Comparative In-vitro Antioxidant and Antimicrobial Activity of Hydroalcoholic Extract of Miliusa tomentosa

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Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

ABSTRACT

Green medicine, the drugs derived from plants attained a wide spread interest, as believed to be safe and dependable, compared with costly synthetic drugs that have adverse effects. The present study aimed to analyze a comparative in vitro free radical scavenging and antimicrobial potentials of leaf hydroalcoholic extract fractions of Miliusatomentosa (Roxb.) J. Sinclair (M. tomentosa, Annonaceae) commonly known as hoom, kari. It is a large deciduous tree, growing up to 20 m tall and are given to children to cure the weakness in summer. The hydroalcoholic extract of leaves of M. tomentosa was studied for antioxidant activity on different in vitro models namely 1,1-diphenyl, 2-picryl hydrazyl (DPPH) assay, Hydrogen peroxide (H₂O₂) and Nitric oxide (NO) radical scavenging method. Agar well diffusion method has been used to determine the antimicrobial activities of plant extracts against Gram-positive bacteria, Gram-negative bacteria (Enterococcus faecalis, Salmonella Bongori, Propionibacterium acnes), and fungus (Aspergillusflavus). In the investigated models, the extract showed dose-dependent free radical scavenging properties. For the DPPH technique, M. tomentosa leaves extract had an IC₅₀ value of 335.92g/ml, which was close to that of ascorbic acid (IC₅₀=17.68g/ml). The IC₅₀ value for hydrogen peroxide was determined to be 96.42g/ml, which compares favourably to ascorbic acid (IC₅₀=36.61g/ml). The IC₅₀ value for nitric oxide was 105.87g/ml, which was close to that of ascorbic acid (IC₅₀=24.63g/ml). The extracts exhibited both antibacterial and antifungal activities against tested microorganisms using standardampicilin, ciprofloxacin, clindamycin and fluconazole (10-30μg/ml).

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The antimicrobial activity was determined by measuring the diameter of the zone of inhibition in terms of millimeter (mm). The antimicrobial activity of hydroalcoholic extract of leaves against all microorganisms was concentration dependent but less than standard drug. On the basis of results obtained, it is suggested that both *M. tomentosa* leaves extracts may be a potential source of natural antioxidants and antimicrobial compounds to be used in the treatment of various oxidative disorders, infectious diseases caused by resistant microorganisms.

Keywords: *Miliusa tomentosa*; annonaceae; antioxidant activity; antimicrobial activity.

1. INTRODUCTION

The study of diseases and their treatment have been existing since the dawn of human civilization. Also for the treatment of a range of diseases, herbal drugs have been used since ancient times as medicines. Medicinal plants have played a key role in world health. The World Health Organisation (WHO) estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs [1]. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. A large proportion of the India population for their physical and psychological health needs depend upon traditional systems of medicine. Medicinal plants have become the focus of intense study in terms of conservation and as to whether their traditional uses are supported by actual pharmacological effects or merely based on folklore [2-4]. Recently, there is great attention towards natural antioxidants from plants. Antioxidants can act as a radical scavenger, promote health, and produce anticancer activity [5]. There is a growing concern about antimicrobial resistance issue [6]. The complications of multidrug resistance enforced scientists to search for new antimicrobial agents from various sources such as medicinal plants [7]. The genus *Miliusa* (Annonaceae) consists about 40 species which grows in tropical rainforest of India, Thailand, South China and North Australia [8]. The different species of *Miliusa* are invariably small to large trees and are found in a wide range of rainforest communities. Only three species of Genus *Miliusa* occur in Australia, which are endemic to that contain two essential oils [9]. The plant is used in folk medicine for different symptoms such as gastropathy and glomerulo nephropathy [10]. In Chinese traditional medicine *M. tomentosa* oil has been found to have both antibacterial and analgesic properties [11]. Knowledge of the chemical constituents of plants is desirable because such information will be valuable for synthesis of complex chemical substances [12]. Two new isoquinoline alkaloids, 2,10- dimethoxy-3,11-dihydroxy-5,6-dihydroprotoberine and 1,9-dihydroxy-2,11 -dimethoxy-4,5- dihydro-7-oxoaporphine, together with thirteen known alkaloids, were isolated from the ethanolic extracts of the stem and leaves of *M. cuneata* (Grail) [1]. Since *M. tomentosa* is one of them, its traditional uses are not reported but its fruits are eaten in some parts of India and its tree yields a pale yellow gum known as karee gum [13]. Literature reviews pointed out that no studies combining the antioxidant and antimicrobial activities of the leaves of *M. tomentosa* have so far been undertaken. In this work, we wanted to evaluate the antioxidant and antibacterial activity of hydroalcoholic extracts of *M. tomentosa* leaves, which is in line with our ongoing interest in pharmacological screening of Indian medicinal plants.

2. MATERIALS AND METHODS

2.1 Plant Material

Leaves of *M. tomentosa* were collected from rural area in month of December 2019. The leaves plant sample were separated and washed with sterile distilled water to remove the adhering dust particles and other unwanted materials. The leaf was air dried under room temperature. The dried plant samples were cut and grinded to make it in powder form. The powdered samples were stored in clean, dry and sterile container for further use.

2.2 Chemical Reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals used in this study were of analytical grade. The pathogenic bacteria and fungus used in the current study obtained from
Microbial Culture collection, National Centre
for cell science, Pune, Maharashtra, India.

2.3 Defatting of Plant Material

Powdered leaves of *M. tomentosa* were shade
dried at room temperature. The shade dried plant
material was coarsely powdered and subjected
to extraction with petroleum ether using
maceration method. The extraction was
continued till the defatting of the material had
been completed.

2.4 Extraction by Soxhlet Method

100 gram of powdered leaves of *M. tomentosa*
was exhaustively extracted with hydroalcoholic
solvent (ethanol-water 80:20) by soxhlet method.
The extract was evaporated above their
boiling points. Finally, the percentage yields were
calculated of the dried extracts [14].

2.5 Antioxidant Activity

2.5.1 DPPH radical scavenging assay

DPPH scavenging activity was measured by
modified method of Olufunmiso *et al.* [15]. DPPH
scavenging activity was measured by the
spectrophotometer. Stock solution (6 mg in
100ml methanol) was prepared such that 1.5 ml
of it in 1.5 ml of methanol gave an initial
absorbance. Decrease in the absorbance in
presence of sample extract at different
concentration (10-100 µg/ml) was noted after 15
minutes. 1.5 ml of DPPH solution was taken and
volume made till 3 ml with methanol, absorbance
was taken immediately at 517 nm for control
reading. 1.5 ml of DPPH and 1.5 ml of the test
sample of different concentration were put in a
series of volumetric flasks and final volume was
adjusted to 3 ml with methanol. Three test
samples were taken and each processed
similarly. Finally the mean was taken. Absorbance at zero
was taken for each
concentration. Final decrease in absorbance
was noted of DPPH with the sample at different
concentration after 15 minutes at 517 nm. The
percentage inhibition of free radical DPPH
was calculated from the following equation:

\[
\text{S\%} = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}\right) \times 100
\]

Where *Abs control = absorbance of the blank
control (containing all reagents except the extract
solution) and Asample = absorbance of the test
sample.

2.5.2 Nitric oxide (NO\(^0\)) radical scavenging

The determination of NO\(^0\) radical scavenging
ability of the extracts is based on the inhibition of
NO\(^0\) radical generated from sodium nitroprusside
in phosphate buffer saline solution by
Griess reagent (1% sulfanilamide, 2% 
orthophosphoric acid and 0.1%
naphthylethlenediaminedihydrochloride). Scavengers
of nitric oxide act against oxygen,
prompting to lessened production of nitrite ions
which can be monitored at 546 nm [16,17].
Briefly, sodium nitroprusside (0.6 ml, 5 mM)
solution was mixed with and without varying the
concentration of the extracts or Ascorbic acid (2
ml, 10-200 µg/ml) and incubated at 25 ± 2°C for 5
h. Incubated solution (2 ml) was mixed with equal
volume of Griess reagent and absorbance of the
purple coloredazo dye chromophore was measured at \(\lambda_{max}546\) nm using UV-Vis
spectrophotometer. The NO\(^0\) radical
scavenging ability was calculated using following
formula:

\[
\text{Scavenging activity (\%)} = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}\right) \times 100
\]

2.5.3 Free radical scavenging activity (FRSA)

using hydrogen peroxide

Scavenging activity of hydrogen peroxide (H\(_2\)O\(_2\))
by the plant extract was determined by the
method of Ruch *et al.* [18]. Ethanolic extract (4
ml) prepared in distilled water at various
concentration was mixed with 0.6 ml of 4 mM
H\(_2\)O\(_2\) solution prepared in phosphate buffer (0.1
M pH 7.4) and incubated for 10 min. The
absorbance of the solution was taken at 230 nm.
Ascorbic acid was used as a positive control
compound. The percentage of inhibition was
calculated by comparing the absorbance values
of the control and test samples using following
equation [19].

\[
\text{S\%} = \left(\frac{\text{Abs control} - \text{Asample}}{\text{Abs control}}\right) \times 100
\]

Where *Abs control = absorbance of the blank
control (containing all reagents except the extract
solution) and Asample = absorbance of the test
sample.
2.6 Antimicrobial Activity

2.6.1 Method of preparation

This agar medium was dissolved in distilled water and boiled in conical flask of sufficient capacity 20 ml. Dry ingredients are transferred to flask containing required quantity (15 ml) of distilled water and heat (40°C) to dissolve the medium completely.

2.6.2 Sterilization culture media

The flask containing medium was cotton plugged and was placed in autoclave for sterilization at 15 lbs /inch² (121°C) for 15 minutes.

2.6.3 Preparation of plates

After sterilization, the media in flask was immediately poured (20 ml/ plate) into sterile Petri dishes on plane surface. The poured plates were left at room temperature to solidify and incubate at 37°C overnight to check the sterility of plates. The plates were dried at 50°C for 30 minutes before use.[10]

2.6.4 Well diffusion method

The well diffusion method was used to determine the antimicrobial activity of the extract prepared from leaves of M. tomentosa using standard procedure [20]. There were 3 concentration used which are 25, 50 and 100 mg/ml for each extracted phytochemicals in antibiogram studies. It’s essential feature is the placing of wells with the antibiotics on the surfaces of agar immediately after inoculation with the organism tested. Undiluted over night broth cultures should never be used as an inoculums. The plates were incubated at 37°C for 24 hr. and then examined for clear zones of inhibition around the wells impregnated with particular concentration of drug.

3. RESULTS AND DISCUSSIONS

The crude extracts so obtained after each of the successive soxhletion extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The yield of hydroalcoholic extracts obtained from the leaves of M. tomentosa was found to be 9.14%w/w. DPPH radical scavenging assay measured hydrogen donating nature of extracts [21]. Under DPPH radical scavenging activity the inhibitory concentration 50% (IC₅₀) value of M. tomentosa hydroalcoholic leaves extract was found to be 335.92μg/ml as compared to that of ascorbic acid (17.68μg/ml). A dose dependent activity with respect to concentration was observed Table 1 & Fig. 1. Extracts showed NO⁺ scavenging effects by competing with oxygen to react with NO directly hence inhibited the nitrite ion formation [22]. M. tomentosa hydroalcoholic leaves extract showed nitric oxide (NO⁻) radical scavenging activity with IC₅₀ value of 105.87μg/ml as compared to that of ascorbic acid (IC₅₀24.63μg/ml) Table 2& Fig. 2. Hydrogen peroxide is generated in vivo by several oxidase enzymes and by activated phagocytes and it is known to play an important role in the killing of several bacterial and fungal strains [23]. There is increasing evidence that, hydrogen peroxide, either directly or indirectly via its reduction product, OH², can act as a messenger molecule in the synthesis and activation of several inflammatory mediators [24]. When a scavenger is incubated with H₂O₂ using a peroxidase assay system, the loss of H₂O₂ can be measured. Table 3 & Fig. 3 show the scavenging ability of M. tomentosa hydroalcoholic leaves extract and ascorbic acid on hydrogen peroxide at different concentrations. Extracts was capable of scavenging hydrogen peroxide in an amount dependent manner at all the tested concentrations. Hydrogen peroxide itself is a rather weak oxidant and most organic compounds (except for some sulfur containing molecules) are virtually inert to attack by it at ordinary environmental or cellular concentrations and temperatures. In the presence of reduced transition metal ions, however, hydrogen peroxide is converted to the much more reactive oxidant, hydroxyl radical in the cells by Fenton reaction. Besides this, studies have shown that other transition metals such as copper (I), cobalt (II) and nickel (II) also take part in the process [23]. Thus, the removing is very important for antioxidant defense in cell or food systems. The antibacterial and antifungal activities were determined using the well diffusion method. This method is highly effective for rapidly growing microorganisms, and the activities of the test extracts are expressed by measuring the diameter of the zone of inhibition. The antimicrobial activity of hydroalcoholic leaves extract of M. tomentosashowed bioactivity by inhibiting growth of microbial species selected for the test as shown in Tables 4 and 5. The zone of inhibition shown by the extracts was comparable to the standard drug. It is effective against Enterococcus faecalis, Salmonella Bongori, and Propionibacterium acne in concentration dependent manner but hydroalcoholic extract shown no activity against Aspergillus flavus.
Table 1. % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using DPPH method

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration (µg/ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>Hydroalcoholic extract</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>44.65</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>48.62</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>65.34</td>
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<tr>
<td>4</td>
<td>60</td>
<td>69.65</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>77.41</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>84.13</td>
</tr>
<tr>
<td>IC 50</td>
<td></td>
<td>67.68</td>
</tr>
</tbody>
</table>

Table 2. % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using NO method

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration (µg/ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>Hydroalcoholic extract</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>47.70</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>52.92</td>
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<tr>
<td>3</td>
<td>60</td>
<td>67.43</td>
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<td>4</td>
<td>80</td>
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<tr>
<td>5</td>
<td>100</td>
<td>74.42</td>
</tr>
<tr>
<td>IC 50</td>
<td></td>
<td>44.63</td>
</tr>
</tbody>
</table>

Table 3. % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using H$_2$O$_2$ Method

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration (µg/ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>Hydroalcoholic extract</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>42.87</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>52.19</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>60.41</td>
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<tr>
<td>4</td>
<td>80</td>
<td>65.78</td>
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<tr>
<td>5</td>
<td>100</td>
<td>68.75</td>
</tr>
<tr>
<td>IC 50</td>
<td></td>
<td>36.61</td>
</tr>
</tbody>
</table>

Fig. 1. % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using DPPH method
Fig. 2. % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using NO method

Fig. 3. % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using *H*₂*O*₂ method

Table 4. Antimicrobial activity of *standard drug* against selected microbes

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of drug</th>
<th>Microbes</th>
<th>Zone of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>1.</td>
<td>Ampicillin</td>
<td><em>Enterococcus faecalis</em></td>
<td>12±0.28</td>
</tr>
<tr>
<td>2.</td>
<td>Ciprofloxacin</td>
<td><em>Salmonella Bongori</em></td>
<td>17±0.15</td>
</tr>
<tr>
<td>3.</td>
<td>Clindamycin</td>
<td><em>Propionibacterium acnes</em></td>
<td>13±0.28</td>
</tr>
<tr>
<td>4.</td>
<td>Fluconazole</td>
<td><em>Aspergillus flavus</em></td>
<td>17±0.81</td>
</tr>
</tbody>
</table>

Table 5. Antimicrobial activity of hydroalcoholic extract of leaves of *M. tomentosa* against selected microbes

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of microbes</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydroalcoholic extract</td>
<td>25mg/ml</td>
</tr>
<tr>
<td>1.</td>
<td><em>Enterococcus faecalis</em></td>
<td>6±0.47</td>
</tr>
<tr>
<td>2.</td>
<td><em>Salmonella Bongori</em></td>
<td>6±0</td>
</tr>
<tr>
<td>3.</td>
<td><em>Propionibacterium acnes</em></td>
<td>8±0.47</td>
</tr>
<tr>
<td>4.</td>
<td><em>Aspergillus flavus</em></td>
<td>-</td>
</tr>
</tbody>
</table>
4. CONCLUSION

From the experiment it has shown that hydroalcoholic extracts have been used in vitro to inhibit the growth of some disease causing bacteria. It can therefore be suggested that plant extracts have great potential as antimicrobial compounds against microorganisms and they can be used in the treatment of infectious diseases caused by resistant microorganisms. They can also be a source of natural antioxidants. Due to their antibacterial and antioxidant activities, M. tomentosa extracts have promising potential as a source of natural antioxidant and antimicrobial agents. Therefore these results are encouraging enough to pursue characterization of these fractions in different other models in detail. Further studies may also be conducted to isolate & purify the active constituents to evaluate others activity.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


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