PHYTOCHEMICAL ANALYSIS AND *IN VITRO* GENOTOXICITY, CYTOTOXICITY, ANTIOXIDANT, ANTIMICROBIAL, ANTIOBESITY ACTIVITY OF *MORINDA TINCTORIA ROXB.*

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ABSTRACT

The medicinal plant *Morinda tinctoria* Roxb. is used in the treatment of various diseases traditionally. Report on bioactive evaluation of *M. tinctoria* Roxb. has led us to the phytochemical examination of *M. tinctoria leaves*. Our objective was to isolate compounds form *M. tinctoria* Roxb. and characterize each compound. The isolated compounds were tested for antioxidant, antimicrobial, antiobesity, cytotoxicity and genotoxicity properties. For this work, we have chosen solvents with increasing polarities. Hexane, chloroform, ethyl acetate and methanol were used in this study. The solvent extracts were analyzed using column chromatography and the isolated compounds were characterized using FTIR, ^{1}H NMR, ^{13}C NMR and mass spectra. The constituents have been characterized and screened for *in vitro* antioxidant, antimicrobial, antiobesity, cytotoxicity and genotoxicity properties. Nine bioactive compounds were isolated and identified: palmitic acid, fucoxanthine, broussin, isoliquiritigenin, cynarin, gallic acid, β-sitosterol, quercetin and oleuropein. The results thus indicate conclusively that *M. tinctoria* leaves possess various potent bioactive compounds and is therefore recommended as a plant of phytopharmaceutical importance.

Keywords: *Morinda tinctoria* Roxb, column chromatograph, Fucoxanthine, Broussin, Isoliquiritigenin, Cynarin, cytotoxicity and genotoxicity

INTRODUCTION

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is also an important cause of morbidity and mortality in immune compromised patients in developing countries[1]. In recent years, attempts have been made to investigate indigenous drugs against infectious diseases. This may help to develop safer antimicrobial drugs[2]. Some examples of plant secondary metabolites are shown in Fig.1, as bioactive materials continue to play a vital role in the maintenance of human health[3-4]. They are also the best sources for obtaining natural antioxidants for various medicinal uses such as aging and diseases related to radical mechanism, such as cancer[5].

Morinda tinctoria Roxb. (Family-Rubiaceae) is distinguished for its traditional use, phytochemical and therapeutic potentials. It is locally known as "Togaru" and is commonly known as Indian mulberry or "Aal" or "Nunaa" in India. *M. tinctoria* is a species of flowering plants and is native to southern Asia. Its synonyms are *Morinda tomentosa H*eyne and *M. pubescens*. The plant is extensively cultivated in India in order to make the morindone dye, sold under the trade name "Suranji". *M. tinctoria* Roxb.'s fruits, flowers, leaves and heartwood have been used in treating several diseases[6]. The leaves of *M. tinctoria* Roxb. have many biological properties like anti-convulsant, analgesic, anti-inflammatory, anti-oxidant, anti-microbial and cytoprotective activities[7-13]. The ashes of *M. tinctoria* leaves are also reported to act as biosorbents in controlling ammonia pollution in waste waters[14]. The leaves yielded ursolic acid[15] and polyphenolic compounds such as quercetin, kaempferol-3-*O*-rutinoside. Acacetine-7-*O*-β- D-glucopyranoside and apigenin 5,7-dimethylether 4'-galactoside have been reported from the leaves and flowers of *M. tinctoria* Roxb[16].

The leaves of *M. tinctoria* possess larvicidal activity[17]. Traditionally, the leaf juice is given orally to children before food for easy digestion[18]. The charred leaves made into a decoction with mustard seeds is a remedy for

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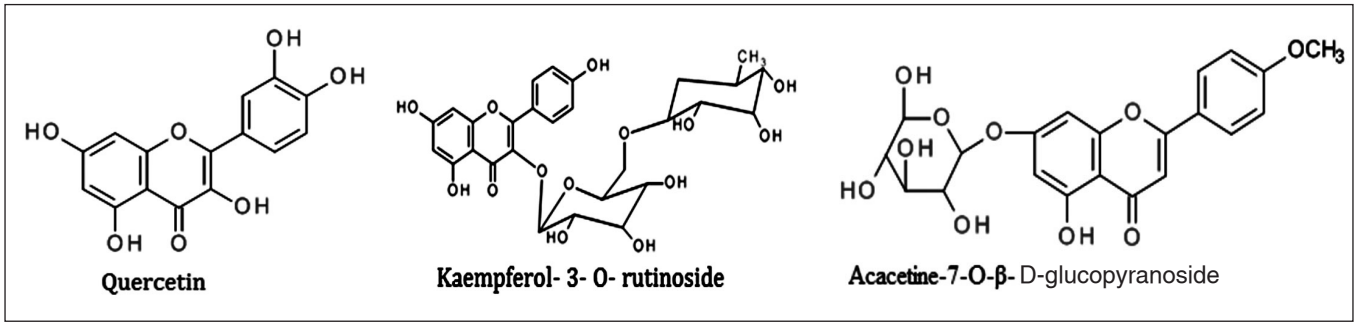

Fig. 1: Some secondary metabolites present in plants

infantile diarrhea. The juice of leaves is used internally as astringent and externally applied for gout and rheumatism. The hexane, dichloromethane and methanol extracts of the leaves were shown to possess antibacterial and antifungal activities[19]. The petroleum ether extract showed anticonvulsant activity[20]. Fruits are mashed and applied to boils[21]. Bark and Heartwood are reported to have antibacterial and anti-fungal activities[22]. *M. tinctoria* also possesses excellent fuel wood characteristics. The fruits and flowers of *M. tinctoria* have been reported to have wound healing properties in rats[23], antioxidant properties[24], antidiabetic, antihyperglycemic and hepatoprotective activities.

Encouraged by these medicinal properties of *M. tinctoria* leaves and as an extension to our work on *M. tinctoria* leaves[25-27], the authors have taken up studies to isolate the bioactive molecules from the leaves and study the phytochemical properties of isolated molecules from *M. tinctoria* and succeeded in isolating nine compounds from different extracts of the leaves. This is the first report on isolation of the compound their biological properties.

MATERIALS AND METHODS

Plant material

The leaves of *M. tinctoria* Roxb. were collected from GITAM University campus, Visakhapatnam, Andhra Pradesh, India. The specimen voucher of the plant material was deposited at Botany Department Herbarium, Andhra University, Visakhapatnam, Andhra Pradesh, India. The herbarium voucher number is A.U/B.D.H/21106.

Extraction

Dry plant material (1 kg) was extracted with 2 L of hexane in a Soxhlet apparatus for 72 h at 50°C. After extraction, the solvent was removed with the aid of a rotary evaporator. The same process was carried out to get chloroform, ethyl acetate, and methanol extracts. The total yield of the extract obtained after removal of solvents was 15 g (hexane), 33 g (chloroform), 20 g (ethyl acetate) and 43.6 g (methanol).

Phytochemical analysis

Freshly prepared leaf extracts of *M. tinctoria* were qualitatively tested for the presence of alkaloids, carbohydrates, glycosides, phytosterols, triterpenes, diterpenes, tannins, fats and oils, saponins, flavonoids, phenols, proteins and amino acids. These were identified by characteristic color changes and precipitation reactions using standard procedures[24].

EXPERIMENTAL

Isolation of bioactive compounds

Chromatography

Column chromatography was carried out on silica gel (60-120 mesh particle size). Thin layer chromatography was carried out on GF-254 pre coated plates. The commercially available solvents were distilled and then used for TLC and column chromatography.

Hexane extract

10 g of the dried extract was column fractionated by wet packing method. About 500 g of silica mesh 60-120 (column chromatography grade) was kept in an oven for 1 h at 105 °C for activation. It was cooled in a desiccator for 1 h. A column of length 150 cm and diameter 7.5 cm was used for doing column chromatography. The column was packed up to 2/3 portion with activated silica by wet packing procedure. The extract was also adsorbed with silica gel before introducing into column. The column was then eluted with hexane and hexane:chloroform in different ratios. Every 30 mL fraction was collected and homogenous fractions were clubbed together after analyzing with TLC. First 30 mL was discarded. The collected fractions were analyzed on TLC with mobile phase hexane: pet ether (60-80): chloroform (5: 2.5:1 V/V/V). Similar fractions 35-54 and 73-83 were clubbed together.

Chloroform extract

About 10 g of the chloroform extract was defatted using hexane for 1 h for the removal of fatty materials. The extract was then dried and weighed about 8.5 g. It was dissolved in chloroform (50 mL) and mixed with silica gel mesh 60-120 and dried; the dried material was subjected to column chromatography. About 500 g of silica mesh 60-120 (column chromatography grade) was kept in an oven for 1 h at 105 °C for activation. It was cooled in a desiccator for 1 h. A column of length 150 cm and diameter 7.5 cm was used for doing column chromatography. The column was packed up to 2/3 portion with activated silica by wet packing procedure. The column was then eluted with the mixture of hexane: chloroform and chloroform: methanol in different ratios. Every 30 mL fraction was collected, and homogenous fractions were clubbed together after analyzing with TLC. First 30 mL did not contain any compound when checked with TLC, hence was discarded.

Different fractions were collected (203 tubes). Each tube was checked using TLC with mobile phase hexane: chloroform: methanol (5.5: 2: 1 V/V/V) so as to analyze the purity of the isolated fractions. Fractions with same bands were clubbed together. Seven similar fractions were collected. Fractions 45-49, 68-75, 102-107, 131-144, 151-171, 183-190 and 195-203 were clubbed together.

Ethyl acetate extract

About 10 g of dried extract was column chromatographed with activated silica gel by wet packing method. Extract was dissolved in chloroform (50 mL) and mixed with silica gel mesh 60-120 and dried; the dried material was subjected to column chromatography. About 500 g of silica mesh 60-120 (column chromatography grade) was kept in oven for 1 h at 105 °C for activation. It was cooled in a desiccator for 1 h. A column of length 150 cm and diameter 7.5 cm was used for doing column chromatography. The column was packed up to 2/3 portion with activated silica by wet packing procedure. The column was eluted with chloroform, chloroform: methanol in different ratios and the fractions were collected. The collected fractions were analyzed by TLC with mobile phase hexane: chloroform: methanol (5.5: 2:1 V/V/V) and the similar fractions were clubbed together. By analyzing the R_f values, it was found that the spots identified from chloroform extract are identical with those of ethyl acetate extract. Thus both the fractions were again combined.

Methanol extract

About 8 g of dried extract was dissolved in acetone (50 mL) and mixed with silica gel mesh 60-120 and dried; the dried material was subjected to column chromatography. About 500 g of silica mesh 60-120 (column chromatography grade) was kept in an oven for 1 h at 105°C for activation. It was cooled in a desiccator for 1 h. A column of length 150 cm and diameter 7.5 cm was used for doing column chromatography. The column was packed up to 2/3 portion with activated silica by wet packing procedure. The column was then eluted with chloroform: methanol as eluent in different ratios and the isolated fractions were analyzed on TLC with mobile phase hexane: chloroform: methanol (5.5: 2:1 V/V/V). Fractions 52-63 and 73-83 were clubbed together respectively. The phytochemical screening results of various chemical constituents is summarized in Table I.

CHARACTERIZATION

Examination of *M. tinctoria* MT-1 (palmitic acid)

Recrystallized from ethanol as colorless needles. It gave brisk effervescence with 5% $NaHCO_3$ solution.

Yield: 75 mg

Molecular Formula: $C_{16}H_{32}O_2$

Melting Point: 59-62 ^{0}C

R_f Value: 0.55 (hexane:petether:chloroform 5:2.5:1 V/V/V)

IR: 3566.70, 1622.28, 1520.04, 1095.67, 972.21, 804.39, 673.22, 597.99 and 468.74 cm^{-1}

^{1}HNMR ($CDCl_3$; 400MHz): δ 10.67, 2.32, 1.30, 1.28, 1.45, 1.26, 0.86.

^{13}CNMR ($CDCl_3$; 100MHz): δ 182.61 (-COOH), 36.67, 34.58, 34.58, 32.32, 32.31, 32.24, 32.09, 32.02, 31.89, 31.71, 27.33, 25.35, 16.78.

MS: [MH^+] 257.05

Examination of MT-2 (Fucoxanthin)

It is a carotenoid and was obtained as pale yellow colour solid

Yield: 75 mg

Molecular Formula: $C_{42}H_{58}O_6$

Melting Point: 166-168 ^{0}C

R_f Value: 0.97 (Hexane: chloroform: methanol-5.5:2:1 V/V/V)

IR: 3607.21, 3545.48, 2926.28, 2854.90, 1635.78, 1153.54, 1097.60, 802.46, 673.22, 659.71, 597.99 and 466.82 cm^{-1}

^{1}H NMR ($CDCl_3$; 400MHz): δ 6.82, 6.56, 6.56, 6.61, 6.30, 6.29, 6.02, 6.13, 6.09, 6.09, 6.19, 4.81, 4.07, 3.44, 3.35, 2.05, 1.99, 2.08, 2.06, 2.08, 2.11, 1.94, 1.52, 1.06, 0.83, 1.23, 1.37, 1.07, 1.08, 1.36, 2.15.

^{13}C NMR ($CDCl_3$; 100MHz): δ202.85, 197.62, 170.44, 145.40, 138.90, 136.92, 136.93, 136.57, 134.33, 132.72, 133.35, 132.52, 128.46, 130.69, 129.05, 123.17, 117.95, 103.23, 72.78, 68.12, 64.12, 66.82, 65.95, 47.17, 45.64, 45.70, 41.70, 40.97, 35.92, 35.25, 31.10, 30.50, 26.55, 21.15, 21.32, 20.85, 17.45, 13.00 and 11.76

Examination of MT-3 (Broussin)

Yield: 45 mg

Molecular Formula: $C_{16}H_{16}O_3$

R_f Value: 0.5 (Hexane: chloroform: methanol-5.5:2:1 V/V/V)

Table I: Phytochemical screening of *M. tinctoria* leaves

S. No	Chemical Constituent	Name of the Test	Hexane	Chloroform	Ethyl acetate	Methanol
1	Alkaloids	Mayer's Test	+	-	+	-
		Dragendorff's Test	+	-	+	-
		Wagner's Test	+	-	-	-
2	Carbohydrates	Molisch's Test	-	-	-	-
		Benedict's Test Fehling's test	-	-	-	-
		Barfoed's test	-	-	-	-
3	Glycosides	Borntrager's Test	+	-	-	-
		Legal Test	-	-	-	-
4	Saponins	Foam Test	-	-	-	-
		Froth Test	+	-	-	-
5	Phytosterols	Salkowski's Test	+	-	+	-
		Leibermann Burchard Test	+	-	+	-
6	Fats and Oils	Stain Test	+	-	-	-
7	Resins	Acteone Water Test	-	-	-	-
8	Phenols	Ferric chloride Test	-	+	+	+
9	Tannins	Alkaline Reagent Test	-	-	-	+
		Gelatin Test	-	-	-	+
10	Flavonoids	Lead Acetate Test	-	+	+	-
		Shinoda Test	-	+	+	-
		Zn-HCl Reagent Test	-	+	+	-
		Alkaline Reagent Test	-	+	+	-
11	Proteins and Amino acids	Xanthoproteic Test	-	-	-	-
		Ninhydrin Test	-	-	-	-
		Biuret Test	-	-	-	-
12	Diterpenes	Copper acetate Test	-	-	-	-
13	Triterpenes	Noller's Test	-	+	-	+
		Tshugajen Test	-	+	-	+

+ Positive test with the reagent indicates presence *- Negative test with the reagent indicates absence*

IR: 3543.55, 1645.43, 1149.68, 1099.52, 802.46, 671.29, 596.06, 468.74 cm^{-1}

^1H NMR (DMSO. d_6; 400MHz): δ 7.26, 7.26, 7.0, 6.82, 6.82, 6.17, 6.30, 4.88, 3.80, 2.90, 2.36, 6.90.

^{13}C NMR ($CDCl_3$): δ 159.31, 155.30, 156.34, 134.10, 130.41, 128.0, 128.0, 114.20, 114.12, 114.12, 108.41, 103.60, 77.59, 55.36, 29.80, 24.46.

MS: $[MH^+]$ 256.95

Examination of MT-4 (Isoliquiritigenin)

Yield: 72 mg

Molecular Formula: $C_{15}H_{12}O_4$

Melting Point: 207-209 ^0C

R_f Value: 0.57 (Hexane: chloroform: methanol-5.5:2:1 V/V/V)

IR: 3607.21, 3545.48, 1622.28, 1153.54, 1097.60, 804.39, 673.22, 661.64, 596.06, 466.82 cm^{-1}

^1H NMR (DMSO. d_6; 400MHz): δ 9.18, 8.76, 8.52 (d, *J*=8.9Hz), 7.99, 7.91, 7.87, 7.84 (d, *J* = 8.5 Hz), 7.59, 7.54, 7.48, 7.45, 7.32, 7.26, 7.23, 7.17, 7.13, 6.92, 6.88 (d, *J* = 8.5 Hz), 3.36, 2.49, 2.07

^{13}C NMR ($CDCl_3$; 100MHz): δ 157.39, 151.13, 149.94, 140.07, 128.86, 128.69, 127.89, 127.21, 116.08, 112.61, 77.7, 77.07, 76.44, 40.44

MS: $[MH^+]$ 257.08

Examination of MT-5 (Cynarin)

It is obtained as pale green color solid.

Yield: 58 mg

Molecular Formula: $C_{25}H_{25}O_{12}$

Melting Point: 225-227 ^0C

R_f Value: 0.52 (Hexane: chloroform: methanol-5.5:2:1 V/V/V)

IR: 3564.77, 1622.28, 1095.67, 804.39, 673.22, 597.99, 466.82 cm^{-1}

^1H NMR (DMSO- d_6; 400MHz): δ7.66, 7.58, 7.60, 7.31, 7.32, 7.09, 7.10, 6.84, 6.86, 6.77, 6.78, 6.44, 6.45, 6.67, 5.93, 4.53, 4.07, 3.71, 2.63, 2.55, 2.41, 2.23, 1.88, 1.64, 6.45.

^{13}C NMR ($CDCl_3$; 100MHz): δ 175.30, 165.60, 166.18, 148.27, 148.27, 147.11, 147.11, 145.18, 143.20, 128.10, 127.03, 122.70, 122.70, 119.50, 116.34, 116.34, 117.43, 115.11, 115.11, 80.17, 73.69, 69.71, 67.64, 36.06 and 33.10

MS: $[MH]^+$ 517.13

Examination of MT-6 (Gallic Acid)

Crystallised from methanol as colourless needles.

Yield: 25 mg

Molecular Formula: $C_7H_6O_5$

Melting point: 238-242 ^0C

IR: 3347.67, 2920.20, 1687.11, 1643.26, 1604.90, 1550.10, 1506.25, 1456.93, 1336.36, 1199.35, 1029.46, 717.08 cm^{-1}

^{1}HNMR (400MHz, $CDCl_3$): δ 7.08, 7.08, 5.93, 5.52 and 5.52.

^{13}C NMR (100MHz, $CDCl_3$): δ 177.72, 147.11, 147.11, 138.62, 130.64, 112.13 and 112.13.

MS: $[MH^+]$ 171.20

Examination of MT-7 (β-Sitosterol)

Crystallised from Chloroform-methanol as colourless needles. It gave positive L.B test for sterols.

Yield: 125 mg

Molecular Formula: $C_{29}H_{50}O$

Melting point: 135-136 ^0C

R_f: 0.67 (Hexane: chloroform: methanol-5.5:2:1 V/V/V)

IR: 3380, 2958.90, 2931.51, 1654.79, 1463.01, 1369.86, 1052 & 953cm^{-1}

^1H NMR (400MHz, $CDCl_3$): δ5.39, 3.57, 2.31, 2.20, 1.95, 1.37, 1.84, 1.09,1.38, 1.28, 1.09, 1.50, 1.44, 1.06, 1.24, 1.70, 1.39, 1.26, 1.13, 1.82, 1.62, 0.82, 0.87, 0.90, 0.89, 0.83 and 0.92.

^{13}C NMR (100MHz, $CDCl_3$): δ140.86, 121.56, 71.33, 56.43, 55.91, 50.07, 46.04, 42.36, 42.28, 39.35, 36.53, 37.32, 35.51, 19.30 (four carbons), 33.93, 31.83, 32.14, 31.71, 29.04, 28.41, 26.34, 23.19, 24.46, 12.08, 11.90.

Examination of MT-8 (Quercetin)

Yield: 49 mg

Molecular Formula: $C_{15}H_{10}O_7$

Melting Point: 314-316 ^{0}C

R_f Value: 0.96 (Hexane: chloroform: methanol 5.5:2:1 V/V/V)

IR: 3386.30, 2926.03, 2361.64, 2334.25, 2252.05, 2126.03, 1654.79, 1594.52, 1495.89, 1353.52, 1304.11, 1161.64, 827.40, 761.64 cm^{-1}

^{1}H NMR (DMSO. d_6; 400Mz): δ 12.50, 10.7, 9.67, 9.48, 9.35, 7.75, 7.51, 6.9, 6.46, 6.25, 3.45, 2.50

^{13}C NMR (DMSO. d_6; 100MHz): δ 175.99, 161.04, 160.89, 156.32, 147.84, 146.94, 145.20, 136.91, 122.18, 120.19, 115.79, 115.25, 103.19, 98.38 and 93.54

Examination of MT-9 (Oleuropein)

Yield: 32 mg

Molecular Formula: $C_{25}H_{32}O_{13}$

Melting Point: 89-90 ^{0}C

R_f Value: 0.43 (Hexane: chloroform:methanol 5.5:2:1 V/V/V)

IR: 3545.48, 1622.28, 1107.24, 804.39, 673.22, 596.06, 459.10 cm^{-1}

1 H NMR (DMSO. d_6+ $CDCl_3$; 400MHz): δ 8.72, 7.84, 7.70, 7.69, 7.60, 7.36, 7.35, 6.34, 4.39, 4.02, 3.19, 2.62, 2.50, 2.05, 1.14

^{13}C NMR (DMSO. d_6+ $CDCl_3$; 100MHz): δ 160.71, 153.95, 134.36, 128.93, 126.08, 124.59, 124.26, 122.29, 121.59, 116.01, 79.11, 78.66, 78.48, 77.81

MS: [MH^+] 541.19

RESULTS

The qualitative phytochemical screening of crude extracts (hexane, chloroform, ethyl acetate, and methanol) of *M. tinctoria* leaves revealed the presence of alkaloids, phenols, flavonoids, tannins, saponins, triterpenes, phytosterols, glycosides, fats and oils.

DISCUSSION

Chemical constituents

Dried leaf powder was extracted by serial exhaustive extraction process with hexane, ethyl acetate, chloroform and methanol. Further, the extracts were examined for bioactivity. So far, we had reported potent anti-microbial, antioxidant, anti-cancer, cytotoxic and genotoxic activities for leaf extracts of *M. tinctoria* Roxb. In the present study our objective was to isolate the bioactive molecules from various extracts of *M. tinctoria* leaves. We have isolated and characterized nine compounds from the hexane, ethyl acetate, chloroform and methanol extracts.

MT-1 was obtained as white crystalline needles, M.P. 59-62 ^{0}C and with molecular formula $C_{16}H_{32}O_2$. It gave brisk effervescence with 5 % $NaHCO_3$ solution, indicating the presence of carboxylic acid. ^{1}H NMR spectrum showed a singlet at δ 10.67 indicating the carboxylic acid proton. The other peaks were at δ 2.32 for methylene protons adjacent to acid group, δ1.30, 1.28, 1.45, 1.26, 0.86, indicating the presence of methylene protons. ^{13}C NMR spectrum showed peaks at δ182.61, indicating the presence of carboxylic acid carbon and δ 36.67 indicated the carbon adjacent to carboxylic carbon. The other peaks were at δ 34.58, 34.58, 32.32, 32.32, 32.24, 32.09, 32.02, 31.89, 31.71, 27.33, 25.35 and 16.78, indicating the carbons C1 to C14. Mass spectrum showed the base peak at m/z 257.05. From the above data MT-1 was characterized as palmitic acid and finally confirmed by comparing with an authenticating sample and co-TLC. Structure is given in Fig. 2.

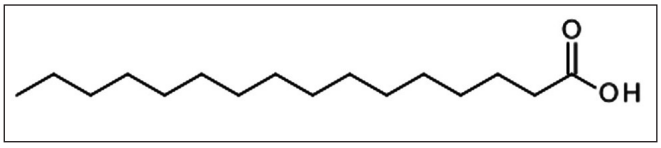

Fig. 2: Palmitic acid

MT-2 was obtained as a pale-yellow solid, M.P. 166-168 ^{0}C, with molecular formula $C_{42}H_{58}O_6$. IR spectrum showed characteristic absorptions at 3607.21 (OH), 3545.48 (OH), 1635.78 (-O-C=O), 1153.54 (-O-) and 1097.60 cm^{-1}. ^{1}H NMR spectrum showed singlets at δ 1.06, 1.23, 0.83, 2.05, 2.08, 2.08, 1.37, 1.07, 1.08 and 2.15, indicating the presence of methyl protons. A singlet at δ2.11 indicates the presence of methyl proton of acetate (O-Ac). Singlet at δ 3.44 indicates the presence of hydroxyl proton adjacent to diene in cyclohexane and 1.52 indicates the presence of hydroxyl proton attached to cyclohexane. Peak at δ 4.81 indicates the presence of CH proton to which acetate group is attached. ^{1}H NMR also showed peaks at δ 6.82, 6.56, 6.61, 6.30, 6.29, 6.02, 6.13, 6.09, 6.09, 6.19 and 3.35 confirming the presence of olefin protons. Peaks at δ 4.07 showed the presence of carbinol hydrogen and peaks at 2.06, 1.99, 1.94 and 1.36 indicate the presence of $-CH_2$ protons in six-member ring. ^{13}C NMR showed peaks at δ 138.90, 123.17, 145.40, 133.35, 133.35, 130.69, 129.05, 136.92, 132.72, 128.46 for unsaturated carbons peaks at δ103.23 indicated carbon atom adjacent to diene peaks at δ 136.93, 136.57,

134.33, and 132.52 indicated the presence of methyl group attached carbon atoms. Peaks at δ 13.0, 11.76, 17.45, 20.85 showed the presence of methyl carbons attached to unsaturated carbon chain. The other peaks at δ 21.15, 26.55, 31.10, and 30.50 indicated the presence of methyl carbons attached to cyclohexane rings. δ 64.12 and 72.78 indicated the presence of carbinol carbons. A sharp peak at δ 197.62 confirmed the presence of carbonyl carbon atom, δ 170.44 confirmed the presence of carbonyl carbon in acetate group and δ 202.85 indicated the presence of diene carbon. Epoxide carbons are shown at δ 66.82 and 65.95. A peak at δ 21.32 indicated the presence of methyl carbon in acetate group and δ 68.12 is for acetate attached carbon atom. The peak at δ 117.95 was for diene attached carbon in cyclohexane ring, δ 47.17, 45.64, 45.70, 41.70 for carbons in cyclohexane ring, δ 35.92 and 35.25 for methyl attached carbons in cyclohexane ring and δ 40.97 for the presence of carbon between carbonyl group and epoxide linkage. Mass spectrum showed a base peak at m/z 692.46. From the above data, the compound was characterized as fucoxanthin and was confirmed by comparing the spectral data with the reported values. Its structure is given in Fig.3.

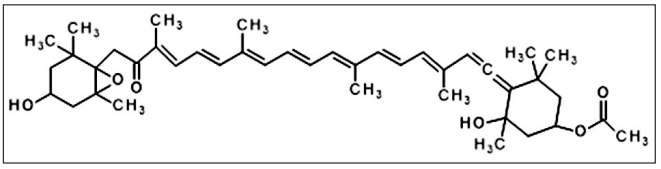

Fig. 3: Fucoxanthin

MT-3 was obtained as colourless liquid, with molecular formula $C_{16}H_{16}O_3$. It gave green colouration with neutral $FeCl_3$ solution, indicating the presence of phenolic group. IR spectrum showed absorption at 3543.55 (-OH), 1149.68 (-O-), and 1645.43 (C-H) cm^{-1}. ^1H NMR spectrum showed δ6.17, 6.30, 7.26, 6.82, 6.82, 7.26, and 6.90, indicating the presence of aromatic protons. A singlet at δ 7.0 indicated the presence of phenolic hydroxyl group. A singlet at δ 3.80 (-O-CH_3) attached to benzene ring and δ4.88 for the proton adjacent to epoxide. ^{13}C NMR showed peaks at δ 155.30, 114.20, 130.41, 108.41, 134.10, 128.0, 128.0, 114.12 and 103.60 indicating the aromatic carbons. Peak at δ 156.34 indicated the carbon attached to hydroxyl group, δ 77.59 indicating the epoxide linkage and δ 29.80 and 24.46 indicated the presence of methylene carbons in six-member cyclic ring. A peak at δ 55.36 indicated the presence of methoxy carbon. Mass spectrum showed a base peak at m/z 256.95. From all the above data, MT-3 was characterized as broussin and confirmed by comparing with the reported spectral data. Its structure is given in Fig. 4.

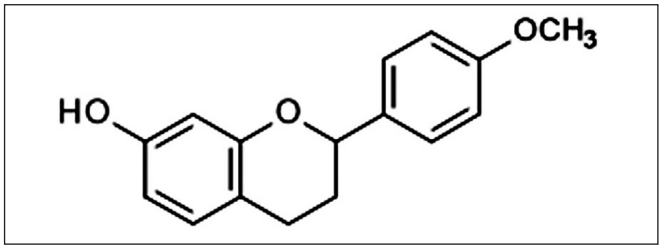

Fig. 4: Broussin

MT-4 was obtained as a yellow colour powder, M.P. 207- 209 ^0C, with molecular formula $C_{15}H_{12}O_4$. IR spectrum showed broad absorption peaks at 3607.21 and 3545.48 cm^{-1}, indicating the presence of aromatic hydroxyl groups. A peak at 1622.28 cm^{-1} indicated the presence of enone. ^1H NMR spectrum showed sharp singlets at δ 9.18, 8.76 and 8.52, indicating the aromatic hydroxyl protons. Multiplets at δ 6.88 to 7.99 indicated the presence of aromatic protons. ^{13}C NMR showed sharp singlets at δ 157.39, 151.13 & 149.94, indicating the aromatic carbons attached to hydroxyl groups and peaks at δ 112.61 to 140.07 indicated the presence of aromatic carbons. Mass spectrum showed a mass ion peak at 257.11. By comparing the above spectral data with the reported values, MT-4 was characterized as isoliquiritigenin. Its structure is given in Fig. 5.

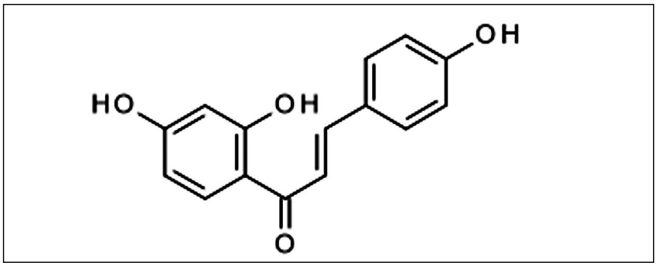

Fig. 5: Isoliquiritigenin

MT-5 was obtained as pale green colour solid, M.P., 225-227 ^0C, molecular formula $C_{25}H_{25}O_{12}$. IR spectrum showed a broad peak at 3564.77 cm^{-1}, indicating the presence of phenolic groups, a broad peak at 1622.28 cm^{-1} indicating the presence of C=O groups and a broad peak at 1095.67 cm^{-1} indicating the presence of ester group. ^1H NMR spectrum showed a multiplet at δ 6.67, 6.77, 6.78, 6.84, 6.86 and 7.09, indicating the presence of aromatic protons. Singlets at δ 7.58, 7.31, 7.66 and 7.10 indicated the presence of aromatic hydroxyl protons and peaks at δ 2.23 and 6.45 indicated the presence of aliphatic hydroxyl protons. Peaks at δ 6.44 and 5.93 indicated the presence of methylene proton adjacent to ester linkage. Peaks at δ 4.07, 3.71, 2.63, 4.53 and 2.41 showed the presence of cyclohexane protons. Peaks at δ 7.60 and peak at 7.32 confirmed the presence of '= CH'

protons. ^{13}C NMR spectrum of MT-5 exhibited a peak at δ 175.30 confirming the presence of aliphatic carboxylic acid. Peaks at δ 148.27 and 147.11 indicated the presence of four aromatic carbon atoms to which hydroxyl groups are attached. Peaks at δ 115.11, 127.03, 122.70, 116.34 and 128.10 indicates the presence of eight aromatic carbons. Peaks at δ 165.60 and 166.18 confirmed the presence of two C=O carbon atoms. Peaks at δ 143.20, 117.43, 145.18 and 119.50 indicated the presence of olefin carbon atoms. ^{13}C NMR also exhibited peaks at δ 80.17, 73.69, 69.71, 67.64, 36.06 and 33.10, indicating the presence of aliphatic carbon atoms. Mass spectrum showed a mass ion peak at 517.13. All the obtained spectral data were compared with that reported in the literature and authenticated by co-TLC. MT-5 was characterized as cynarin. Its structure is given in Fig. 6.

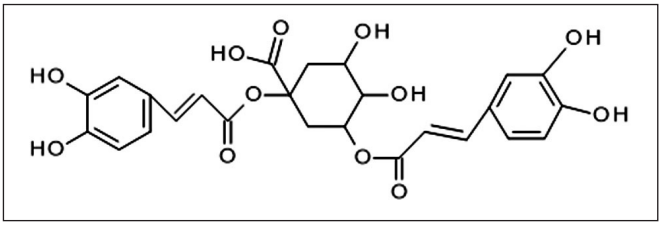

Fig. 6: Cynarin

MT-6 was obtained as colourless needles from methanol, M.P. 238- 242 ^0C, with molecular formula $C_7H_6O_5$. It gave green colouration with neutral $FeCl_3$ solution, indicating the presence of phenolic group. It gave brisk effervescences with 5 % $NaHCO_3$ solution, indicating the presence of carboxylic acid. IR spectrum showed characteristic absorptions at 3347.67 (OH), 1687.11 (C=O), 1604, 1456 and 717 cm^{-1}. ^1H NMR spectrum showed a singlet at δ 7.08, indicating the presence of two aromatic protons. Singlet at δ 5.93 was assigned to phenolic hydroxyl in para position and δ 5.52 was assigned to meta hydroxyl proton. ^{13}C NMR showed peaks for aromatic carbons at δ 112.13, 112.13, 130.64, 138.62, 147.11 and 147.11 and carbonyl of the carboxylic acid group at δ 177.72. From the above data, MT-6 was identified as gallic acid and finally confirmed with an authentic sample of gallic acid. Its structure is given in Fig. 7.

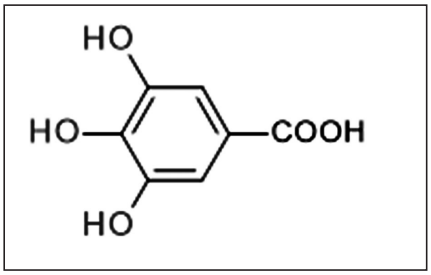

Fig. 7: Gallic acid

MT-7 was obtained as colourless crystalline needles from chloroform-methanol, M.P. 135-136 ^0C and with molecular formula $C_{29}H_{50}O$. It gave positive Liebermann-Burchard test for steroids. Its IR spectrum showed prominent absorption at 3380.82 cm^{-1}, assigned to hydroxyl group. ^1H NMR spectrum showed a multiplet at δ 5.39 accounting for one olefinic proton and another multiplet at δ 2.31 assigned to methine proton geminal to β-OH. A peak at δ3.57 indicated the presence of carbinol proton. The signals at δ 0.82, 0.87, 0.90, 0.89, 0.83 and 1.09 were assigned to six methyl groups. A singlet at δ1.37 indicated the presence of beta-hydroxy group. The signals at δ 1.84, 1.42, 1.44, 1.40, 1.78, 1.70, 1.95, 1.65 and 1.82 were assigned to nine methylene protons. From the above data, MT-7 was found to be identical with the reported values of β-sitosterol. ^{13}C NMR assignments of MT-7 exhibited peaks at δ 71.33 for carbinol carbon, δ 140.86 and 121.56 for olefenic carbons, δ 19.30, 12.08 and 11.90 for methyl carbons, δ 42.36 for methine carbon, δ 36.53, 42.28, 35.5129.04 and 23.19 for methyl attached carbons, δ 31.71, 56.43, 55.91, 50.07, 39.35, 37.32, 32.14, 31.83, 24.46 and 28.41 for carbons in cyclohexane and cyclopentane rings and δ 46.04, 33.93 and 26.34 for side chain carbons. The ^{13}C NMR values are well in agreement with the reported values. MT-7 was identified as β-sitosterol.Its structure is given in Fig. 8.

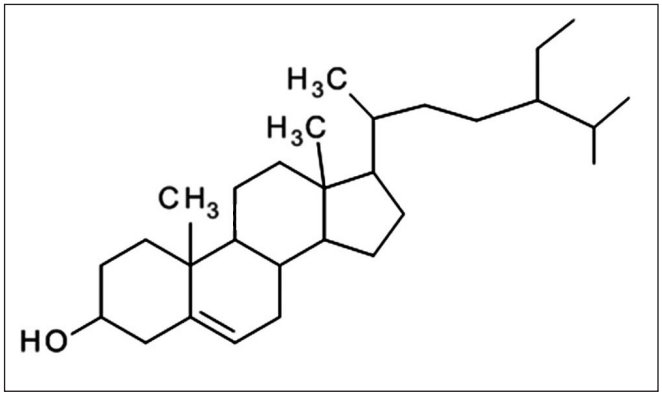

Fig. 8: β-Sitosterol

MT-8 was obtained as a pale yellow coloured solid, M. P. 314-316 ^0C, molecular formula $C_{15}H_{10}O_7$. It gave green colouration with neutral $FeCl_3$ solution, indicating the presence of phenolic group. IR spectrum showed prominent absorptions at 3386.30 cm^{-1}(OH), 1654.79 cm^{-1} (C=O) and 1200.0 cm^{-1} (-O-). ^1H NMR spectrum showed δ6.25, 6.46, 6.90, 7.51, 7.75, indicating the presence of five aromatic protons. Singlets at δ 12.50, 10.7, 9.35 and 9.67 confirmed the presence of four phenolic hydroxyl protons and peak at δ 9.48 indicated the presence of olefinic hydroxyl group. ^{13}C NMR showed peak at δ

175.99, indicating the presence of carbonyl group in six-member ring. Peaks at δ 156.32, 93.54, 98.38, 103.19, 122.18, 115.25, 115.79 and 120.19 indicated the presence of aromatic carbons. A peak at δ 147.84 showed the presence of olefinic carbon atom adjacent to epoxide and the peaks at δ 136.91 was assigned to hydroxyl group attached olefinic carbon atom. Peaks at δ 161.04, 160.89, 146.94 and 145.20 indicated the presence of hydroxyl group attached aromatic carbons. From the above data and comparing with the reported spectral data, MT-8 was characterized and confirmed as quercetin. Its structure is given in Fig. 9.

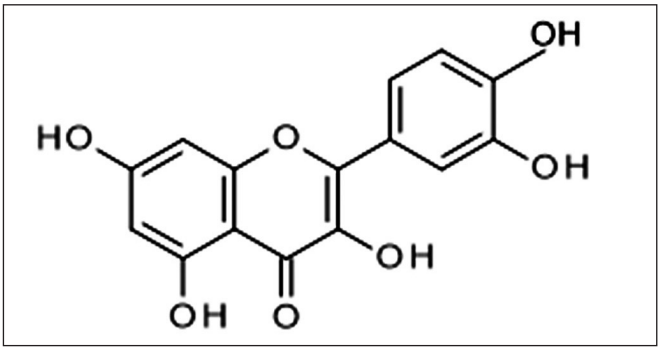

Fig. 9: Quercetin

MT-9 was obtained as a brownish yellow powder, M.P: 89-90 ^{0}C, molecular formula $C_{25}H_{32}O_{13}$. IR spectrum showed a broad peak at 3545.48 cm^{-1}, indicating the presence of phenolic groups, a broad peak at 1622.28 cm^{-1} indicating the presence of C=O group and a broad peak at 1107.24 cm^{-1} indicating the presence of ether group. ^{1}H NMR spectrum showed a sharp singlet at δ 8.72, indicating the presence of aromatic hydroxyl protons. A multiplet at δ 7.35 to 7.84 indicated the presence of aromatic protons and a singlet at δ 6.34 indicated the presence of cyclic hydroxyl groups. ^{13}C NMR spectrum showed peaks at δ 160.71 and 153.95 indicating the presence of phenolic carbon atoms and peaks at δ 121.59 to 134.36, indicating the presence of aromatic carbons. Mass spectrum showed mass ion peak at 541.19. All the above data was consistent with that reported in the literature. MT-9 was characterized as oleuropein. Its structure is given in Fig.10.

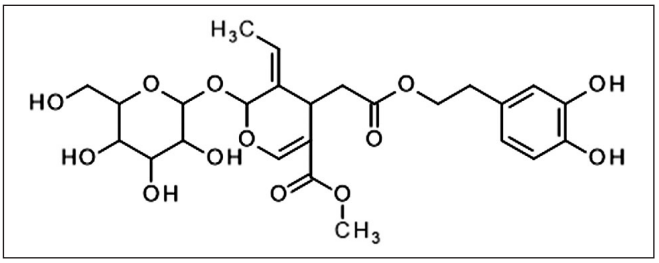

Fig. 10: Oleuropein

CONCLUSION

We herewith report the isolation and characterization of nine bioactive compounds from hexane, chloroform, ethyl acetate and methanol extracts of the leaves of *M. tinctoria* Roxb., whose structures have been characterized as palmitic acid (MT1), fucoxanthin (MT2), broussin (MT3), isoliquiritigenin (MT4), cynarin (MT5), gallic acid (MT6), β-sitosterol (MT7), quercetin (MT8) and ooleuropein (MT9) on the basis of extensive 1D (1H and 13C) NMR as well as high resolution mass spectral (HRMS) data. This is the first report on isolation of bioactive compounds from *M. tinctoria* Roxb. leaves. The presence of bioactive compounds might justify the age-old ethno-medical practices on this species. However, further biomedical studies are required to standardize its use in herbal remedies.

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