Evaluation of \textit{IN-VITRO} Antimycobacterial Activity of \textit{Caesalpinia bonduc} Seed Coat Extracts

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Abstract:
Tuberculosis (TB) mainly causes due to the mycobacterium species which is characteristics of both bacteria as well as fungi. Bacterial resistance developed against \textit{Mycobacterium tuberculosis H37Rv} due to drug and combination of drug therapy (MDR-TB), causes a serious problem. So its need to investigate a new lead molecule or herbal remedies which are effective against mycobacterium species. In present investigation an attempt has made to assess the medicinal and anti-mycobacterial properties of natural products obtained from a plant, easily available in Marathwada region of Maharashtra. In this study aqueous and ethanolic seed coat extract of \textit{Caesalpinia bonduc} (\textit{Caesalpiniaeae}) has been evaluated for anti-mycobacterial activity to substantiate the traditional claims. Both aqueous & alcoholic extracts were screened against \textit{Mycobacterium tuberculosis H37Rv} strain using Micro Plate Alamar Blue Assay (MABA) method. Various concentrations of the extracts in the range of 0.78, 3.125, 6.25, 12.5, 25, 50 and 100 μg/ml from the plant species were used to determine their respective Minimum Inhibitory Concentrations (MICs). The ethanolic extract showed moderate, whereas aqueous extract exhibited significant inhibition against \textit{M. tuberculosis}. Standard compound Pyrazinamide showed inhibition at concentration 100, 50, 25, 12.5, 6.25 and 3.125μg/ml concentration where as aqueous extract was found to be equipotent to standard with MIC 6.25μg/ml.

Keywords: Anti-mycobacterial, Phytochemical constituents, Micro-plate Alamar Blue Assay (MABA), Seed coat extract, Alkaloids

Received 24 Sept 2016 Received in revised form 12 Oct 2016 Accepted 14 Oct 2016

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INTRODUCTION
\textit{Caesalpinia bonduc} is a wild highly thorny shrub, belonging to the family \textit{Caesalpiniaeae}, is a prickly shrub widely distributed all over the world specially India, Sri Lanka and Andaman and Nicobar Islands, in India specially found in tropical regions, near the sea-costs, especially Bengal, Bihar, Mumbai, Rajasthan and whole of Southern India [1]. The plant grows all over in India, grows in shade as well as in open. It is a free-flowering and free-fruiting plant without periodicity. It is a large straggling, thorny shrub, the branches are armed with hooks and straight, hard yellow prickles. The leaves are compound. The flowers are pale yellow in color, in supra-axillary racemes at the top. The fruits are inflated pods, covered with prickles, 6 cm long, and 1-2 seeds per pod [2,3]. The seeds are globular, hard, bluish grey in color with a smooth shiny surface.

The seeds contain various chemical constituents such as furanoditerpene’s, \(\alpha\)-caesalpin, \(\beta\)-caesalpin, \(\gamma\)-caesalpin, \(\delta\)-caesalpin, \(\epsilon\)-caesalpin, and caesalpin-F; fatty acids. Palmitic, stearic, octadeca-4-enoic, octadeca-2, 4-dienoic, lignoceric, oleic and linoleic acids, phytosterin, \(\beta\)-sitosterol, homoisoflavone bonducellin, amino acids, aspartic acid, arginine and citrulline, carbohydrates: starch and sucrose, arotene, glycoside-bonducin, gums, and resins [4].

The seed of \textit{Caesalpinia bonduc} claimed a purgative, Anthelmintics, anti-malarial, anti-inflammatory action [5]. The oil from seed is used in convulsion and paralysis. The roots are considered as anthelmintics and decoction of the root prescribed in fever. The leaves of \textit{Caesalpinia bonduc} used in treatment of small pox, disorder of liver. The flower is bitter cures kapha and vata.
Fruit of *Caesalpinia bonduc* shows action aphrodisiac and hyperacidity. *Caesalpinia bonduc* seeds have been attributed with anti diabetic, anti-inflammatory, anti-oxidant, anti-bacterial, anti-filarial, anti-tumor, antileptotic, anxiolytic, immunomodulatory properties in the folklore medicine [6]. Literature survey revealed that there is little focus on phytochemical investigation and biological activity of seed coat extract of *Caesalpinia bonduc*, based on the facts it was planned to undertake investigation of seed coat extract.

Natural products are a proven template for the development of new scaffolds of drugs and they have received considerable attention as potential anti-TB agents. The emergence of pathogenic microbes with increased resistance to established antibiotics provides a major incentive for the discovery of new antimicrobial agents [7-9].

In present study we have screened *Caesalpinia bonduc* seed coat aqueous and ethanolic extract for phytochemical investigation and in-vitro anti-mycobacterial activity against *Mycobacterium tuberculosis* H37Rv.

**Experimental:**

**Plant material:**
Seed of *Caesalpinia bonduc* were collected from Marathwada region of Maharashtra. The seeds were preserved and authenticated by Chatrapati Shivaji Science College, Department of Botany, Osmanerga, Osmanabad (Auth 11-36, 11-198, 8-68). The seed dried at 45°C in hot air oven and seed coat was powdered, passed through sieve (mesh no 80). Powdered seed coat was stored in plastic containers.

**Preparation of extract:**
The plant material was dried and seeds were broken so as to separate the kernel and the seed coat. The seed coat was coarsely powdered and extracted with water and 95% ethanol in a Soxhlet extractor; further, the extract was filtered and concentrated on rota-evaporator, to get a sticky reddish brown extract which was obtained 12.3% w/w (*C. bonduc* seed coat extract [CBSCE]).

**Phytochemical Investigation:**
The powdered seed coat was subjected to systemic phytochemical screening by successively extracting them in water and ethanol testing for the presence of chemical constituents.

**Qualitative chemical examination of extract**
The sequential extraction of seed coat powder was carried out using water & ethanol by Soxhlet extraction method. All the extracts obtained from solvent extraction are further subjected to phytochemical analysis [10-12].

**Detection of alkaloids**
Extractions were dissolved individually in dilute hydrochloric acid and filtered. The filtrates were tested carefully with alkaloid reagents.

a) Mayer's Test: Filtrates were treated with Mayer's reagent (potassium mercuric iodide). The formation of a yellow cream precipitate indicated the presence of alkaloids.

b) Wagner's Test: Filtrates were treated with Wagner's reagent (iodine in potassium iodide) and observed. Formation of brown or reddish brown precipitate indicated the presence of alkaloids.

c) Dragendorff's Test: Filtrates were treated with Dragendorff's reagent (solution of potassium bismuth iodide). Formation of red precipitate indicated the presence of alkaloids.

d) Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Formation of yellow colored precipitate indicated the presence of alkaloids.

**Detection of carbohydrates**
Extractions were dissolved individually in 5 ml of distilled water and filtered. The filtrate was used to test the presence of carbohydrates.

a) Molisch's Test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml concentrated sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicated the presence of carbohydrates.

b) Benedict's test: Filtrates were treated with Benedict's reagent and heated on water bath. Formation of an orange red precipitate indicated the presence of reducing sugars.
c) Fehling’s Test: Filtrates were hydrolyzed with dilute hydrochloric acid, neutralized with alkali and heated with Fehling’s A and B solution. A red precipitate was formed which indicated the presence of carbohydrates.

d) Barfoed’s test: Filtrates were treated with Barfoed’s reagent and heated on water bath. Formation of an orange red precipitate indicated the presence of reducing sugars.

Detection of proteins and amino acids
a) Millon’s Test: The extracts were treated with 2 ml of Millon’s reagent. The formation of white precipitate, which turned to red upon heating, indicated the presence of proteins and amino acids.
b) Biuret Test: The extracts were treated with 1 ml of 10% sodium hydroxide solution by mild heating. A drop of 0.7% copper sulphate solution to the above mixtures was added. The formation of purplish violet color indicated the presence of proteins.
c) Ninhydrin Test: To the extract, 0.25% ninhydrin reagent was added and boiled for few minutes. Formation of blue color indicated presence of amino acid.

Detection of glycosides
Extracts were hydrolyzed with dilute hydrochloric acid and the hydrolysate was subjected to glycosides tests.

a) Modified Borntrager’s Test: The extracts were treated with ferric chloride solution and heated on boiling water bath for about 5 mins. The mixture was cooled and shaken with equal volume of benzene. The benzene layer was separated and treated with half of its volume of ammonia solution. The formation of rose pink or cherry red color in the ammoniacal layer indicated the presence of glycoside.
b) Libermann’s Test: The extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was added through the sides of the test tube. The form of brown or pink colored rings at the junction confirmed the presence of glycosides respectively.
c) Killer Killani Test: 0.5g of dried extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solutions. This was then laid with 1 ml of concentrated sulphuric acid. A brown ring obtained at the presence of glycosides.

Anti-mycobacterial activity:
*Mycobacterium tuberculosis* reference strain H37Rv was used to evaluate the preliminary screening of crude extracts. Micro-plate Alamar Blue Assay (MABA) [13,14] method was used for the study. The reagent allows the detection of microbial growth in micro titer plates without the use of spectrophotometer. DMSO was used as control and Pyrazinamide, Streptomycin and ciprofloxacin were used as standard. The extracts were considered active (have inhibitory activity) for the well of the plate with unchanged color or the blue, non-fluorescent, oxidized form and if the color of the reagent is changed to pink (fluorescent) the extract is inactive or the microorganism is considered resistant strain to the plant extract.

Minimum inhibitory concentration (MIC) was determined for those extracts showing inhibitory effect. The MIC was conducted at various concentrations of 0.78, 3.125, 6.25, 12.5, 25, 50, 100 mg/ml of the extracts.

RESULTS AND DISCUSSION
The powdered seed coat material of *Caesalpinia bonduc* aqueous and ethanolic extracts was tested positive for steroids, triterpenes, alkaloids, saponins, tannins, anthraquinones and flavonoids. The phytochemical analysis of these extract are summarized in (Table 1). These results agreed with the literature review on the plant which showed these chemical constituents to be present.

Against H37RV a standard strain of Mycobacterium tuberculosis, a clinical sensitive and a clinical resistant strain of *Mycobacterium tuberculosis* H37Rv. Moreover, our findings concerned to antimycobacterial screening against a clinically sensitive and clinically resistant standard strain of *M. tuberculosis* H37Rv as (Table 2) demonstrates that, the minimum inhibitory concentration of the aqueous and ethanolic extracts of plant species showed inhibitory effect on *M. tuberculosis* H37Rv MIC (25µg/ml) for ethanolic extract whereas at concentration of 6.25 µg/ml for aqueous extract (Figure 1).
Hence, the results clearly indicate that aqueous extracts showed significant inhibition compared with ethanolic which was moderate. The results of antimycobacterial testing of plant extracts that would eventually give leads for probable drug development.

Antimicrobial screening for the phytochemicals from plant extracts then represents a starting point for antimicrobial drug discovery especially, anti-mycobacterial drugs. There are reports from folklore which claim that extracts of Caesalpinia bonduc are used by tribal’s and natives in cases of Tb infection, but to our knowledge such anti M tuberculosis activity is reported for the first time using seed coat extract of cesalpenia bonduc in vitro with remarkable anti Mycobacterial potential [15-17].

Table 1: Qualitative chemical analysis of Aqueous and Ethanol extracts of C. bonduc seed coat

<table>
<thead>
<tr>
<th>Test</th>
<th>Aqueous extract</th>
<th>Ethanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Mayer test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II. Dragendorff’s test</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>III. Wagner Test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IV. Hager’s test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Molisch’s test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II. Benedict’s Test</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>III. Fehling’s Test</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IV. Barford Test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein &amp; amino acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Million Test</td>
<td></td>
<td></td>
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<tr>
<td>II. Biuret Test</td>
<td></td>
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</tr>
<tr>
<td>III. Ninhydrin test</td>
<td>+</td>
<td></td>
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<tr>
<td>Glycoside</td>
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<td></td>
</tr>
<tr>
<td>I. Borntrager’s test</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>II. Libermann’s test</td>
<td>+</td>
<td></td>
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<tr>
<td>III. Killer-Killani test</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

(+) indicates presence while (-) indicates the absence of the components

Table 2: The Seed coat extract with anti-mycobacterial activity against Mycobacterium tuberculosis H37Rv

<table>
<thead>
<tr>
<th>Sample</th>
<th>Minimum Inhibitory concentration Range in µg/ml</th>
</tr>
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<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>S</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>S</td>
</tr>
<tr>
<td>Ethanolic Extract</td>
<td>S</td>
</tr>
</tbody>
</table>

R-Resistant to strain, S-sensitive to the strain

Figure 1: Anti-mycobacterial activity of extract. P-Pyrazinamide, A-Aqueous Extract, E-Ethanolic Extract
CONCLUSION
The above result showed that, *Caesalpinia bonduc* seed coat have shown promising anti-mycobacterial activity in that, the ethanolic extract of seed coat showed moderate, whereas aqueous significant inhibition against *M. tuberculosis*. The Phytochemical analysis of the seed coat extract suggested that both aqueous and ethanolic extract may contain an active chemical constituents such as furanoditerpene's: α-caesalpin, β-caesalpin, γ-caesalpin, δ- caesalpin ε-caesalpin, and caesalpin-F; fatty acids: palmitic, stearic, octadeca-4-enoic, octadeca-2, 4-dienoic,lignoceric, oleic and linoleic acids, phytosterin, β-sitosterol, homoisoflavone bonducellin; amino acids: aspartic acid, arginine, and citrulline; carbohydrates: starch and sucrose; β-carotene, glycoside-bonducin gums and resins.

Further detailed Phytochemical analysis and activity studies need to be carried out using crude solvent extracts as well as further purified constituents to comprehend their role in anti-tuberculosis activity and develop suitable drugs so that the most deadly disease in the world can be combated. The present study also could pave the way towards possibility to obtain anti mycobacterial moieties against other Mycobacterial species.

REFERENCES