Phytochemical and \textit{in vitro} Antimicrobial assay of Fruit extracts of \textit{Morinda tinctoria} Roxb

S. Ramesh\textsuperscript{1*}, R. Muthubalaji\textsuperscript{1} and R. Elangomathavan\textsuperscript{2}

\textsuperscript{1}Department of Microbiology, PRIST University, Thanjavur - 614 904, Tamil Nadu, India
\textsuperscript{2}Department of Biotechnology, PRIST University, Thanjavur - 614 904, Tamil Nadu, India

*Corres.auther : marineramesh2020@gmail.com
Tel.:+91999761 93870, Fax: +91 4362 265150

Abstract : The aim of the present work is to perform physicochemical analysis, phytochemical screening and antimicrobial activity of \textit{Morinda tinctoria} fruits at different maturity stages. Physicochemical parameters were studied as per WHO recommended guidelines for standardization. The fruits were extracted by different solvents and tested for preliminary phytochemical screening and antimicrobial activity against selected pathogenic microorganisms. Physicochemical parameters such as ash values, moisture content and extractive values were determined. The results of phytochemical analysis revealed the presence of various secondary metabolites. Antimicrobial activity revealed that different extracts of \textit{M. tinctoria} fruits exhibited inhibitory effects against the pathogens. In the present study, \textit{S. typhi} (16 mm) and \textit{K. pneumoniae} (18 mm) were inhibited by ethanol and methanol extract of mature fruit extracts. The result of physicochemical analysis is useful in developing standards for sample identity and purity of \textit{M. tinctoria} fruits. The different extracts of \textit{M. tinctoria} showed the presence of various secondary metabolites and the extracts were found to be effective against the tested microorganisms. It can be concluded that the fruits of \textit{M. tinctoria} would be helpful in the development of phyto-medicine against microbial infections.

Keywords: \textit{Morinda tinctoria}, maturity stages, physicochemical parameters, phytochemical screening, antimicrobial activity.

Introduction

In recent years, there has been a rising attention in drugs from medicinal plant origin in compare to the synthetics which are considered as unsafe to humans\textsuperscript{[1]}. The medicinal plants have been in existence for thousands of years\textsuperscript{[2]}. These medicinal plants derived drug is a key resource in developing countries to fight with serious disease\textsuperscript{[3]}. Medicinal plant remains the source of inspiration of novel drug compounds as they afford key chemical structure for the progress of new antimicrobial drugs as well as phytomedicine. The most important bioactive compounds of plant are alkaloids, flavonoids, tannins and phenolic compounds\textsuperscript{[4]}. These phytochemicals are antibiotic principles of plants and have been reported to possess anti-bacterial, anti-fungal
and anti-inflammatory activities [5]. Thus, medicinal plants play an important role in developing of newer drugs because of their effectiveness, less side effects and relatively low cost when comparing with synthetic drugs [6].

*Morinda tinctoria* Roxb. belongs to the family of Rubiaceae that grows wild and is distributed throughout Southeast Asia. It is commercially known as Nunaa and is indigenous to tropical countries. *M. tinctoria* is considered as an important folklore medicine. In the traditional system of medicine, leaves and roots of *M. tinctoria* are used as astringent, deobstrent and to relieve pain in the gout [7]. There is a greater demand for fruit extract of *Morinda* species in treatment for different kinds of illness such as arthritis, cancer, gastric ulcer and other heart disease [8,9]. The ashes of *M. tinctoria* leaves are also reported to act as biosorbents in controlling ammonia pollution in waste waters [10]. The major components have been identified in the Nunaa plant which includes octoanicacid, potassium, vitamin C, terpenoids, scopeotin, flavones, glycosides, linoleic acid, anthraquinones, morindone, rubiadin and alizarin [11]. In this work, we evaluated the physiochemical properties, phytochemical and *in vitro* antimicrobial screening of various maturity stages (immature, midmature, mature) of *M. tinctoria* fruit extracts.

**Materials and methods**

**Collection of fruits**

Different maturity stages (immature, midmature and mature) of *M. tinctoria* fruits were collected from Thanjavur, Tamil Nadu, India.

**Physicochemical analysis**

The physicochemical parameters (total ash, water soluble ash, acid insoluble ash and moisture) and extractive values (water, alcohol, methanol, ethyl acetate and chloroform) were determined using a standard procedure [12].

**Preparation of fruit extracts**

*M. tinctoria* fruits at maturity stages were washed with tap water followed by washing with distilled water. The fruits were peeled and the core was cut into small pieces and kept for shade drying and the dried fruit was then finely powdered using a mixer.

The powdered fruit material (15g) was extracted with 100 ml of water, ethanol, methanol, ethyl acetate and chloroform separately. The contents were kept as such in room temperature for 48 hour with constant stirring at regular intervals. After the incubation period, the contents were filtered through Whatmann No.1 filter paper. Then filtrates were vacuum dried using rotary evaporator and concentrates were stored at 4°C. The residues were re-dissolved with the appropriate solvents from which they were prepared and used for further studies.

**Preliminary phytochemical analysis**

Qualitative phytochemical analyses were performed in various extracts of *M. tinctoria* fruits at different maturity stages to determine the presence of phytochemicals like carbohydrate, protein, tannin, flavonoid, saponin, steroids, alkaloids and glycosides as described by standard procedure [13].

**Test organisms**

The microbial cultures such as *Escherichia coli* MTCC-433, *Klebsiella pneumoniae* MTCC-432, *Salmonella typhi* MTCC-733, *Aspergillus niger* MTCC-10180 and *Aspergillus flavus* MTCC-9064 were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

**Preparation of inoculum**

The bacterial cultures were inoculated into nutrient broth and incubated for 24 hour at 37°C. Fungal cultures were inoculated into rose bengal broth and incubated for 48 hour at 37°C. The turbidity of the medium indicates the growth of organisms.
Antimicrobial studies

The agar well diffusion method was employed for the determination of antimicrobial activity of the extracts.[14] Lawn culture of *E. coli*, *K. pneumonia* and *S. typhi* were spread on nutrient agar and *A. niger* & *A. flavus* were spread on rose bengal agar using sterile cotton swabs. The wells (6 mm in diameter) were cut from the agar plates using a cork borer. 30µl of the extracts (7mg/ml) were poured into the well using a sterile micropipette. The plates were incubated at 37±2°C for 24 hours for bacterial activity and 48 hours for fungal activity. The plates were observed for the zone formation around the wells. The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. Two common antibiotics streptomycin and fluconazole were used as positive control for the assay.

Results

Physicochemical parameters

The physiochemical parameters such as total ash, water soluble ash, acid insoluble ash, moisture content and extractive values were measured (Table 1 and Fig. 1). The total ash value, water soluble ash and acid insoluble ash of dried fruits of *M. tinctoria* was found to be in the range of 3.5% - 7%, 1% - 6.5% and 2.5% - 8% respectively. The moisture content was estimated to be in the range between 2.5% and 5.5%.

Table 1. Physicochemical parameters of *M. tinctoria* fruits at different maturity stages

<table>
<thead>
<tr>
<th>Parameters (% w/w)</th>
<th>IM</th>
<th>MM</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ash</td>
<td>6.5</td>
<td>7.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Water soluble ash</td>
<td>2.0</td>
<td>6.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Acid insoluble ash</td>
<td>7.0</td>
<td>2.5</td>
<td>8.0</td>
</tr>
<tr>
<td>Moisture content (% dry weight basis)</td>
<td>5.5</td>
<td>2.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

IM = immature, MM = midmature, M = mature

Figure 1. Extractive values of different maturity stages of *M. tinctoria* fruits using various solvents
Preliminary phytochemical analysis

Different maturity stages of *M. tinctoria* fruits were investigated for the presence of various secondary metabolites using different solvents (Table 2). Aqueous extract showed the presence of carbohydrate, protein, alkaloids, saponin, glycosides, tannins, flavonoids and steroids. Ethanol extract showed the presence of all the tested metabolites except saponins. Proteins were not identified in the methanol extract. In ethyl acetate extract, alkaloids were not detected, whereas saponins were found to be present only in the mature fruit extract. Carbohydrates, proteins, tannins and steroids were present in chloroform extract, while flavonoids, saponins, glycosides and alkaloids were absent.

Table 2. Phytochemical analysis of extracts of *M. tinctoria* fruits at different maturity stages

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Aqueous</th>
<th>Ethanol</th>
<th>Methanol</th>
<th>Ethyl acetate</th>
<th>Chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IM</td>
<td>MM</td>
<td>M</td>
<td>IM</td>
<td>MM</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = Present, - = Absent, IM = immature, MM = midmature, M = mature

Antimicrobial activity

The antimicrobial activity was investigated in various maturity stages of *M. tinctoria* fruits (Fig. 2). Mature aqueous extract of *M. tinctoria* fruit showed mild inhibition against *E. coli* (5 mm), whereas midmature ethyl acetate and immature chloroform extracts exhibited 11 mm and 13 mm zone of inhibition respectively. Mature (16 mm) and midmature (13 mm) aqueous extract showed positive results for *K. pneumoniae*, whereas midmature ethyl acetate extract exhibited 12 mm zone of inhibition against this organism. The chloroform extracts of immature (12 mm), midmature (14 mm) and mature (12 mm) fruits inhibited the growth of *S. typhi*. Mature aqueous (13 mm) and ethyl acetate (11 mm) extracts also displayed the inhibitory zone against *S. typhi*. Aqueous extract displayed a mild inhibition against *A. niger* (11 mm) and *A. flavus* (8 mm). The zone of inhibition was observed to be 14 mm in midmature ethyl acetate extract against *A. flavus*.

The ethanol and methanol fruit extracts displayed an inhibitory zone against *E. coli* whereas, immature ethanol and immature methanol extracts showed 15 mm and 12 mm zone of inhibition respectively. Similarly, midmature ethanol and mature methanol extracts displayed 14 mm, 12 mm zone of inhibition respectively. *K. pneumoniae* was found to be susceptible to all the methanol and ethanol fruit extracts. Immature methanol, midmature and mature ethanol extracts displayed 17 mm, 15 mm, 17 mm zone of inhibition respectively. In midmature and mature methanol extracts, the zone of inhibition was found to be 18 mm and 12 mm respectively against *K. pneumoniae*. *S. typhi* was inhibited by mature ethyl acetate (16 mm) and methanol (16 mm) fruit extracts, whereas 13 mm and 14 mm inhibition zone was observed in immature and midmature ethanol extracts respectively. The zone of inhibition was found to be 16 mm in immature and 11 mm in mature ethanol extracts against *A. niger* and *A. flavus* respectively.
Figure 2. Antimicrobial activity of extracts of *M. tinctoria* fruits at different maturity stages (1-aqueous; 2-ethanol; 3-methanol; 4-ethyl acetate; 5-chloroform)

**Discussion**

Standardization is very much essential in order to assess the purity, quality control and identification of the sample. Determination of different physicochemical parameters is most important for the standardization of drug and establishing its pharmacological efficacy. Hence these studies help in identification and authentication of the plant material \cite{15, 16}. In the present study, the physicochemical parameters were determined in order to develop the standardization of *M. tinctoria* fruits. Physicochemical studies such as, ash values, moisture content and extractive values were determined. Ash values were used to detect the presence of siliceous contamination. Ash value which is simply represents inorganic components naturally occurring in crude drug, and also various impurities like carbonate, oxalate, and silicate \cite{17}. Lower ash value indicates its suitability to be used as a medicine. In this work, the ash values are of fruits were found to be in low percentage which is helpful for the identification of plant materials. Moisture content of drugs could be at minimal level to discourage the growth of bacteria, yeast, or fungi during storage, as the general requirement for moisture content in crude drug is not more than 14% w/w \cite{18}. In this study, the percentage of moisture content on all the maturity stages were found to be less than 14%, thus by ensuring the contamination will not occur during the period of storage. Extractive values are useful to evaluate the chemical constituent of drugs \cite{17}. It is also useful in determination of specific constituents soluble in particular solvents. In the present investigation, the water soluble extractive values were found to be 26.5% in immature fruits which shows the presence of polar compounds. Ethanol soluble extractive values are high in immature fruits which indicate the higher solubility of the chemical constituents in that particular solvent. Chloroform soluble extractive values were found to be 14% which indicates the presence of semi polar constituents in the fruits.

The medicinal plants are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, steroids, tannins and saponins. The therapeutic value of the medicinal plants lies in the secondary metabolites present in it. Recently a number of plants have been reported to have antimicrobial potential \cite{19, 20}. In the present investigation, phytochemical analysis conducted on the different maturity stages of fruit extracts showed the presence of various phytochemicals such as alkaloids, glycosides, flavonoids and steroids which are known to possess therapeutic values. Many of the tannin containing plants are used in medicine as astringent. It can be employed for the treatment of burns as they precipitate the proteins of exposed tissue to form a protective covering. It has been found to have antiviral, antibacterial, anti-inflammatory, antiulcer, and antioxidant properties for therapeutic applications \cite{21}. In our findings, the presence of tannins was observed in all the fruit extracts of various solvents. Saponins are considered as a key ingredient in Chinese medicine and responsible for many biological effects which include, antimicrobial, anti-inflammatory, and hemolytic effects \cite{22}. Similarly, saponins were found to be present in aqueous and methanol extracts of fruits thus by supporting our findings on the usefulness of *M. tinctoria* fruits in therapeutic applications. Alkaloid which is one of the major phytochemical groups in plants has remarkable effect on humans and they possess anti-inflammatory and anti-asthmatic properties \cite{23}. We confirmed the presence of alkaloids in all the maturity stages of fruits in...
aqueous, ethanol and methanol extracts in our study. Flavonoids are the major group of phenolic compounds which are reported for their antiviral, antimicrobial and spasmylytic activity. They are significant for prevention of diseases associated with oxidative damage of membrane, proteins and DNA. In human diet, flavonoids may diminish the risk of various cancers. They are the potent water soluble antioxidants and free radical scavengers, which put off the oxidative cell damage and have strong anti-cancer activity. Steroids are known to be important for their cardiotonic activities. Steroids have been reported to have antibacterial properties. Glycosides are known to lower the blood pressure. The presence of steroids and glycosides were observed in ethanol and methanol extract of fruits in the present study. Overall, in the present study, the phytochemical analysis exhibited the presence of diverse phytochemical components which are supporting the antimicrobial efficacy of different maturity stages of *M. tinctoria* fruits.

The increased occurrence of resistance to commonly used antibiotics, leads to the search for new, effective and inexpensive drugs in the management of infectious diseases. Many higher plant producing extractable organic substances which can be economically useful as pharmaceuticals. In this work, antimicrobial efficacy of *M. tinctoria* fruits was studied. As a source for the extraction of the metabolites, we used the aqueous, ethanol, methanol, ethyl acetate and chloroform solvents. Among the various source, ethanol and methanol extracts showed high degree of antimicrobial activity against selected pathogens. We observed the antimicrobial activity against *K. pneumoniae*, *S. typhi*, *E. coli*, *A. niger* and *A. flavus* in different maturity stages of *M. tinctoria* fruits in our study. Extracts were quantitatively assessed on the basis of zone of inhibition. All the fruit extracts studied in present investigation showed varying degree of inhibitory effect against selected pathogens. The inhibitory effect of this medicinal plant on microorganisms may be due to the presence of the phytochemical components. There are reports that plants rich in tannins have potent antimicrobial activity due to their character, which allows them to react with proteins to form stable water soluble compounds thereby inhibiting the growth of bacteria by directly damaging its cell membrane. Possibly this may be the mechanism of action of ethanol and methanol extract, which also showed the presence in phytochemical analysis in our study. Flavonoids and saponins have also been reported to have antimicrobial properties. *K. pneumonia* is an important cause of human infectious and several diseases such as, urinary tract infections, nosocomial infections, pneumonia and soft tissue infections. The disease caused by *K. pneumonia* can result in death of patients who are immunodeficient. In this study, ethanol and methanol extract of *M. tinctoria* fruits at different maturity stages displayed antibacterial activity against the *K. pneumonia*. It is suggested that the fruits can be used to treat urinary tract infections, nosocomial infections, pneumonia and soft tissue infections. The pathogen *S. typhi* is known to cause fever and food borne illness. In our experiments, chloroform, ethanol and methanol extracts of fruits of *M. tinctoria* showed the inhibitory activity against bacteria *S. typhi* and thus by confirms the presence of active constituents in the fruits. Virulent strains of *E. coli* can cause neonatal meningitis, urinary tract infections and gastroenteritis. In our study, ethanolic extract of fruits of *M. tinctoria* displayed the inhibitory activity against *E. coli*. It is suggesting that, the fruits can be used to treat urinary tract infections and neonatal meningitis. The antifungal activity of ethanol, methanol, aqueous, ethyl acetate and chloroform extracts of *M. tinctoria* fruits were evaluated by measuring the zone of inhibition. Antifungal activity denoted that the tested fungal strains are susceptible to ethanol extract. Thus the present study confirms the potential value of *M. tinctoria* fruits by the presence of various compounds.

In conclusion, physicochemical studies would be helpful in identification and authentication of the *M. tinctoria* fruits. The presence of various secondary metabolites such as, flavonoids, tannins, saponins, and alkaloids in different maturity stages of fruits conform the antimicrobial efficacy against selected pathogenic organism. Hence, it can be concluded that, the different maturity stages of *M. tinctoria* fruits would direct to the establishment of some compounds that could be used to invent new and more potent antimicrobial drugs of natural origin.

### References


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