Phytochemical constituent | Test used | Result of PZME | Result of PZE |
|---|---|---|---|---|
| 1 | Alkaloids | Mayer's test | - | - |
| 2 | Steroids | Liebermann-buchard test | - | - |
| 3 | Terpenoids | Liebermann-buchard test | - | + |
| 4 | Cardiac glycosides | Keller killani test | + | + |
| 5 | Flavanoids | Lead acetate test | + | + |
| 6 | Phenolic compounds | Ferric chloride | + | + |
| 7 | Carbohydrates | Iodine test and Molisch's test | + | + |
| 8 | Saponins | Foam test | + | + |
| 9 | Tannins | Lead acetate | - | + |

Methanolic extract of *Plumbago zeyalanica* leaves both dried and fresh ( PZME and PZE) are evaluated by alamar blue assay in which the fresh extract (PZE) inhibited the growth of *M.tuberculosis*H37Rv ATCC 27294 sensitive strain at MIC values 50 and 100µg/ml respectively and dried extract showed inhibition at 100µg/ml(Tableno2), we for the first time showed that fresh leaves of *Plumbago zeyalanica* more active against the *M.tuberculosis*H37Rv strain , however the activity in *P. zeyalanica* is reported for the first time

In this we have tested the extracts at a concentration of 100µg/ml. Now we are trying to purify the active anti TB principles from the extracts using techniques like Preparative TLC, Column chromatography, HPLC. Since (fresh and dried) leaves methanolic extract showed activity against the *M. tuberculosis* H37Rv strain . It is possible that the active compounds could be flavonoids, alkaloids and phenolic compounds. Once purified these compounds are expected to exert their anti TB activity at much lower concentration

**CONCLUSION:**

With increasing rates of tuberculosis worldwide and the rise of MDR-TB and XDR-TB, there is a need for novel antitubercular agents. Using the MABA as a screening tool this study assessed anti mycobacterial properties of extracts of *Plumbago zeyalanica*. This

study serves to validate traditional knowledge and adds to the growing literature on botanical sources identified as providing important novel antitubercular compound.

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CONFLICT OF INTEREST REPORTED: NIL ; SOURCE OF FUNDING: NONE REPORTED