ANTIMICROBIAL, ANTIOXIDANT AND ANTI α-GLUCOSIDASE ACTIVITIES OF THE LEAF EXTRACT FROM MUSSAENDA ROXBURGHII HOOK. F. (RUBIACEAE)

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ABSTRACT

Context: The genus Musaenda (Rubiaceae) includes 200 species distributed in hilly tracks of north eastern India and in tropics of the old world. Musaenda roxburghii Hook. f. mainly grown in Tripura and other north eastern states of India has been used as traditional medicine.

Objectives: The objective of this study was to evaluate in vitro antimicrobial, antioxidant and anti-α-glucosidase activities of the leaves extract from Musaenda roxburghii.

Methods: Antimicrobial assay was done by disc diffusion technique using six human pathogenic bacteria, Shigella dysenteriae 1, Vibrio cholerae non.0139(L4), Vibrio cholerae non.0139(CSK6669), Streptococcus pneumoniae, Staphylococcus aureus and Escherichia coli. In vitro antioxidant potential was studied using DPPH (1,1-diphenyl-2-picryl hydrazyl) and superoxide radical scavenging property with Butylated Hydroxy Anisole (BHA) and Ascorbic acid as positive control, respectively. In vitro α-glucosidase (E.C No.3.2.1.20) inhibitory activity was determined using Acarbose as positive control.

Results: Among the two fractions, maximum antimicrobial activity was against V. cholerae non.0139(CSK6669) at 500 µg/disc with n- butanol fraction and with chloroform fraction it was against S. aureus and E. coli at 1 mg/disc concentration. At 100µg/ml concentration both n-butanol and chloroform fractions showed significant antioxidant activity against DPPH and superoxide radicals. Both the fractions also exhibited moderate inhibition of α-glucosidase activity at 500 µg/ml of n-butanol fraction and 250 µg/ml of chloroform fraction.
Conclusion: The results indicated that leaf extract of Mussaenda roxburghii has antimicrobial, antioxidant and α-glucosidase inhibitory activity. Phytochemical investigation of these extracts is needed to find out potent antimicrobial, anti-diabetic phytochemicals for their use as herbal drugs.

Keywords: leaf extract, antimicrobial, antioxidant, antidiabetic, butanol, Mussaenda roxburghii.

INTRODUCTION

According to the World Health Organization (WHO), about 80% of the world population use plant based drugs for their treatment of diseases (http://www.who.int/mediacentre/factsheets/fs134/en/). Scientific experiments for isolation of antimicrobial phytochemicals were first documented in the late 19th century. Naturally occurring antimicrobials have been derived from plants, animal tissues, and microorganisms [1].

The genus Mussaenda (Rubiaceae) is an important source of bioactive phytochemicals, particularly iridoids, triterpenes and flavonoids. Earlier work on Mussaenda frondosa reported antimicrobial, analgesic [2], antioxidant [3], wound healing [4] activities and on Mussaenda erythrophylla antihelmintic activity [5]. Whole plant Mussaenda roxburghii has been used as natural medicine for various ailments in eastern India and as well as in south-east Asia [6]. It is distributed in the Eastern and Central Nepal and in moist hilly places of eastern India and adjoining Bhutan, Bangladesh, and Myanmar. A paste of the root is applied to the eruption to treat on tongue [6].

Free radicals are associated with various physiological and pathological events such as inflammation, aging, mutagenicity and carcinogenicity. Free radicals are generated in the body during different biological reactions [7]. These free radicals or Reactive Oxygen Species (ROS) are capable of damaging DNAs, proteins, carbohydrates and lipids. Antioxidants play an important role in scavenging ROS which are produced in various extracellular and intracellular biochemical reactions. The free radicals play an important role in human health preventing ROS derived disease. Plant products (edible or medicinal) are one of the rich sources of antioxidants, which have been substantially investigated by different researchers time to time and reported in literature [8].
Diabetes has not yet been completely curable with the currently available anti-diabetic agents. Insulin therapy remains the only satisfactory approach in diabetic mellitus, even though it has several drawbacks like insulin resistance [9]. Herbal drugs are gaining popularity in the treatment of diabetic mellitus [10]. The major advantages of herbal medicine seem to be their efficacy, low incidence of side effects, and low cost.

In this study our attempt was to evaluate the antimicrobial, antioxidant and anti-diabetic efficacy of the n-butanol and chloroform fractions of the leaf extract from *Musaenda roxburghii*.

**MATERIALS AND METHODS**

**Collection of plant materials**

The plant *Musaenda roxburghii* (*Rubiaceae*) was collected from hilly region of Kailasahar and Khayerpur of Tripura, India and was identified by Prof. B. K. Datta, Taxonomist, Department of Botany, Tripura University.

**Preparation of plant extracts**

The fresh plant leaves were washed thoroughly with water to remove adhering dirt and shade dried. Completely dried leaves were cut into small pieces and grinded and passed through molecular sieve (mess size 40) in order to obtain uniform size of powdered material. Powdered materials were soaked with methanol at room temperature for 48 hours and methanol extract was concentrated under reduced pressure to a semisolid material. The crude extract was suspended in distilled water (20 gm/lit.) and stirred well for proper mixing. The water soluble material was separated and was fractionated into chloroform, ethyl acetate and n-butanol soluble fractions (5 x 200ml of each solvent). All the fractions were concentrated and only chloroform and n-butanol fractions were used for this study because of their substantial amounts.

**Microbial samples**

Six microbial strains *S. dysenteriae* 1, *V. cholerae* non.0139 (L4), *V. cholerae* non. 0139(CSK6669), *S. pneumoniae*, *S. aureus* and *E. coli*. collected from NICED, Kolkata and AGMC, Agartala were used for antimicrobial assay.

**Chemicals**

The required chemicals nitroblue tetrazolium(NBT), reduced nicotinamide adenine
dinucleotide (NADH), phenazonium methosulphate (PMS), p-nitrophenyl α-D-glucopyranoside (p-NPG), α-glucosidase, sodium carbonate (Na₂CO₃), nutrient agar and dimethyl sulfoxide (DMSO) from SISCO Research Laboratories Pvt. Ltd, Mumbai, India; BHA and methanol from MERCK Chemicals; acarbose from Buyers Pharmaceuticals and DPPH (1, 1-diphenyl-2-picryl hydrazyl) from Sigma-Aldrich were purchased. All chemicals used were of analytical grade.

**Antibacterial Activity**

The anti-bacterial activity of the fractionated leaf extract of *Mussaenda roxburghii* was tested against six microorganisms by the disc diffusion method. Different concentrations of the CHCl₃ fractions (100 µg/disc, 500 µg/disc, 750 µg/disc, 1 mg/disc) and of n-butanol fraction (1 µg/disc, 10 µg/disc, 100 µg/disc, and 500 µg/disc) from methanol extract were prepared with dimethyl sulphoxide (DMSO). The test microorganisms (freshly cultured in Luria Bertani broth) were seeded into respective medium (SRL Agar) by spreading of 40 µl of each strain in spread plate method. The autoclaved paper discs (5 mm in diameter and 0.4 mm in thickness) were then placed in petri dishes (100 mm in diameter) containing test microorganism in agar media followed by addition of different concentrations of extracts on each disc. DMSO was also applied as control for each extract. Antibiotic, Ofloxacin, (2 µg/disc) was used as positive control against each strain. The plates were incubated at 37 °C for overnight. The antimicrobial activities were evaluated by measuring zone of inhibition in mm. The mean of the readings were recorded after repeating each experiment five times.

**Antioxidant activity**

Both the chloroform and n-butanol fractions were assayed for radical scavenging property using superoxide and DPPH radical. All the experiments were performed five times and the results were averaged.

**Superoxide radical scavenging activity**

Superoxide scavenging activity of *Mussaenda roxburghii* extract was measured following the method of Robak et. al. with some modifications. All the assayed solutions were prepared by mixing 100 mM phosphate buffer (pH 7.4), 200 µl of NBT (156 µM), 200 µl of NADH (468 µM) and 600 µl of extracts to produce final concentrations of 0.5-250 µg/ml. The reaction was started by adding 20 µl of PMS (60 µM) and the mixture was then incubated at 25 °C for 5 min and measured the absorbance at 560 nm with a spectrophotometer. Same procedures were used for control using distilled water instead of the extracts and butylated
hydroxyl anisole (BHA) dissolved in methanol for positive control \(^{13}\). The percentage of inhibition was calculated by using the following formula.

\[
\text{% of inhibition} = \left(\frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}}\right) \times 100
\]

**DPPH radical scavenging activity**

DPPH radical scavenging activity was measured according to the method of Cotelle N et al. with some modifications \(^{14}\). In brief 750µl reaction mixture containing 50µl of DPPH (100 µM in methanol) and 700 µl of extracts (at various concentrations; 1-500µg/ml) was incubated at 37ºC for 30 min and absorbance was measured at 517 nm using UV-VIS Spectrophotometer. DPPH radical scavenging percentage was calculated by comparing the results of the test with those of the control (not treated with extract) using above mentioned formula. Ascorbic acid was used as positive control for the same concentration of both the fractions of extract.

**Anti-diabetic activity**

The two fractions were also tested for anti-diabetic activity following \(\alpha\)-glucosidase inhibitory assay protocol and all experimental results were averaged after performing each experiment for five times.

**\(\alpha\)-Glucosidase inhibitory assay**

100 µl of 3 mM p-nitrophenyl glucopyranoside (p-NPG) in 0.2 M sodium phosphate buffer (pH 6.8) was added as a substrate to the mixture of 50 µl of \(\alpha\)-glucosidase (0.15 unit/ml) and 50 µl of extracts (at various concentrations; 1-500 µg/ml) to start the reaction. The reaction was allowed to run at 37 ºC for 15 min and stopped by the addition of 750 µl of 0.1 M Na\(_2\)CO\(_3\). \(\alpha\)-Glucosidase activity was assessed by measuring the release of p-nitrophenol from p-NPG at 405 nm using a UV-Vis spectrophotometer \(^{15}\). Acarbose was used as positive control. All tests are performed independently for five times (n=5) and results were expressed as mean ±SD. The inhibition percent was calculated by the same formula used above.

**Statistical analysis**

All the statistical analysis was done using Microsoft Office Excel 2007 software. Statistical significance was assessed by standard deviation and Student’s t-test and p-value < 0.05 is considered to be significant.
RESULTS AND DISCUSSION

Antimicrobial activity

Discovery of potent antimicrobial phytochemicals from the plants used in traditional medicines and the mechanism of their mode of action become an urgent attention of chemists and pharmacologists\(^{[16]}\). The development of resistant strains of bacteria has increased the need for new antibiotics\(^{[17]}\). In this context, we have studied antibacterial activity against some human pathogenic bacteria \(S.\ dysenteriae\) 1, \(V.\ cholerae\) non0139 (L4), \(V.\ cholerae\) non.0139 (CSK6669) and \(E.\ coli\).

Experimental data in the present study revealed that \(Mussaenda\ roxburghii\) extract possessed potential antibacterial activity against, \(S.\ dysenteriae\) 1, \(V.\ cholerae\) non.0139 (L4), \(V.\ cholerae\) non.0139 (CSK6669) and \(E.\ coli\) as shown in Table-1a and Table-1b. Both the fractions of \(Mussaenda\ roxburghii\) showed significant antibacterial activity against almost all the pathogenic microbial strains within the range of 5mm to 12mm zone of inhibition with different concentration of the extract. The \(n\)-butanol fraction showed highest inhibition zone as 10.33 ± 0.056 mm against \(V.\ cholerae\) non.0139 (CSK6669) at 500 µg/disc concentration and the lowest as 8.5 ± 0.353 mm against \(E.\ coli\) at the same concentration (Table-1a).

Table 1a: Antibacterial activity of \(n\)-butanol fraction of the leaf extract against some human pathogenic microorganisms.

<table>
<thead>
<tr>
<th>Microorganisms were taken for test.</th>
<th>Zone of Inhibition after 24 hours incubation. (mm in 5 repeats ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1µg/disc</td>
</tr>
<tr>
<td>(S.\ dysenteriae) 1</td>
<td>2.5±0.353</td>
</tr>
<tr>
<td>(V.\ cholerae) non.0139(L4)</td>
<td>-</td>
</tr>
<tr>
<td>(V.\ cholerae) non.0139(CSK6669)</td>
<td>-</td>
</tr>
<tr>
<td>(E.\ coli)</td>
<td>5.0±0</td>
</tr>
<tr>
<td>(S.\ aureus)</td>
<td>5.5±0.353</td>
</tr>
<tr>
<td>(S.\ pneumonae)</td>
<td>-</td>
</tr>
</tbody>
</table>

‘-’ not significant

With the chloroform fraction, the highest antibacterial activity was shown with an inhibition zone of 12.67 ± 0.057 mm against \(S.\ aureus\) and \(E.\ coli\) at 1mg/disc concentration of extract.
while least activity was recorded against *V. cholerae* non.0139 (CCK 6669) with a measured zone of inhibition as 10.33±0.056 mm at the same concentration. Chloroform fraction with concentration of 100 µg/disc showed inhibition only against *V. cholerae* non.0139 (L4) (Table-1b).

**Table 1b: Antibacterial activity of chloroform fraction of the leaf extract against some selected pathogenic microorganisms.**

<table>
<thead>
<tr>
<th>Microorganisms were taken for test.</th>
<th>Zone of Inhibition after 24 hours incubation. (mm in 5 repeats ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100µg/disc</td>
</tr>
<tr>
<td><em>S. dysenteriae</em> I</td>
<td>-</td>
</tr>
<tr>
<td><em>V. cholerae</em> non.0139(L4)</td>
<td>05.66±0.057</td>
</tr>
<tr>
<td><em>V. cholerae</em> non.0139(CSK6669)</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>-</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>-</td>
</tr>
</tbody>
</table>

**Antioxidant assay**

Free radicals are chemical entities that exist separately with one or more unpaired electron. The generation of free radicals takes place via thousands of cellular reactions and thus their accumulation to a high concentration causes extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals [18, 19]. The uptake of one electron by molecular oxygen results in the formation of the superoxide anion radical by the action of oxidative enzymes as well as via non-enzymatic reaction such as auto oxidation by catecholamine [20]. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation [21]. DPPH is a stable free radical that accepts an electron or hydrogen radical and becomes a stable diamagnetic molecule [20, 22]. The DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting
decolorization is stoichiometric with respect to the number of electrons captured. We have investigated antioxidant activity of leaf extract of M. roxburghii via superoxide and DPPH radical scavenging assay. A strong positive correlation has been reported between total polyphenol content and DPPH free radical scavenging activity\textsuperscript{[23, 24]}.

**Superoxide radical scavenging activity**

Our assay study indicated that the superoxide radical scavenging activities of n-butanol and chloroform fractions of M. roxburghii leaf methanolic extract and the reference compound were increased markedly with increasing concentrations. Experimental data revealed that n-butanol fraction showed highest % inhibition (44.15 ± 3.94 %) at the concentration of 100 µg/ml compared to that of positive control; BHA which showed maximum inhibition (43.768 ± 1.83 %) only at 250 µg/ml concentration.

On the other hand the chloroform fraction showed 64.452 ± 2.42 % inhibition at 100 µg/ml concentration of the extract. These results indicated that the chloroform fraction have better superoxide radical scavenging activity than the n-butanol fraction. Figure-1a and figure-1b diagrammatically illustrated the comparative percentage of radical scavenging activity between BHA, n-butanol and chloroform fractions respectively.

![Figure 1a: Bar diagram showing the antioxidant activity (Superoxide radical scavenging activity) of both butanol fraction of leaf extract and BHA (as positive control) and their comparison. * indicates p ≤ 0.05, ** indicates p ≤ 0.01, *** indicates p ≤ 0.001.](image1a)

![Figure 1b: Bar diagram showing the antioxidant activity (Superoxide radical scavenging activity) of both chloroform fraction of leaf extract and BHA (as positive control). * indicates p ≤ 0.05, ** indicates p ≤ 0.01, *** indicates p ≤ 0.001.](image1b)
DPPH radical scavenging assay
In this study we observed that the n-butanol fraction showed DPPH reduction significantly with a high value of 67.556±1.523% at 100 µg/ml and a low value of 16.436±1.86% at 15 µg/ml using ascorbic acid as positive control where reduction was 40.022 ± 1.28 % and 13.756 ± 1.27% respectively. The chloroform fraction showed maximum reduction (61.398 ± 1.88 %) at 100 µg/ml and a minimum (25.014 ± 1.61 %) at 25 µg/ml concentration while ascorbic acid showed 40.152 ± 1.28 % and 26.308 ± 2.08 % of DPPH reduction at respective concentrations. Figure-2a and 2b illustrate the results of comparative DPPH radical scavenging activity for both the fractions with ascorbic acid.

**Figure 2a:** Bar diagram showing the antioxidant activity (DPPH radical scavenging activity) of both n-butanol fraction of leaf extract and ascorbic acid (as positive control). * indicates p ≤ 0.05, ** indicates p ≤ 0.01, *** indicates p ≤ 0.001.

**Figure 2b:** Bar diagram showing the antioxidant activity (DPPH radical scavenging activity) of both chloroform fraction of leaf extract and ascorbic acid (as positive control). * indicates p ≤ 0.05, ** indicates p ≤ 0.01, *** indicates p ≤ 0.001.

Antidiabetic assay
One therapeutic approach for treating diabetes is to decrease the postprandial hyperglycaemia. This is done by retarding the absorption of glucose through the inhibition of the carbohydrate hydrolysing enzymes α-glucosidase in the digestive tract [15]. Inhibitors of this enzyme delayed carbohydrate digestion, causing a reduction in the rate of glucose absorption and consequently reducing the postprandial plasma glucose rise. Inhibitors of intestinal α-glucosidase have been used in the treatment of noninsulin-dependent diabetes mellitus (NIDDM) and many of the commonly used antidiabetic drugs possess this property [25]. In the present study we observed that chloroform and n-butanol fractions of leaf extract
of M. roxburghii showed significant α-glucosidase inhibitory activities and this may be due to some active constituent present in the leaf of the plant.

**α – Glucosidase inhibitory assay**

*In vitro* anti diabetic activity was determined by calculating the percentage of α–glucosidase inhibition with *n*-butanol and chloroform fractions of *M. roxburghii* leaves extract. The chloroform fraction showed highest inhibition *viz.* 84.294 ± 0.826 % at a concentration of 500 µg/ml of extract while acarbose as positive control, under similar conditions showed 85.07 ± 2.74 % inhibition. Similarly *n*-butanol fraction showed maximum inhibition of 78.716 ± 3.212 % at 250 µg/ml concentration, which was comparable to that of acarbose having 83.19 ± 3.37 % inhibition at the same concentration. These results indicated that the leaf extract have good α–glucosidase inhibitory activity. Comparative bar diagram for α–glucosidase inhibitory assay for both the fractions are illustrated in Figure 3a and 3b.

**CONCLUSION**

We have investigated the antimicrobial, antioxidant and antidiabetic activities of *n*-butanol and chloroform fractions of *M. roxburghii* leaf extract. In comparison of the two fractions, chloroform fraction showed comparatively better activity than the *n*-butanol fraction. Based on these results, *M. roxburghii* can be considered as a potential source of natural antibacterial, antioxidant and antidiabetic agent for pharmaceutical formulations. Isolation of
biologically active compounds from this plant is in process. Bioactive compounds will be studied in animal model.

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REFERENCE


