BIOASSAY- GUIDED ISOLATION OF NICOTIFLORIN FROM *MORINDA TINCTORIA* ROXB.

Atish K. Sahoo[a]*, Raj K. Hota[b]

[a]*M. S. Ramaiah College of Pharmacy (M.S.R.C.P.), Department of Pharmaceutical Chemistry, M. S. Ramaiah College of Pharmacy, M. S. R. Nagar, M. S. R. I. T Post, Bangalore- 560 054, Karnataka, India.

[b] Institute of Technical Education and Research (ITER), Bhubaneswar-751030, Orissa, India.

Summary

Based on the results of antioxidant activity of ethyl acetate extracts (EAMT); chloroform (CHMT) and n-butanol (BUMT) extracts of *Morinda tinctoria*, bioassay guided isolation of the ethyl acetate extract of *M. tinctoria* leaves was carried out by silica gel column chromatography for its potent antioxidant activity. It afforded a light yellowish powder, which was identified as nicotiflorin. This compound exhibited antioxidant activity (IC_{50} values of 28.12 ± 2.72 µg/ml and 46.43 ±1.51 µg/ml in DPPH and nitric oxide scavenging assay respectively) which was comparable to rutin, taken as a standard.

key words: *Morinda tinctoria* Roxb., Nicotiflorin, Antioxidant activity, Rutin

*Correspondence:

Dr. Atish Kumar Sahoo
Associate Professor,
M. S. Ramaiah College of Pharmacy (M.S.R.C.P.),
Department of Pharmaceutical Chemistry,
M. S. R. Nagar, M. S. R. I. T Post, Bangalore- 560 054,
India.
Phone: +91-80-23607537
Fax: +91-80-23607537
Email: atish_kumar1976@yahoo.co.in

Introduction

Morinda tinctoria Roxb., (Family Rubiaceae); commonly known as Aal or Indian Mulberry, a small or, middle sized tree, is extensively cultivated in India. It has been reported that African aborigines have used Indian Mulberry medicinally. Traditionally the Nakkala tribe in South India use the decoction of garlic mixed with *Andrographis paniculata* and *Morinda tinctoria* root for fever (1). The leaves have been used for various purposes traditionally for e.g. charred leaves made into a decoction with mustard is a favorite domestic remedy for infantile diarrhoea, expressed juice of leaves is externally applied for gout to relieve pain, and leaves are administered internally as a tonic and febrifuge (2). Besides its leaf juice is given to children before food for easy digestion (3). Phytochemical screening of the extracts showed the presence of flavonoids, tannins, steroids in the ethyl acetate extract. Based on the previous report on traditional usage and phytochemical screening of the extracts, the present study has undertaken to isolate and evaluate its *in vitro* antioxidant activity through High throughout screening (HTS).

Materials and Methods

Chemicals and Instruments

All chemicals were of analytical and highest purity and were purchased from Sigma Chemical Co. (St., Louis, USA), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA). Hi-Media, India Ltd. and, Merck Co. (Germany). The melting point was determined using a Kofler micro-hotstage melting-point apparatus. IR spectroscopy was performed on a JASCO- 410 FT-IR spectrophotometer, using KBr disks. ^{1}H-NMR (500 MHz) spectra were recorded on a Bruker AM600 FT-NMR spectrometer with tetramethylsilane (TMS) as internal standard. EIMS (The negative ion high resolution EIMS spectrum) was obtained using was taken in Shimadzu-qp-1000 (Quadraphor) Mass Spectrometer.

Plant materials

The leaves of *Morinda tinctoria* Roxb. (Family Rubiaceae); were collected from hilly areas of Ooty, Tamil Nadu and the authenticity of the leaf specimen was confirmed through Government Arts College, Ooty, Tamil Nadu. A voucher specimen has been kept in our laboratory for future references.

Preparation of the extracts of M. tinctoria

The extraction process was based on the method of (4) with a slight modification. The air-dried leaves (2 kg) were roughly grounded and defatted by soxhlet extraction with hexane. Then the dried plant material was macerated with 80% methanol and the macerated materials were evaporated to dryness (26%, w/w). Then the dried extract was dissolved in distilled water and filtered after 24 h. The filtrate was successively separated with chloroform (3%, w/w); ethyl acetate (4%, w/w); and n-butanol (6%, w/w). The ethyl acetate extracts (EAMT); chloroform (CHMT) and n-butanol (BUMT) extracts of MT were obtained and kept in a desiccator for future use.

Determination of Total phenolic content

Total soluble phenolics in EAMT of *M. tinctoria* were determined with Folin–Ciocalteu reagent according to the method of (5). Briefly, extract solution was transferred into 100 ml Erlenmeyer flask then final volume was adjusted to 46 ml by addition of distilled water. Afterward, 1 ml of Folin–Ciocalteu reagent (FCR) was added into this mixture and after 3 min 3 ml of Na_2CO_3 (2%) was added. Subsequently, mixture was shaken on a shaker for 2 h at room temperature and then absorbance was measured at 760 nm. The concentration of total phenolic compounds in different extracts of *M. tinctoria* was determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol graph. The equation is given below;
Absorbance = 0.001 x Pyrocatechol (µg) + 0.0033.

Determination of free radical scavenging activity

1. DPPH radical scavenging activity
The free radical scavenging capacity of the extracts was determined using DPPH. A methanol DPPH-solution (0.15%) was mixed with serial dilutions (10, 20, 40, 60, 80 and 100 µg/ml) of fractions CHMT, EAMT and BUMT of *M. tinctoria.* Because of the better enzyme inhibitory activity, the concentration range of different extracts of *M. tinctoria* was considered for this assay. After 10 min the absorbance was read at 515 nm using a spectrophotometer (Perkin-Elmer). The % of inhibition was calculated by using following equation;

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The inhibition curve was plotted and IC_{50} values obtained (6, 7). Rutin was used as a standard.

2. Inhibition of nitric oxide radical
Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (8, 9). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the drug in different concentrations CHMT, EAMT and BUMT of *M. tinctoria* was incubated at 25 ^{0}C for 150 min. At intervals samples (0.5 ml) of incubation solution were removed and 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was measured at 546 nm. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test compounds. The % of inhibition was calculated by using following equation;

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

3. Column chromatography of the ethyl acetate extract of M. tinctoria

A glass column, 75 cm in length, 5.5 cm in side diameter fitted with a stopcock was used. Silica gel 230-400 mesh size, 0.040-0.063 mm (E. Merck and Co. Ltd) was activated by heating at 120 ºC for one hour and was used as adsorbing material. The solvent system of chloroform: ethyl acetate (10:0 to 0:10) was used. The ethyl acetate fraction (9 g) was subjected to chromatography on a silica gel column using gradient elution of chloroform: ethyl acetate (10:0 to 0:10) (chloroform: 10; chloroform-ethyl acetate: 9:1; 8:2; 7:3, 6:4; 5:5; 4:6; 3:7; 2:8; 1:9 v/v; ethyl acetate:10). Samples were collected and monitored by TLC (n-butanol-acetic acid-water; 4:1:5; UV-254 nm). Similar samples were combined to three fractions (Fr I: 27-35; Fr II: 48-63; Fr III: 67-77). Each fraction was tested for *in vitro* antioxidant activity. Among these fractions, Fraction I showed the most significant *in vitro* antioxidant activity by DPPH and nitric oxide assay. Fraction I was further purified to yield compound 1 (0.00445%; w/w) a yellowish powder which subsequently studied for its antioxidant activity. Rutin was used as standard in this work.

Statistical Analysis

The data on all antioxidant activity tests are the average of triplicate analyses. Statistical analysis of variance was performed by one way ANOVA procedures followed by Dunnett's test. Values of P-values <0.05 were regarded as significant, P-values <0.01 regarded as very significant and P-values <0.001 regarded as most significant. IC_{50} values were calculated from the concentration-effect linear regression curve.

Results

1. Total phenolic content

In the *M. tinctoria* leaf fraction, the total phenolic content was found out. The EAMT (198.77 µg/mg; 19.87%; w/w), CHMT (109.3 µg/mg, 10.93%; w/w) and BUMT (87.8 µg/mg; 0.87%; w/w) of *M. tinctoria;* which are pyrocatechol equivalent.

2. DPPH radical scavenging activity

The percentage inhibition of DPPH radicals by CHMT, EAMT and BUMT fractions of methanol extract leaves of *M. tinctoria* was shown in Table 1. Rutin was used as a standard and its IC_{50} value determined to be 25.55 µg/ml, whereas the IC_{50} value of CHMT, EAMT and BUMT was found to be 98.96 µg/ml, 56.49 µg/ml and 66.55 µg/ml respectively as shown in Table 1. EAMT has shown a significant (P <0.01) inhibition of enzymes which comparable with rutin.

3. Inhibition of nitric oxide radical

Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by CHMT, EAMT and BUMT extract of leaves of *M. tinctoria* (Table 1), where rutin was used as a reference compound and its IC_{50} value was found to be 26.65 µg/ml. The concentration of CHMT, EAMT and BUMT needed for 50% inhibition was found to be 260.44 µg/ml, 194.99 µg/ml and 222.41 µg/ml respectively.

Table 1. Inhibitory effect (IC_{50}) extracts of *M. tinctoria* on DPPH, nitric oxide scavenging *in vitro* antioxidant assay.

Parameters	**CHMT (µg/ml)**	**EAMT (µg/ml)**	**BUMT (µg/ml)**	**Standard (µg/ml)**
DPPH radical scavenger	98.96*± 3.87	56.49**± 1.83	66.55**± 2.14	25.55±0.85 (rutin)
Nitric oxide radical scavenger	260.45**± 8.78	194.69**± 10.32	222.42**± 14.46	26.65± 2.14(rutin)

Chloroform fractions (CHMT), ethyl acetate fraction (EAMT) and n-butanol extracts of *M. tincoria* (BUMT). *Represents P<0.05 and **Represents P<0.01. Statistical analysis of variance was performed by one way ANOVA procedures followed by Dunnett's test. All groups CHMT, EAMT and BUMT were compared with standard drug rutin.

4. Physico–chemical characterization of compound-1

Compound-I: Yellow powered material; odourless; It was practically insoluble in water, freely soluble in ethyl acetate and methanol; m.p.178-180°C.

R_f= 0. 39; BAW; n-butanol: acetic acid: water (4:1:5, v/v).

UV (MeOH): Absorbtion peaks 257-358nm arising from the flavonols and it is suggested that the glycosidation of the C-3 (OH) position.

5. Spectral data of compound-1

The IR spectra were recorded in the region of 4000 cm-1 to 550 cm^{-1}.

Compound-1showed absorption bands at γ = 3400, 1710, 1670, 1510, 1441, 1276, 1192, 1010, 885 cm^{-1}; indicated the presence of hydroxyl group (OH_{st} 3400 cm^{-1}) and a double bond (-C=CH-$_{st}$ at γ= 1710 cm^{-1}) and also has shown the presence of carbonyl (-C=Ost 1670 cm^{-1}) group.

The compound-1 on Mass spectra was found to be the fragmentation pattern m/z 580(3), 287(53), 269(16), 259 (12), 245 (15).

The ^{1}H-NMR (500 MHz, $CDCl_3$) spectral data of compound-1 showed the presence of methyl group at δ 0.98 (3H, d, J= 7.5 Hz) shows the presence of methyl group at C-6 of rhamnose sugar moiety; δ 4.36 (1H, br, s, J= 7.11) shows the peak at C-1 of rhamnose sugar moiety; δ 5.30 (1H, d, J=8.0 Hz) the peak appears at C-1 of glucose moiety; δ 6.10 (1H, s, H-6) the peak appears at C-6; δ 6.35 (1H, s, H-8) the peak appears at C-8; δ 6.85 (2H, d, J=8.0 Hz, H-3 and H-5′) the peak appears at C-3 and C-5′; δ 7.98 (2H, d, J=8.0 Hz, H-2′ and H-6′) the peak appears at C-2′ and C-6′ at different ppm.

Table 2. *In vitro* antioxidant assay of ethyl acetate extract (EAMT), isolated fractions and nicotiflorin of *Morinda tinctoria* by DPPH and nitric oxide radical scavenging method, where rutin is considered as standard.

In Vitro assay	EAMT (µg/ml)	Fraction-I (µg/ml)	Fraction-II (µg/ml)	Fraction-III (µg/ml)	Compound-I (Nicotiflorin) (µg/ml)	Standard Rutin (µg/ml)
DPPH radical scavenger	56.49* ± 3.83	48.22* ± 4.74	97.45* ± 7.32	65.47* ± 4.45	28.12* ± 2.72	14.61± 0.75
Nitric oxide radical scavenger	174.69* ± 2.89	52.43* ± 2.51	159.02± 6.88	105.47* ± 3.25	46.43* ±3.51	23.55± 0.44

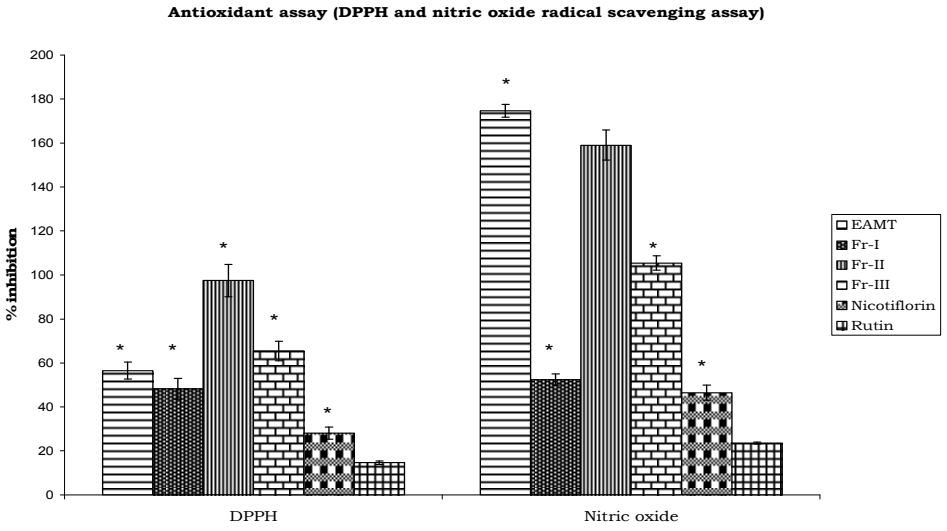
*****Represents P <0.05; the data on all antioxidant activity tests are the average of triplicate analyses. Statistical analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Student's t test, P-values <0.05 were regarded as significant. IC_{50} values were calculated from the concentration-effect linear regression curve. Nicotiflorin was compared with the standard drug rutin and the rest groups EAMT, fractions-I, II and III was compared with compound Nicotiflorin.

Discussion

M. tinctoria different leaf extracts was screened for total penolic contents, the EAMT has shown more %phenolic contents than the CHMT and BUMT extracts. The antioxidant result has shown that the DPPH and the nitric oxide scavenging activity of EAMT extract of *M. tinctoria* has shown potent enzyme inhibitory activity at its IC_{50}-value than the rest CHMT and BUMT extracts. Hence EAMT extract of *M. tinctoria* was considered for the isolation by column chromatography technique so as to find out the exact phytoconstituent which is responsible for the *in vitro* antioxidant activity which was further supported by the phytochemical screening as EAMT has shown the presence of phenols, flavonoids and saponins. By the column chromatography, Samples were collected and monitored by TLC (n-butanol-acetic acid-water; 4:1:5; UV-254 nm). Similar samples were combined to three fractions (Fr I: 27-35; Fr II: 48-63; Fr III: 67-77). Each fraction was tested for *in vitro* antioxidant activity. Among these fractions, Fraction I showed the most significant *in vitro* antioxidant activity by DPPH and nitric oxide assay. Fraction I was further purified to yield compound 1 (0.00445%; w/w) a yellowish powder. Form the Physicochemical characterization as well as spectral evidence compound-1 was confirmed as kaempferol-3-O-rutinoside (Nicotiflorin) (Fig 2); which further supports the literature (10, 11). Nicotiflorin was subsequently studied for its antioxidant activity where rutin was used as standard in this work. The results indicate that fraction I exhibited a greater antioxidant activity on DPPH and nitric oxide radicals than the ethyl acetate extract (EAMT) and other fractions, which were comparing with those of flavonoid glycosides (rutin). The IC_{50} values were shown in Table 2 and Fig

1**.** It is well known that the Nicotiflorin (flavan-3-ol) derivatives may act as potential antioxidants in plasma to modulate cellular oxidative stress and prevent the oxidation of low-density lipoprotein (LDL) (12, 13, 14). The flavonoidal glycosides (rutin) was reported to play an important role in the prevention of lipid peroxidation and cardiovascular disease (15, 16, 17), however, both rutin, fraction I and isolated phytoconstituent (Nicotiflorin) showed distinguished scavenging activity on DPPH and nitric oxide radicals in our work. A large number of these flavan-3-ol compounds are known to possess strong antioxidant properties, which further supports the plant *Morinda tinctoria* Roxb. antioxidant nature. Hence the scientific evaluation supports the long history of use of *Morinda tinctoria* various traditionally purposes for different disorders.

Fig. 1. *In vitro* antioxidant assay of EAMT, isolated fractions and nicotiflorin of *Morinda tinctoria* by DPPH and nitric oxide radical scavenging method, where rutin is considered as standard. Where, ethyl acetate extract of *M. tinctoria* is EAMT, and isolated fraction are fraction-I, II and III, nicotiflorin and rutin.



*****Represents P <0.05; The data on all antioxidant activity tests are the average of triplicate analyses. Statistical analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Student's t test, P-values <0.05 were regarded as significant. IC_{50} values were calculated from the concentration-effect linear regression curve. Nicotiflorin was compared with the standard drug rutin and the rest groups EAMT, fractions-I, II and III was compared with compound Nicotiflorin.

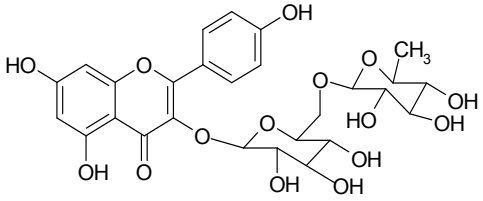


Fig 2. The isolated compound- I (Nicotiflorin)

References

1. Vedavathy S. Tribal medicine–the real alternative. Indian J Trad Knowledge 2002; 1: 25-31.
2. Kirtikar KR, Basu BD. Indian Medicinal Plants. Vol 2, 2nd ed. Bishen Singh Mahendra Pal Sing; 1935. pp. 1294-1295.
3. Muthu C, Allanar M, Raja N, Ignacimuthu S. Medicinal plants used by traditional healers in Kancheepuram District of Tamil Nadu, India. Indian J Ethnobiol Ethnomed 2006; 2: 43; doi: 10.1186/1746-4269-2-43.
4. Kanyanga C, Tess DB, Aleidis L, Qimin L, Luc P, Mangda C, Dirk Vanden B, Kabangu K, Lutete T, Arnold V. Flavonoid-O-glycosides from the leaves of *Morinda morindoides*. Phytochemistry 1995; 38: 1301-1303.
5. Slinkard K, Singleton VL. Total phenol analyses: automation & comparison with manual methods. Am J Enology Viticulture 1977; 28: 49-55.
6. Viturro C, Molina A, Schmeda HG. Free radical scavengers from *Mutisia friesiana* (Asteraceae) and *Sanicula graveolens* (Apiaceae). Phytother Res 1999; 13: 422– 424.
7. Mukherjee PK. Plant products with hypocholesterolemic potentials. Adv Food Nutr Res 2003; 47: 278-324.
8. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JK, Tannenbaum SR. Analysis of nitrate, nitrite and 15N in biological fluids. Anal Biochem 1982; 126: 131-136.
9. Raja S, Nazeer Ahamed KFH, Kumar V, Mukherjee K, Bandyopadhyay A, Mukherjee PK. Antioxidant effect of *Cytisus scoparius* against carbon tetrachloride treated liver injury in rats. J Ethnopharmacol 2007; 109: 41–47.
10. Chaurasia N, Wichtl M. Flavonolglykoside aus *Urtica dioca*. Planta Medica 1987; 53: 432-434.
11. Iwashina T, Konta F, Kitajima J. Anthrocyanins and flavonols of *Chimonanthus praecox* (Calycanthaceae) as flower pigments. J Jap Bot 2001; 76: 166-172.
12. Mangiapane H, Thomson J, Salter A, Brown S, Bell GD, White DA. The inhibition of the oxidation of low-density lipoprotein by (+)-catechin, a naturally occurring flavonoid. Biochem Pharmacol 1992; 43: 445-450.
13. Tijburg LB, Mattern T, Folts JD, Weisgerber UM, Katan MB. Tea flavonoids and cardiovascular diseases: a review. Crit Rev Food Sci Nutr 1997; 37: 771-785.
14. Yang CS, Landau JM. Effects of tea consumption on nutrition and health. J Nutr 2000; 130: 2409-2412.
15. Hertog MGL, Holmann PDH. Potential health effects of the dietary flavonol quercetin. Eur J Clin Nutr 1996: 50; 63-71.
16. Lekse JM, Xia L, Stark J, Morrow JD, May MJ. Plant catechols prevent lipid peroxidation in human plasma and erythrocytes. Molecular Cellular Biochemistry 2001; 226: 89-95.
17. Tripathi YB, Chaurasia S, Tripathi E, Upadhyay A, Dubey GP. *Bacopa monniera* Linn. as an antioxidant. Mechanism of action. Indian J Exp Biol 1996; 34: 523-526.