ANTIBACTERIAL ACTIVITY AND GC-MS ANALYSIS OF METHANOLIC EXTRACT FROM STEM BARK AND LEAVES OF *MITRAGYNA PARVIFOLIA* (ROXB.) KORTH

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**ARTICLE INFO**

**Article history**
Received 21/01/2014  
Available online 04/02/2014

**Keywords**
*Mitragyna parvifolia*, Antibacterial Activity, GC-MS analysis, Mitraphylline.

**ABSTRACT**

*Mitragyna parvifolia* is important medicinal tree belongs to family Rubiaceae. It is traditionally mainly known for its antimicrobial and anti-inflammatory properties. The antibacterial activity with its Minimum Inhibitory Concentration (MIC) were determined by disk diffusion testing against *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, the investigation was also carried out to determine the possible bioactive components of leaf and bark methanolic extract using Gas Chromatograph and mass spectrometer (GCMS). The highest antibacterial potential demonstrated against *E. coli* with 31±1.4 mm and 31.5±2.12 mm zone of inhibition by leaves and bark extract respectively and interestingly which is found to be more than Chloramphenicol. The MIC for both extracts was 12 mg/ml for the same bacterial strain. The lowest MIC of both extract was seen against *B. subtilis*. The major compounds identified in the extracts of leaf and bark of were isobutanoic acid, 2-ethylhexyl ester (19.36%), 4 methyl mannose (53.13%), mitraphylline (21.59%), isomitraphylline (3.37%) and 1, 2 Hydrazine dicarboxylic acid, diethyl ester (3.50%), α, α-dimethyl mucconic acid (22.97%), isobutanoic acid, 2-ethylhexyl ester (43.83%), α-D-glucopyranoside, α-D-glucopyranosyl (27.21%) respectively. Among these compounds found in the leaf of *M. parvifolia*, a pentacyclic oxindole alkaloid mitraphylline is known for its anti-inflammatory, antiproliferative activities. Our investigation conducted on different parts of this plant validate the traditional usage of this plant as antimicrobial and several compounds identified by GC-MS analysis are principal factors for significant antibacterial activities.

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*Please cite this article in press as Pashupat Vasmatkar et al. Antibacterial activity and gc-ms analysis of methanolic extract from stem bark and leaves of Mitragyna parvifolia (Roxb.) Korth.Indo American Journal of Pharm Research.2014:4(01).*
INTRODUCTION

The exploration for newer antibiotic sources is very crucial as there is account on rising incidence of multi-drug resistant strains of bacteria as well as the recent appearance of strains with reduced vulnerability to antibiotics [1]. The organisms akin to Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa are concerned to cause harsh infections in individual, as they are found in various environmental habitats [2,3] and these phytopathogens cause infection in any plant tissue it invades [4].

Figure 1, Natural habitat of Mitragyna parvifolia

*Mitragyna parvifolia* (family Rubiaceae) is average to large tree found growing through the drier parts of India, Pakistan and Srilanka (Figure 1). It is endorsed with a variety of medicinal properties and is widely used by tribal, inhabitant community and other conventional medicine practitioners for its therapeutic properties such as pain reliever, antimicrobial, anticonvulsant, antioxidant [5], antipyretic, antiarthritic [6], antidiabetic [7], anti-inflammatory, antinociceptive [8], anxiolytic effect [9]. The fever, colic, muscular pain, burning sensation, poisoning, gynecological disorders, cough, edema and as an aphrodisiac are cured with its bark and roots. The quantities of breast milk in lactating mothers are enhanced by its fruit juice and it has been also used to treat malaria, diarrhea and for the expulsion of worms [10, 11]. Its leaves are conventionally used to treat wounds, ulcers, to alleviate pain, swelling and thus for better healing [12-15]. Though the plant has great potential for antimicrobial activity, no one so far reported the simultaneously study of antibacterial activity of leaves and bark methanolic extracts and determination of potential antibacterial activity of *M. parvifolia* extracts could be more informative for the future use in the controlling of bacterial diseases.

Plant based antimicrobial compounds have enormous therapeutic potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials. Hence, the present study was conducted to investigate the potent role of methanolic leaves and bark extracts of *M. parvifolia* as an antibacterial agent against three human pathogenic bacterial strains. Although the antimicrobial properties of this plant have been reviewed in the past, the mechanism of action has not been studied in great detail due to the very little knowledge about its chemical constituents. In latest years GC-MS studies have been progressively more applied for the analysis of medicinal plants as this practice has proved to be a valuable method for the analysis of non polar components, volatile essential oil, fatty acids, lipids and alkaloids. Considering the large number of different groups of chemical compounds present in methanolic extract of this plant, it is most likely that their antibacterial activity is attributable to many of these and other new compounds other than those observed by scientists until now, which might be identified and characterized from other plants but not from *M. parvifolia*. Any such new compounds are screened and their structures are elucidated in this plant by latest techniques may give better understanding for folklore use of this plant. We can further find out new drugs and also make new synthetic compounds especially for the control of multi drug resistant bacteria and emerging microbes.

MATERIALS AND METHODS

Collection and extraction of plant materials

The fresh leaves and stem bark of *M. parvifolia* were collected from the Agro forestry Research Centre, GBPUAT, Pantnagar in the month of February. Plant materials were washed, air dried and powdered mechanically. 50 g of powder were extracted with methanol and extracts were filtered. Filtrates were then concentrated to dryness under reduced pressure at 50°C using a rotary evaporator to get crude extracts. Dried residues of leaves and bark extracts were dissolved separately in the appropriate quantity of methanol.
Microorganism used

The methanolic extracts of leaves and bark of *Mitragyna parvifolia* were individually tested against one Gram positive (*Bacillus subtilis*), and two Gram negative (*Escherichia coli, Pseudomonas aeruginosa*), isolated and identified in the lab.

Determination of antibacterial activity of the extracts

Antibacterial activities of extracts were evaluated by paper disc diffusion method [16, 17] with some modifications. 100 μl inoculate of each test bacterial strain in lag phase was swabbed on nutrient agar plate and kept for 10 min for adsorption. Four paper discs having 6 mm diameter are placed per NA plate and the extract (25, 50, 75 and 100 mg of dry extract/ml in methanol) were added to the four different discs on solidified medium. Antibacterial assay Plates were incubated at 37 °C for 16 h. A clear area with no growth around each disc signifies zone of inhibition and the diameter of such zones were measured in millimeter (mm). Chloramphenicol (25 μg/disc) and methanol (25 μl/disc) were used as positive and negative control respectively with triplicate experimentation for statistical analysis.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) in mg/ml was determined by selecting the lowest concentration of each extract that have zone of inhibition. Eight paper discs were placed on each NA plate with pre-swarbed inoculate. Each extract of 2, 4, 6, 8, 10, 12, 14 and 16 mg of dried extract/ml methanol applied on eight different discs in increasing order. MIC plates were incubated at 37 °C for 16 h. The least concentration of the samples with no visible growth was taken as the MIC [18, 19].

Preparation of extract and GCMS conditions

The collected leaves and bark were cleaned shade dried, powdered and extracted with methanol and filtered. The filtrate was then concentrated under reduced pressure to get the viscous residue. The methanolic extracts of the plant were used for GC-MS analysis. GC-MS analysis was carried out on a regular Perkin Elmer system comprising Auto System XL Gas Chromatograph and mass spectrometer with total GC running time was 40 min.

Identification of components

Interpretation on mass spectrum GC-MS was conducted using the database of national Institute Standard and technology (NIST). The Name, Molecular weight and structure of the components of the test materials were ascertained by comparing the spectrum of the unknown component with the spectrum of the known components stored in the NIST library.

RESULTS

Antibacterial activity and MIC of *M. pavifolia*

Results of antibacterial activity and MIC were tabulated in Table 1. The results reveal that the both extracts of *Mitragyna parvifolia* exhibited antibacterial activity in methanol solvents against all three tested bacteria (Fig. 2 and 3). Leaves extract recorded significant inhibition zone of 26±2.8 mm against *P. aeruginosa*, 31±1.4mm (*E. coli*) and 30±0.7 mm (*B. subtilis*) at 100 mg/ml concentration. At lowest concentration 25mg/ml leaves extract most effective against *E. coli* with 23±1.4 mm of inhibition zone.

Table 1: Antibacterial activity of *Mitragyna parvifolia* leaves and bark methanolic extracts against Bacterial strains

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Escherichia coli</em></th>
<th><em>Bacillus subtilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>25</td>
<td>17±1.4</td>
<td>23±1.4</td>
<td>13±1.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>19±1.4</td>
<td>27±2.1</td>
<td>24±0.7</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>25.5±0.7</td>
<td>29±1.4</td>
<td>28±0.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>26±2.8</td>
<td>31±1.4</td>
<td>30±0.7</td>
</tr>
<tr>
<td>Bark</td>
<td>25</td>
<td>21±0</td>
<td>21±1.4</td>
<td>12±0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>23±1.4</td>
<td>24.5±0.07</td>
<td>13±1.4</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>24.5±2.1</td>
<td>29.5±0.07</td>
<td>21±1.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>25±1.4</td>
<td>31.5±2.12</td>
<td>22±2.8</td>
</tr>
<tr>
<td>CONTROL</td>
<td>Chloramphenicol</td>
<td>26±2.8</td>
<td>25±1.4</td>
<td>26±0.7</td>
</tr>
<tr>
<td></td>
<td>methanol</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
The inhibitory zone of the bark extract recorded 25±1.4 mm against P. aeruginosa, 31.5±2.1 mm (E. coli) and 22±2.8 mm (B. subtilis) at highest 100 mg/ml concentration. The maximum zone of inhibition was showed by M. parvifolia methanol extract of leaves (31±1.4) and bark (31.5±2.12) against E. coli as compared to other two bacterial strains. The bark extract showed least activity against B. subtilis with mean zone of inhibition of 22±2.8 mm at the same concentration. At lowest concentration of bark extract 25 mg/ml it shows maximum inhibition against E. coli. Interestingly it was found that both extract at their highest as well as lowest concentration most effective against E. coli (Fig. 2b).

Minimum inhibition concentration of leaves extract of M. parvifolia is higher for P. aeruginosa and B. subtilis compared to bark extract showed in Table 2.

Leaves extract of M. parvifolia showed MIC of 14 mg/ml against P. aeruginosa, (Fig. 4c), 12 mg/ml (E. coli) (Fig. 4b) and 8 mg/ml (B. subtilis) whereas the bark extract showed MIC of 8 mg/ml against P. aeruginosa (Fig. 4c), 12 mg/ml against E. coli (Fig.
4a) and 4 mg/ml against *B. subtilis* (Fig. 4b). Surprisingly ethanolic extract of *Mitragyna parvifolia* fruit failed to show any activity against Gram positive and Gram negative bacterial strains [20].

Table 2: Minimum inhibitory concentrations (MICs) of methanolic extract of leaves and bark of *M. parvifolia*

<table>
<thead>
<tr>
<th>Extract</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Escherichia coli</em></th>
<th><em>Bacillus subtilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>14</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Bark</td>
<td>8</td>
<td>12</td>
<td>4</td>
</tr>
</tbody>
</table>

3.2 GCMS analysis

Chromatogram of both extracts showed the presence of 6 major peaks and is (figure 5 and 6). The spectral analysis of the extracts revealed the presence of four compounds by forming four major peaks in each extract. The active principles in leaves and bark extracts with their retention time (RT), molecular formula, molecular weight (MW) and concentration (Peak area %) are presented in table 3 and 4 respectively.

Figure 5: GC chromatogram of methanolic leaves extract of *M. parvifolia*

Figure 6: GC chromatogram of methanolic bark extract of *M. parvifolia*
In the *M. parvifolia* leaves extract 4 methyl mannose (53.13%) was the prominent constituent followed by mitraphylline (21.59%), isobutanoic acid 2-ethylhexyl ester (19.36%), and isomitraphylline (3.37%).

### Table 3: Major bioactive compounds identified in the methanolic leaves extract of *M. parvifolia*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>RT</th>
<th>Name of the compound</th>
<th>Molecular formula</th>
<th>MW</th>
<th>Peak area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.971</td>
<td>Isobutanoic acid, 2-ethylhexyl ester</td>
<td>C_{12}H_{23}O_{2}</td>
<td>200</td>
<td>19.36</td>
</tr>
<tr>
<td>2</td>
<td>19.061</td>
<td>4 Methyl mannose</td>
<td>C_{7}H_{14}O_{6}</td>
<td>194</td>
<td>53.13</td>
</tr>
<tr>
<td>3</td>
<td>30.703</td>
<td>Mitraphylline</td>
<td>C_{21}H_{32}O_{4}N_{2}</td>
<td>368</td>
<td>21.59</td>
</tr>
<tr>
<td>4</td>
<td>30.007</td>
<td>Isomitraphylline</td>
<td>C_{21}H_{32}O_{4}N_{2}</td>
<td>368</td>
<td>3.37</td>
</tr>
</tbody>
</table>

### Table 4: Major bioactive compounds identified the methanolic bark extract of *M. parvifolia*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>RT</th>
<th>Name of the compound</th>
<th>Molecular formula</th>
<th>MW</th>
<th>Peak area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.899</td>
<td>1, 2 Hydrazine dicarboxylic acid</td>
<td>C_{6}H_{12}O_{3}N_{2}</td>
<td>176</td>
<td>3.50</td>
</tr>
<tr>
<td>2</td>
<td>11.141</td>
<td>α, α-dimethyl mucconic acid</td>
<td>C_{6}H_{17}O_{4}</td>
<td>170</td>
<td>22.97</td>
</tr>
<tr>
<td>3</td>
<td>16.934</td>
<td>Isobutanoic acid, 2-ethylhexyl ester</td>
<td>C_{12}H_{23}O_{2}</td>
<td>200</td>
<td>43.83</td>
</tr>
<tr>
<td>4</td>
<td>18.328</td>
<td>α-D-glucopyranoside, α-D-glucopyranosyl</td>
<td>C_{12}H_{22}O_{11}</td>
<td>342</td>
<td>27.21</td>
</tr>
</tbody>
</table>

In *M. parvifolia* bark extract, 1, 2-Hydrazine dicarboxylic acid (3.50%), α, α-dimethyl mucconic acid (22.97%), isobutanoic acid, 2-ethylhexyl ester (43.83%) and α-D-glucopyranoside, α-D-glucopyranosyl (27.21%). Among identified compound in bark extract, isobutanoic acid, 2-ethylhexyl ester (43.83%) was the major component followed by the α-D-glucopyranoside, α-D-glucopyranosyl (27.21%), α, α-dimethyl mucconic acid (22.97%) and 1, 2- Hydrazine dicarboxylic acid (3.50%).

### DISCUSSION

The MIC (12.5mg/ml) of methanolic bark extract of *Mitragyna parvifolia* was recorded against various human pathogens such *B. subtilis*, *P. aeruginosa* and *E. coli* [21]. No literature on the MIC of *Mitragyna parvifolia* leaves has been reported till now.

Gram-negative bacteria are frequently reported to have developed multi drug resistance to many of the antibiotics currently available in the market of which *Escherichia coli* is the most prominent [22, 23]. However, *M. parvifolia* is still of special interest for further investigations in this regard as in the bark and leaves, which showed exceptionally stronger activity against *E. coli* as compared to Gram-positive bacteria, a trend is generally not observed for other species of plants.

*E. coli* was more resistance to the standard antibiotic comparatively to other strains, found to be more susceptible to leaves extract as compared to standard antibiotic (Table & figure 2). Whereas *P. aeruginosa* found to be equally susceptible to the leaves extract and standard antibiotic. Further these results conclusively indicates that *E. coli* and *B. subtilis* are developing resistance against commonly used antibiotics but are also more susceptible to bark and leaves extracts of *M. parvifolia*.

Indol alkaloids [24] and triterpenoid saponins [25, 26] with mitragynine, an indole alkaloid, were reported as the major phytochemical constituents of the plant. It has been observed that environmental factor has a vital role to play in modifying the alkaloid content and the structure [27, 28]. Though various indolic and oxindolic alkaloids have been reported from the species, only six alkaloids, all oxindolic isomitraphylline, isomitraphylline, pteropodine, isopteropodine, speciophilline and uncarine F have been reported from the Lucknow region [29].

In the *M. parvifolia* leaf extract 4 methyl mannose (53.13%) was the prominent constituent followed by mitraphylline (21.59%), isobutanoic acid 2-ethylhexyl ester (19.36%), and isomitraphylline (3.37%). One main alkaloid of Uncaria tomentosa bark was isolated and identified as mitraphylline and it has anti-tumoral effects [30], anti-inflammatory activity [31], antigenotoxic, antioxidant and lymphocyte induction effects [32]. Two alkaloids, isomitraphylline and mitraphylline in the leaves of *M. parvifolia* and the mitraphylline were the main alkaloid constituent [33].

Among identified compound in bark extract, isobutanoic acid, 2-ethylhexyl ester (43.83%) was the major component followed by the α-D-glucopyranoside, α-D-glucopyranosyl (27.21%), α, α-dimethyl mucconic acid (22.97%) and 1, 2- Hydrazine dicarboxylic acid (3.50%). α-D-glucopyranoside, α-D-glucopyranosyl commonly known as trehalose and has the ability to protect cellular membranes and labile proteins against damage and denaturation as a result of desiccation and oxidative stress and is also used in the treatment for dry eye syndrome [34].

Furthermore trehalose has applications in the food, cosmetic and medical industries [35-37]. Among the identified compounds, the presence of 4 methyl mannose in leaf and the presence of 1, 2 Hydrazine dicarboxylic acid, α, α-dimethyl mucconic acid, α-D-glucopyranoside, α-D-glucopyranosyl in bark reported for the first time.

### CONCLUSION
Today, most pathogenic organisms are becoming resistant to antibiotics [38]. The results of the study also support the traditional application of the plant as expulsion of worms. These studies suggest that the both extracts of Mitragyna parvifolia possess compounds with antibacterial properties that can be used as antibacterial agents. Four compounds were identified from methanolic extract of leaves and bark of M. parvifolia. Among the identified compounds, like mitraphylline and isomitraphylline were reported as therapeutic compounds and the presence of 4 methyl mannose in leaves and 1,2 Hydrazine dicarboxylic acid, α, α-dimethyl mucconic acid, α-D-glucopyranoside, α-D-glucopyranosyl in bark reported for the first time.

Future research is needed to design this plant as a drug after completing the molecular level research work.

Authors’ Statements
Competing Interests
The authors declare no conflict of interest.

REFERENCES


