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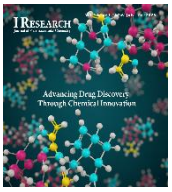

In-vitro Antioxidant and Anti-Haemolytic Activity of the Ethanolic Extract of *Morinda citrifolia L.*

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ABSTRACT

Morinda citrifolia L. (Rubiaceae), commonly known as noni, is a small evergreen tree traditionally used across the Indian coastline and the wider Indo-Pacific region for a broad range of ailments. The present study evaluated the preliminary phytochemical profile, in vitro antioxidant activity, and in vitro anti-haemolytic activity of the ethanolic extract of the leaves and fruits of *M. Citrifolia*, following authentication of the plant material by the Department of Botany, The American College, Madurai. The extract was obtained by Soxhlet extraction and screened qualitatively for major phytochemical classes. Antioxidant activity was assessed by the potassium ferricyanide reducing-power method, and protection of human erythrocytes against hydrogen-peroxide-induced haemolysis was assessed using quercetin as the reference standard. Preliminary screening indicated the presence of steroids, cardiac glycosides, phenolics, terpenoids, resins, carbohydrates, flavonoids, reducing sugars and saponins, with alkaloids and anthraquinones absent. The ethanolic extract exhibited concentration-dependent reducing power, although lower than that of ascorbic acid, and showed a concentration-dependent reduction in hydrogen-peroxide-induced haemolysis of erythrocytes, comparable in trend to quercetin. These findings support the traditional use of *M. citrifolia* as a source of natural antioxidant and membrane-protective constituents, and provide a basis for further isolation and in vivo evaluation of its bioactive phytoconstituents.

Introduction

Medicinal plants have formed the foundation of traditional healthcare systems for millennia and continue to be an important source of lead compounds for modern drug discovery. Plants synthesise a wide range of secondary metabolites — alkaloids, flavonoids, terpenoids, glycosides, and phenolic compounds — many of which underlie the antimicrobial, anti-inflammatory, antioxidant and anticancer properties reported for medicinal species.

Morinda citrifolia L. (Rubiaceae), widely known as noni or Indian mulberry, is a small evergreen tree distributed throughout the tropical Indo-Pacific, including the coastal belts of Tamil Nadu, Karnataka and Kerala. It has a long history of use in Polynesian and South and Southeast Asian traditional medicine for a wide range of conditions, and contemporary reviews describe reported antibacterial, antiviral, antifungal, anti-inflammatory, hypotensive, anticancer and immune-enhancing effects across its fruit, leaf, root and stem-bark preparations.

Recent phytochemical and pharmacological reviews attribute much of this activity to the plant's flavonoid (kaempferol, rutin), iridoid (asperuloside, asperulosidic acid) and phenolic (scopoletin) constituents [1,2,3]. Antioxidant activity in particular has been reported for extracts of the leaf, fruit and root of *M. citrifolia* using a range of solvents and assay systems, with reported activity broadly comparable to standard antioxidants such as ascorbic acid and vitamin C under specific extraction conditions [4]. Free-radical-mediated oxidative damage to the erythrocyte membrane is a recognized model for evaluating the biological relevance of plant antioxidants, since protection against hydrogen-peroxide- or peroxyl-radical-induced haemolysis reflects the ability of an extract to intercept reactive oxygen species at a lipid membrane interface [8].

Despite this body of literature, systematic reports combining preliminary phytochemical screening with both a reducing-power-based antioxidant assay and an erythrocyte-based anti-haemolytic assay on the ethanolic extract of *M. citrifolia* collected from the Madurai region are limited. The present work was therefore undertaken to (i) authenticate and extract the plant material, (ii) carry out preliminary qualitative phytochemical screening and thin-layer chromatography, (iii) determine total phenolic and flavonoid content, and (iv) evaluate the in vitro

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antioxidant and anti-haemolytic activity of the ethanolic extract.

Plant Profile

Botanical name: *Morinda citrifolia* L.

Family: Rubiaceae

Common names: Noni, Indian mulberry

(English); Manjanathi, Nuna (Tamil).

M. citrifolia is a small evergreen tree or shrub, 3–10 m in height at maturity, found up to about 400 m above sea level and often thriving near volcanic soils, pastures, coastal forests and waste ground. The leaves are opposite, glossy and pinnately veined (20–45 cm long); the flowers are small, white and borne in ovoid to globose heads; and the fruit is a fleshy, yellowish-white syncarp, 5–10 cm long, that becomes soft and pungent on ripening. The species is distributed throughout tropical regions worldwide and is common along the coastlines of Tamil Nadu, Karnataka and Kerala.

Fig. 1. Morinda citrifolia L. — Leaves and Developing fruit.

Materials and Methods

Collection and Authentication of Plant Material

Fresh leaves and fruits of *M. citrifolia* were collected locally and identified as *Morinda citrifolia* (family Rubiaceae) by Dr. D. Stephen, Assistant Professor, Department of Botany, The American College, Madurai, who issued an authentication certificate for the specimen used in this study.

Dr. D. STEPHEN, M.Sc., Ph.D.,
ASST. PROFESSOR,
DEPARTMENT OF BOTANY

THE AMERICAN COLLEGE,
MADURAI - 625 002.
Mobile No: 9944793299

AUTHENTICATION CERTIFICATE

This is to certify that the plant specimen brought to me by the Final year B.Pharm students M. THARALESHWARI (Reg.No: 560020509181), K. THEIVANAI (Reg.No:560020500584), I. THEMBAVANI (Reg.No: 560031000585), G.THIRUMOORTHY RAGAVAN (Reg.No. 560020500586), M. UMADEVAN (Reg.No: 560020508587), Department of Pharmaceutical Chemistry, K.M.College of Pharmacy, Uthangudi, Madurai, has been identified as *Morinda citrifolia*, belongs to the family **Rubiaceae**.

Date :
Place :

Dr. D. STEPHEN, M.Sc., Ph.D.,

Fig. 2. Authentication certificate issued by the Department of Botany, The American College, Madurai.

Extraction

Shade-dried leaves and fruits were pulverised to a coarse powder using a mechanical grinder. The powdered material was packed into a cellulose extraction thimble and subjected to continuous hot extraction in a 1 L Soxhlet apparatus using approximately 400 mL of ethanol as solvent, at a bath temperature of 50–60 °C for 18 hours. The resulting extract was concentrated by distillation of the solvent under reduced conditions at approximately 20 °C, and the concentrated ethanolic extract was stored in an airtight container at 4 °C until further use.

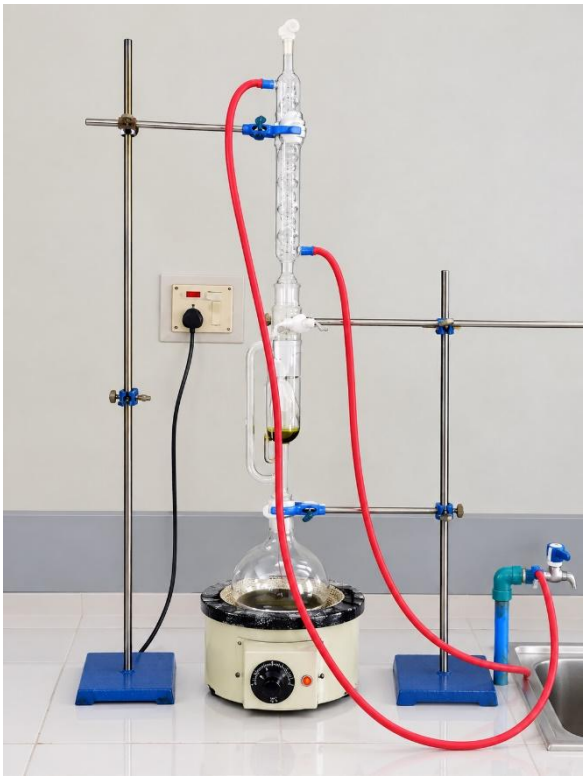

Fig. 3. Soxhlet extraction setup used for ethanolic extraction of M. citrifolia leaves and fruits.

Preliminary Qualitative Phytochemical Screening

The ethanolic extract was screened for the presence of major phytochemical classes using standard qualitative tests: Liebermann–Burchard reaction (steroids), Keller–Kiliani test (cardiac glycosides), ferric chloride test (phenols and tannins), Salkowski test (terpenoids), Mayer's test (alkaloids), acetic anhydride/sulphuric acid test (resins), Molisch's test (carbohydrates), ammonia/sulphuric acid test (flavonoids), Borntrager's test (anthraquinones), a reducing-sugar test, and the froth test (saponins), following established phytochemical methods.[9,10]

Thin-layer Chromatography

Thin-layer chromatography was performed on silica-gel-coated glass plates using n-butanol : acetic acid : distilled water as the mobile phase, with gallic acid as the reference standard. Spots were visualised after spraying with ferric

chloride–α-naphthol reagent, and retention factor (Rf) values were calculated as the ratio of the distance travelled by the compound to the distance travelled by the solvent front.

Total Phenolic Content

Total phenolic content was estimated colorimetrically using potassium ferricyanide and ferric chloride, with gallic acid as the standard, following the general principle of the Folin–Ciocalteu-type colorimetric approach to phenolic estimation. Absorbance was recorded at 680 nm against a reagent blank.[11]

Total Flavonoid Content

Total flavonoid content was determined by the aluminium chloride colorimetric method, using quercetin as the reference standard and measuring absorbance at 510 nm after colour development.

***In vitro* Antioxidant Activity (Reducing Power Assay)**

The reducing power (ferric-reducing antioxidant) capacity of the extract was determined by the potassium ferricyanide method. Different volumes (0.5–2.5 mL) of the ethanolic extract and of the ascorbic acid standard were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, vortexed and incubated at 50 °C for 20 minutes. Trichloroacetic acid (10%, 2.5 mL) was added to terminate the reaction, and the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was mixed with deionised water and 0.1% ferric chloride, and the absorbance of the resulting bluish-green complex was read against a blank using a UV–visible spectrophotometer. An increase in absorbance indicates an increase in reducing power and, by extension, antioxidant activity.[12]

***In vitro* Anti-Haemolytic Activity**

Anti-haemolytic activity was assessed using a hydrogen-peroxide-induced erythrocyte haemolysis model. A 4% erythrocyte suspension was prepared in phosphate-buffered saline (0.2 M, pH 7.4). To 2 mL of this suspension, increasing volumes of the ethanolic extract (or of quercetin as reference standard) were added and the volume made up to 5 mL with buffered saline; the mixture was incubated for 5 minutes at room temperature before the addition of 0.5 mL H_2O_2 solution to induce oxidative membrane damage. Tubes were centrifuged at 1000×g for 10 minutes and the absorbance of the supernatant was read at 580 nm. Complete (100%) haemolysis was defined using a distilled-water control. Percentage haemolysis was calculated as:

$$\% \text{ Haemolysis} = [(\text{Absorbance of control} - \text{Absorbance of extract}) / \text{Absorbance of control}] \times 100$$ [8,13]

Results

Phytochemical screening

Table 1: Summarises the results of qualitative phytochemical screening of the ethanolic extract.

Chemical test	Result	Inference
Liebermann–Burchard reaction	+ve	Presence of steroids
Keller–Kiliani test	+ve	Presence of cardiac glycosides
Ferric chloride test	+ve	Presence of phenolic compounds / tannins
Salkowski test	+ve	Presence of terpenoids
Mayer's test	−ve	Absence of alkaloids
Resin test	+ve	Presence of resins
Molisch's test	+ve	Presence of carbohydrates
Flavonoid test	+ve	Presence of flavonoids
Borntrager's test	−ve	Absence of anthraquinones
Reducing sugar test	+ve	Presence of reducing sugars
Froth test	+ve	Presence of saponins

Steroids, cardiac glycosides, phenolic compounds, terpenoids, resins, carbohydrates, flavonoids, reducing sugars and saponins were detected, while alkaloids and anthraquinones were not detected under the conditions tested.

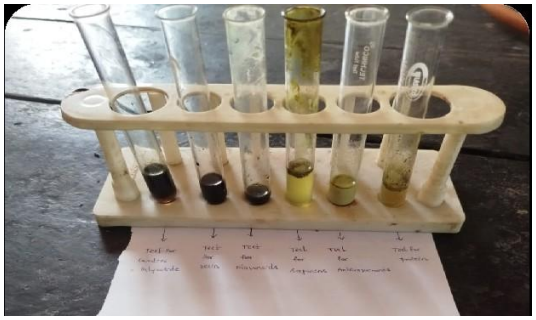

Fig. 4. Colour reactions obtained during preliminary phytochemical screening of the ethanolic extract.

Thin-layer Chromatography

Under the mobile phase used (n-butanol : acetic acid : water), the test spot showed an Rf value of 0.84 (distance travelled by compound / solvent front = 5.5/6.5), compared with an Rf of 0.92 for the gallic acid standard (6.0/6.5).

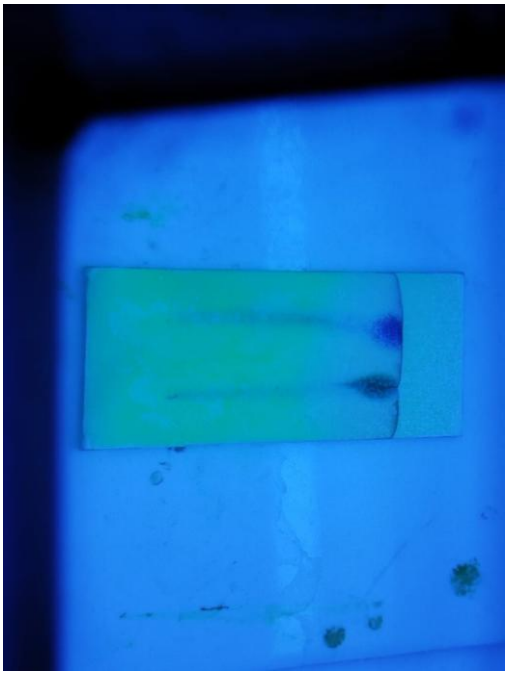

Fig. 5. TLC plate of the ethanolic extract (left band) and gallic acid standard (right band) visualized under UV light after derivatization.

Total Phenolic and Flavonoid Content

Using the recorded absorbance values at 680 nm (blank 0.29, standard 0.31, sample 0.35), the total phenolic content of the extract, expressed relative to the gallic acid standard, was calculated as 0.9 mg gallic acid equivalents (GAE) per mL of extract. For total flavonoid content, calculations from the NaOH-developed reaction (blank 1.52, standard 1.36, sample 1.22) and the corresponding aqueous reaction (blank 1.49, standard 1.33, sample 1.32) gave values of 0.0375 mg and 0.02125 mg quercetin equivalents (QE) per mL of extract, respectively.

***In vitro* Antioxidant (Reducing Power) Activity**

Table 2: shows the absorbance (480 nm) of the standard (ascorbic acid) and the test ethanolic extract across increasing volumes.

Sample	0.5 ml	1.0 ml	1.5 ml	2.0 ml	2.5 ml
Standard (Ascorbic acid)	0.16	0.18	0.21	0.22	0.28
Test (Ethanolic extract)	0.18	0.19	0.19	0.19	0.21

The standard (ascorbic acid) showed a concentration-dependent increase in absorbance from 0.16 to 0.28, confirming progressively increasing reducing power. The ethanolic extract of *M. citrifolia* showed a smaller but still concentration-related increase in absorbance (0.18 to 0.21), indicating measurable but comparatively lower reducing power than the standard across the concentration range tested.

***In vitro* Anti-Haemolytic Activity**

Table 3: presents percentage haemolysis for quercetin (standard) and the ethanolic extract of *M. citrifolia* (test) with increasing concentration.

Concentration (mL)	log (Conc.)	Standard (quercetin) % haemolysis	Test (*M. citrifolia* extract) % haemolysis
0.5	−0.3010	87	70
1.0	0.0000	80	71
1.5	0.1760	82	68
2.0	0.3010	80	66
2.5	0.3979	78	62

With this caveat noted, both the standard and the test extract showed a broadly concentration-dependent decrease in percentage haemolysis, i.e. increasing protection of the erythrocyte membrane against H2O2-induced oxidative damage as concentration increased. The ethanolic extract of *M. citrifolia* showed lower percentage haemolysis than quercetin at every concentration tested, indicating comparatively strong membrane-protective (anti-haemolytic) activity for the extract under these conditions.

Discussion

The phytochemical screening confirms that the ethanolic extract of *M. citrifolia* is rich in steroids, cardiac glycosides, phenolic compounds, terpenoids, flavonoids and saponins, and is consistent with previous phytochemical surveys of noni leaf and fruit extracts, which similarly report flavonoid, iridoid and phenolic acid constituents as the principal contributors to the plant's reported bioactivity.[1,2,3]

The reducing-power assay demonstrated that the ethanolic extract of *M. citrifolia* possesses concentration-dependent antioxidant activity, consistent with reports of antioxidant activity in noni leaf, fruit and root extracts using a range of solvents and assay systems, though the magnitude of activity observed here was lower than that of the ascorbic acid standard, in keeping with the general

pattern reported for crude ethanolic noni extracts relative to purified reference antioxidants.[4]

In the anti-haemolytic assay, the extract reduced hydrogen-peroxide-induced haemolysis of erythrocytes in a concentration-dependent manner, and compared favourably with quercetin, a well-characterised membrane-protective flavonoid used as reference standard in erythrocyte-based antioxidant assays. This protective effect is most plausibly attributed to the phenolic and flavonoid constituents identified in the phytochemical screening, which can intercept reactive oxygen species before they initiate lipid peroxidation of the erythrocyte membrane.[8]

Taken together, these results are consistent with the wider literature on *M.citrifolia*, which reports antibacterial, antifungal and antioxidant activity for extracts of its leaf, fruit, root and stem bark, and support the traditional use of the plant as a source of natural antioxidant principles.[5,6,7]

The present study is limited to in vitro assays on a crude ethanolic extract; the specific phytoconstituents responsible for the observed effects were not isolated or quantified individually, and the sample size for each assay was limited to a single extract batch without biological replicates. Further work should therefore include bioassay-guided fractionation to identify the active constituents, dose–response and IC50 determination, and confirmation of antioxidant and cytoprotective activity in an in vivo model.

Conclusion

The ethanolic extract of *Morinda citrifolia L*. and fruits was found to contain steroids, cardiac glycosides, phenolic compounds, terpenoids, flavonoids, reducing sugars and saponins, and demonstrated concentration-dependent in vitro antioxidant (reducing power) activity and in vitro protection of erythrocytes against hydrogen-peroxide-induced haemolysis. These findings support further investigation of *M. citrifolia* as a source of natural antioxidant and membrane-protective agents, with scope for future work on isolation of the responsible constituents and in vivo validation.

Acknowledgement

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