ANTI TUBERCULOSIS ACTIVITY AND GC-MS ANALYSIS OF
ETHYL ACETATE EXTRACTS OF WRIGHTIA TINCTORIA BARK

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ABSTRACTS

Wrightia tinctoria (WT) is a known rich medicinal plant in India. In this study, bioactive compounds of WT bark were extracted; isolated and evaluated. Dried WT bark was successively extracted with different solvents in Soxhlet apparatus. All the extracts were in vitro tested for their potential against M. tuberculosis. Promising extracts were subjected to column with for the fractionation of the compounds and were analyzed by GC-MS. Ethyl acetate extract showed MIC 6.25 µg/ml against M. tuberculosis which was significantly different from other extract of WT bark. Proximate analysis results showed the presence of Alkaloids, Carbohydrates, Proteins, Glycerol, Resins, Tannins, Sterols Cardiac Glucosides and Triterpinse. Chromatogram was analyzed and matched with NIST library. As per the matching, Methoxyacetic acid 3-tridecyl ester, Quinoline, 2,7-dimethyl, Hexadecane, 4,5-dimethyl pentadecane, Nonadecane and Docosane were identified in fraction 1. Similarly in fraction 2, 3, 4 and 5 peaks were analyzed and characterized as Benzene,1,3-dichloro, Dodecaflne, Benzothiazole, Tetradecane, Ethano 1,2-(Octadecyloxy), Octadecane, Dibutyl phthalate, Eicosane, Benzene 1,3-dinitro, Heptadecane 2,6,10,15-Tetramethyl, 1-Hexadecanol, Tridacane-4-methyl, Phthalic acid butyl, 2-pentyl ester and Eruvic acid presence was confirmed. Current study is focused on characterization of constituents responsible for the anti-tuberculosis activity of the plant.

KEYWORD: Bioactive compound, ethanomedicine, tuberculosis.
Abbreviation
WT = *Wrightia tinctoria*
GC-MS = Gas Chromatography Mass Spectroscopy
TLC = Thin layer Chromatography
EAE = Ethyl Acetate Extract
NIST = National Institute Standard and Technology
REMA = Risazurin Microtiter Assay Plate Method
TDA = Toluene-diamine
EI = Electron Ionization
PCI = Positive Chemical Ionization
NCI = Negative Chemical Ionization
RT = Retention Time
MF = Molecular Formula
MW = Molecular Weight
Amu = Atomic mass unit

1. INTRODUCTION

*Wrightia tinctoria* (Roxb.) (WT) is a small deciduous tree belonging to family Apocynaceae, distributed in deciduous forest of India. It is being used in tribal areas and in traditional systems of medicine, commonly called as “jaundice curative tree” in south India. Already reported constituents in bark are stigmasterol, lupeol, indirubin, alkaloids, terpene, wrightial, tryptanthrin, indole and some flavonoids. WT bark is being used by the tribal people of Chhattisgarh and Madhya Pradesh region for the treatment of cough, cold, jaundice, eye complaint, throat swelling, various type of cancer, urinary trouble, pathri, skin trouble, dysentery and Tuberculosis. WT has been assigned to have stomachic, analgesic and antidiabetic, anti-inflammatory, antidiuretic, antituberculosis and antiulcer. Hence the objective of the present study is to identify the possible bioactive chemical constituents responsible for the activity with the aid of GC-MS technique.

2. MATERIAL AND METHODS

2.1 Collection of plant material: Plant bark was collected from Wadrafnagar (Madhna), District Balrampur, Chhattisgarh, India tribal area. Plant material was authenticated by Prof. K.P. Sahu, Department of Botany, Govt. Model Science College Jabalpur, India.
2.2 Preparation of extracts: Various organic solvents of increasing polarity were used for extraction according to the methodology of Indian pharmacopoeia.\textsuperscript{[14]} Shed dried bark was subjected to pulverization to get coarse powder. The powdered material was subjected to soxhlet extraction separately and successively with petroleum ether, ethyl acetate, methanol and water. All the extract were concentrated to dryness under reduce pressure by using flask evaporator at controlled 40\(^{\circ}\)C temperature.

2.3 Bioactivity test: Anti tuberculosis activity of all WT bark extracts was performed in Lowenstein –Jensen (L-J) Medium in collaboration with CDRI Lucknow against \textit{M. tuberculosis} with two control drugs, Isoniazid and Rifampicin. On the basis of results obtained from this study potential extract was selected for the further investigations using GC-MS.

2.4 Proximate analysis: Different tests were performed for the detection of alkaloids, Carbohydrates, Proteins, Lipids, Saponins, Flavonoids, Resins, Tannins, Sterols, Cardian glucosides Coumerins, Anthraquinone and Triterpines as per the standard methods of AOAC, 1995.

2.5 Fractionation: Ethyl acetate extract was selected for the further analysis as it showed significantly high level activity against \textit{Mycobacterium tuberculosis} as compared to other WT crude bark extracts. Crude ethyl acetate extract was subjected to column (1000x40mm) packed with silica gel (100-200 mesh size) for fractionation. Elutes were collected and continuously monitored by performing TLC to avoid the mixing of fractionating compounds.

2.6 GC-MS analysis of material

2.6.1 Instruments: Agilent 5975C TDA series gas chromatography/mass spectroscopy selective detector system offer high performance and flexibility with many options. Gas chromatograph Agilent 7890A is the auto sampler, oven temperature is ambient +4- 450\(^{\circ}\)C and 20/21 negative ramps allowed and mass selective detector includes standard mode –EI, optional mode-PCI, NCI and EI acquisition with CI source, EI ion source type-non coated inert EI source, Ion source temperature-150\(^{\circ}\)C to 350\(^{\circ}\)C, Quadrupole temperature-106-200\(^{\circ}\)C, mass filter-monolithic hyperbolic quadrupole, minimum mass-1.6u, maximum mass-1050u, mass axis stability- better than 0.10u/48h, detector- triple axis.
2.6.2 Method of GC-MS analysis and chromatographic condition: Ethyl acetate extract (1µl) of WT was used in GC-MS analysis. GC-MS of ethyl acetate extract was performed using Agilent 7890A GC-MS instrument with 5975C MS column (5% poly siloxane) and 30×250µm×0.25µm size. Oven temperature was programmed as follows: Isothermal temperature was 5ºC/min and held for 1.75 min then increased to 275ºC at the rate of 8ºC/min and kept constant for 5min. The run time was 25 min. Ionization of sample components were performed on EI mode (70eV).

2.6.3 Identification of bioactive compounds: Identification of bioactive compounds and interpretation of mass spectrum (GC-MS) was conducted using the database of National Institute of Standard and Technology (NIST). Present spectrum of unknown compound was compared with the spectrum of the known compound in the NIST library. The name, structure and molecular weight of the compounds in sample material were ascertained.

3. RESULT
Bioactivity test of all the crude extracts of WT bark against *M. tuberculosis* screened out ethyl acetate extract as potential amongst all as its MIC value was calculated as 6.25µg/ml while other extracts showed MIC more than 25 µg/ml  

\[ F_{(P<0.01)} = 152, \ df = 10, \ SE_{(d)} = 0.0002, \ LSD_{(P<0.05)} = 0.0011 \]  

(Table 01). Proximate analysis results showed the presence of Alkaloids, Carbohydrates, Proteins, Lipids, Saponins, flavonoids, Resins, Tannins, Sterols Cardiac Glucosides, Triterpenes Coumerines and Anthraquinone are listed in table-02.

<table>
<thead>
<tr>
<th>Table 1: Bioactivity of WT bark extracts against <em>M. tuberculosis</em></th>
<th>Table 2: proximate analysis of WT bark extract in ethyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatments</strong></td>
<td><strong>MIC of plant extract (In µg/ml)</strong></td>
</tr>
<tr>
<td><strong>WT (Bark Extract)</strong></td>
<td><strong>PEE</strong></td>
</tr>
<tr>
<td></td>
<td><strong>EAE</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ME</strong></td>
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<tr>
<td></td>
<td><strong>WE</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Isoniazid</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Rifampicin</strong></td>
</tr>
<tr>
<td></td>
<td><strong>F&lt;sub&gt;(P&lt;0.01)&lt;/sub&gt;</strong></td>
</tr>
<tr>
<td></td>
<td><strong>df</strong></td>
</tr>
<tr>
<td></td>
<td><strong>SE_{(d)}</strong></td>
</tr>
<tr>
<td></td>
<td><strong>LSD_{(P&lt;0.05)}</strong></td>
</tr>
<tr>
<td></td>
<td><strong>PEE</strong></td>
</tr>
<tr>
<td></td>
<td><strong>EAE</strong></td>
</tr>
<tr>
<td></td>
<td><strong>ME</strong></td>
</tr>
</tbody>
</table>
In chromatogram of fraction 1 (WT/EAE-01; Figure-1) six peaks were analyzed and identified as Methoxyacetic acid 3-tridecyl ester (2.11% peak percent area), Quinoline,2,7, dimethyl (1.77%), Hexadecane (11.33%), 4,5-dimethyl pentadecane (16.20%), Nonadecane (5.50%) and Docosane (1.90%).

![Figure 1. GC of WT bark extract in ethyl acetate extract fraction-1 (WT/EAE-01)](image1)

In fraction 2 (WT/EAE-02; Figure-2) 8 peaks were analyzed and identified as Benzene,1,3-dichloro (3.40%), Dodecane (8.75%), Benzothiazole (3.66%), Tetradecane (11.64%), Ethanol, 2 (Octadecyloxy) (14.01%), Octadecane (16.01%), Dibutyl phthalate (40.11%) and Eicosane (5.48%).

![Figure 2. GC of WT bark extract in ethyl acetate extract fraction-2 (WT/EAE-02)](image2)
In fraction 3 (WT/EAE-03; Figure-3) three peaks were analyzed and identified as Benzene 1,3-dinitro (12.17%), Heptadecane 2,6,10,15-Tetramethyl (19.90%) and 1-Hexadecanol (8.14%).

![Fig 3. GC of WT bark extract in ethyl acetate extract fraction-3 (WT/EAE-03)](image)

In fraction 4 (WT/EAE-04; Figure-4) two peaks were analyzed and identified as Tridacane-4-methyl (11.08%), Phthalic acid butyl, 2-pentyl ester (29.40%).

![Fig 4. GC of WT bark extract in ethyl acetate extract fraction-3 (WT/EAE-04)](image)
In fraction 5 (WT/EAE-05; Figure-5) only one peak could be analyzed and as per the fragmentation pattern, presence of Erucic acid (9.15%) was confirmed. All the compounds identified are listed in Table 03.

![Fig 5. GC of WT bark extract in ethyl acetate extract fraction-5 (WT/EAE-05)](image)

Table 3: Shows compounds identified in ethyl acetate extracts of WT bark using GC/MS.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>S. No.</th>
<th>Run Time (in minute)</th>
<th>Chemical name of isolated compound</th>
<th>Molecular formula</th>
<th>MW (amu)</th>
<th>Peak area (percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>12.75</td>
<td>Methoxyacetic acid 3-tridecyl ester</td>
<td>C_{16}H_{32}O_{3}</td>
<td>272</td>
<td>3.52</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>13.63</td>
<td>Quinoline, 2,7, dimethyl</td>
<td>C_{11}H_{11}N</td>
<td>157</td>
<td>1.93</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>15.51</td>
<td>Hexadecane</td>
<td>C_{16}H_{34}</td>
<td>226</td>
<td>14.27</td>
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<tr>
<td>4</td>
<td>4</td>
<td>18.26</td>
<td>4,5-dimethyl pentadecane</td>
<td>C_{17}H_{36}</td>
<td>240</td>
<td>17.85</td>
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<td>5</td>
<td>5</td>
<td>24.42</td>
<td>Nonadecane</td>
<td>C_{19}H_{40}</td>
<td>268</td>
<td>6.52</td>
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<tr>
<td>6</td>
<td>6</td>
<td>27.63</td>
<td>Docosane</td>
<td>C_{22}H_{46}</td>
<td>310</td>
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<td>Fraction 2</td>
<td></td>
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</tr>
<tr>
<td>7</td>
<td>7</td>
<td>6.37</td>
<td>Benzene, 1,3-dichloro</td>
<td>C_{6}H_{12}Cl_{2}</td>
<td>146</td>
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<td>8</td>
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<td>9.57</td>
<td>Dodecane</td>
<td>C_{12}H_{26}</td>
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<td>9</td>
<td>9</td>
<td>10.24</td>
<td>Benzothiazole</td>
<td>C_{7}H_{5}N_{S}</td>
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<tr>
<td>10</td>
<td>10</td>
<td>12.74</td>
<td>Tetradecane</td>
<td>C_{14}H_{30}</td>
<td>198</td>
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<tr>
<td>11</td>
<td>11</td>
<td>15.49</td>
<td>Ethanol, 2 (Octadecyloxy)</td>
<td>C_{20}H_{42}O_{2}</td>
<td>314</td>
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<tr>
<td>12</td>
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<td>18.22</td>
<td>Octadecane</td>
<td>C_{18}H_{38}</td>
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<tr>
<td>13</td>
<td>13</td>
<td>20.90</td>
<td>Dibutyl phthalate</td>
<td>C_{16}H_{32}O_{4}</td>
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<td>14</td>
<td>14</td>
<td>24.41</td>
<td>Eicosane</td>
<td>C_{20}H_{42}</td>
<td>282</td>
<td>5.40</td>
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<td>Fraction 3</td>
<td></td>
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<tr>
<td>15</td>
<td>15</td>
<td>13.72</td>
<td>Benzene 1, 3-dinitro</td>
<td>C_{6}H_{4}N_{2}O_{4}</td>
<td>168</td>
<td>10.44</td>
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<tr>
<td>16</td>
<td>16</td>
<td>18.17</td>
<td>Heptadecane 2, 6, 10, 15-Tetramethyl</td>
<td>C_{21}H_{44}</td>
<td>296</td>
<td>19.90</td>
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<tr>
<td>17</td>
<td>17</td>
<td>27.58</td>
<td>1-Hexadecanol, 2-methyl</td>
<td>C_{17}H_{36}O</td>
<td>256</td>
<td>8.14</td>
</tr>
</tbody>
</table>
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4. DISCUSSION

Proximate analysis of SA nuts powder showed the presence of proteins in very high percentage (19.8%) but was less as compared to 26.4% protein reported in SA nuts but edibility of protein present could not be tested. Fats, fibers, carbohydrates, ash moisture contents were comparable. The total nutritive value estimated was 359.2 kcal/100g as compared to 587 kcal energy reported.\(^{[15]}\)

Despite intense hard work to control TB disease with synthetic drugs number of people affected with drug resistant and multi drug resistant TB is increasing every year worldwide.\(^{[16]}\) For treating drug-resistant strains of \textit{M. tuberculosis}, high doses and lengthy therapy is practiced which is causing toxicity and its various adverse effects in patients. Thus, the need to search for new effective anti-TB agents has become very necessary. Using medicinal plants for the treatment of TB offers a great hope to fulfill these needs because of their chemical diversity and they have been used for curing diseases for many centuries. In addition, natural herbs continue to play a great significant role in the drug discovery and development of highly active antimycobacterial metabolites and they can be used as pure compounds or as crude materials.\(^{[17]}\)

Antituberculosis activity of this plant extract is previously not reported in available literature so the results cannot be compared. Other plants, \textit{Alatonia scholaris}\(^{[18]}\), \textit{Marrubium vulgare}\(^{[19]}\), \textit{Piper nigrum} seeds\(^{[20]}\), \textit{Azadirecta indica}\(^{[21]}\), \textit{Terminalia avicennioides}\(^{[22]}\) are reported for their potential against tuberculosis.

Although whole spectrum could not be completely analyzed but what done was surprising in results. Previously no results are discussed in any literature about the chemical composition of WT bark but 3-O-Methyl-d-glucose (51.44%) is reported as major compound along with 21 minor compounds from WT leaves in ethanolic extract with GC-MS analysis.\(^{[23]}\) Interestingly no similar compound was found in WT bark extract as reported in leaves. Both the reasons, either solvent difference or chances of absence of compounds in bark those reported in leaves may valid.
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Dodecan (4.90% peak area) is reported in this study is responsible for this activity related to Human tyrosyl-DNA phosphodiesterase I (Tdp1). This enzyme is a rational anticancer target because this enzyme is involved in the repair of DNA lesions created by the trapping of human DNA topoisomerase I (Top1) due to anticancer agents such as camptothecins.[24]

Benzothiazole (3.27% peak area) is a heterocyclic compound which possess various biological activities and still of great scientific interest now a days. They are widely found in bioorganic and medicinal chemistry with application in drug discovery. Benzothiazole moieties are part of compounds showing numerous biological activities like anti-fungal, anti-cancer, antiinflammatory, anti-diabetic, anti-convulsant, antimicrobial, diuretic, anti-tubercular, schitosomicidal and anthelmintic activities.[25] Benzothiazole is used in research as a starting material for the synthesis of various bioactive structures. Due to its important pharmaceutical utilities, the synthesis of derivative compounds is of considerable interest.

Dibutyl phthalate (40.11% peak area) is reported for its anti-proliferative activity was present in very high concentration.[26] Eicosane (5.40% peak area) is as ester derivative also reported for its anticancer and cytotoxicity activity which increases apoptosis in gastric cancer cells significantly.[27] Erucic acid (9.15% peak area) is a member of omega 9 fatty acid is metabolized to oleic acid. Dietary erucic acid commonly called as Lorenzo's oil is used in the management of the adrenoleukodystrophy as a gylceryl trierucate form.[28] Compounds identified in WT bark extract in ethyl acetate are not reported for their anti-tuberculosis activity. Only WT leaves extract were tried against *M. tuberculosis* but results were negative.

5. ACKNOWLEDGEMENT

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