

## Research Article

# EVALUATION OF *IN VITRO* AND *IN VIVO* ANTI-OXIDANT POTENTIAL OF *Morinda reticulata* GAMBLE TUBERS IN WISTAR ALBINO RATS SUBJECTED TO CCL<sub>4</sub> AND PARACETAMOL INDUCED HEPATOTOXICITY

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## ABSTRACT

The aim of present study is to explore the antioxidant potential of ethanol and aqueous extracts of *Morinda reticulata* Gamble by *in vitro* and *in vivo* methods. *In vitro* antioxidant activity of benzene, chloroform, ethanol and aqueous extracts of *M. reticulata* was studied by DPPH, metal chelating, super oxide free radical scavenging assay, hydroxyl radical scavenging assay and Reducing power assay where ascorbic acid was used as a standard antioxidant. Oxidative stress in wistar rats was induced by two different models using administration of CCl<sub>4</sub> (1.5 mL/kg, p.o) and paracetamol (2g/kg) on seventh day of study. Ethanol and aqueous extracts were given twice daily for one week. Silymarin (25mg/kg, p.o) was given as a standard drug. *In vitro*, IC<sub>50</sub> values were least with ethanol and aqueous extracts when compared with other extracts. Ethanol extracts exhibited similar free radical scavenging effect as that of standard ascorbic acid. *In vivo* antioxidant activity was assessed by the measurement of melondyaldehyde (MDA), Reduced Glutathione (GSH), Super oxide dismutase (SOD) and Total protein levels were estimated from the liver tissue homogenate. The level of MDA (3.27±2.18) was significantly (p<0.001) increased whereas decreased level of total protein, GSH and SOD were found in CCl<sub>4</sub> and paracetamol control group. Seven days extracts treatments restored these altered parameters in to normal where ethanol extract treatment group showed significant (p<0.001) result than aqueous extract. HPTLC study showed that presence of six phytoconstituents with corresponding Rf value. The study report concluded that *M. reticulata* has very good antioxidant effect against CCl<sub>4</sub> and paracetamol induced liver damage with oxidative stress. Probable mechanism behind this protection against oxidative damage produced by CCl<sub>4</sub> and paracetamol is its phytoconstituents.

**Keywords:** *M. reticulata*, *in vitro*, *in vivo*, Sylmarin, MDA

## INTRODUCTION

Hepatic system plays a pivotal role in the metabolism of chemicals and foods for the regulation of internal environment (Periasamy *et al.*, 2012). Because of its anatomical location and its great capacity for xenobiotic metabolism, it is frequently a target for toxic chemicals. Most of these toxic chemicals have been reported to generate free radicals and reactive oxygen species which are the major culprits in liver pathogenesis (Carmel *et al.*, 2011). Drug exposure, ionizing radiations and environmental pro-oxidant pollutants induce

free radical formation (Ragip *et al.*, 2008). Lipid peroxidation initiated by free radicals is considered to be deleterious for cell membranes and has been implicated in a number of pathological situations. An imbalance between too much oxidative stress (OS) and too little antioxidant defence has been suggested to cause a variety of liver diseases. Antioxidants are radical scavengers, which protect the human body against free radicals (Mohammad *et al.*, 2010). On the basis of evidence obtained from experimental research, antioxidants appeared promising

in prevention and treatment of hepatic damage. Recent studies have suggested that natural antioxidants in complex mixtures ingested with the diet are more efficacious than pure compounds in preventing oxidative stress related pathologies due to particular interactions and synergism. It is evident that there is an increasing demand to evaluate the antioxidant properties of direct plant extract and there is a need for screening more bioactive plant products with antioxidant properties (Carmel *et al.*, 2011). The present study was aimed to investigate, free radical scavenging potential of *Morinda reticulata* Gamble belongs to Rubiaceae family. It is a highly endangered medicinal plant of Southern Western Ghats of India (Rahul *et al.*, 2012). In Malayalam it is called Neyvalli. It is an arborescent climber, growing to a height of 20m. They are mainly used for treating jaundice (Thangaraj *et al.*, 2014; Ijiru *et al.*, 2011), back pain (Ijiru *et al.*, 2011), blood purification (Thangaraj *et al.*, 2014, Ijiru *et al.*, 2011) and also used as laxative (Rahul *et al.*, 2012) due to abundance of anthraquinone derivatives.

## MATERIALS AND METHODS

### Collection of plant material and preparation of extract

The tubers of *Morinda reticulata* Gamble (MR) was collected from the vicinity of kazhakoottam, Thiruvananthapuram, Kerala, India in the month of December 2014. The plant was identified and authenticated by Prof. Ramanibhai, HOD Department of Botany, Christian College, Kattakkada, Thiruvananthapuram. The herbarium specimen of the same is deposited at the Department of Pharmacognosy, Sree Krishna College of Pharmacy and Research centre, Parassala, Thiruvananthapuram (H.S.P.No: SKCPRC /COGNOSY /HS2014/003). Shade dried tubers of MR were cleaned, dried at room temperature and coarse powdered. The powdered drug was successively extracted in a Soxhlet apparatus with solvents of increasing polarity using petroleum ether, benzene, chloroform, ethanol and water. The extract was concentrated to dryness under reduced pressure and controlled temperature to get the dried crude extract.

### Experimental animals

Adult of both sexes of Wistar rats with body weights of 180-230g was used in the study. The animals were maintained under standard environmental conditions (23-25°C, 12h/12h light/dark cycle) and had free access to standard rodent pellet diet and water *ad libitum*. The animals were acclimatized to the laboratory conditions for a week before the commencement of the study. Use of animals as per the experiment was approved by the institutional Animal Ethics Committee (Ref No: Joshila J U/M.Pharm/KUHS/2014/a&b).

### Preliminary phytochemical analysis

All extracts except petroleum ether extract were subjected to preliminary phytochemical studies to identify the presence of various phytoconstituents (Kokate, 2007).

### In vitro antioxidant studies

The following assay methods were performed as per Raju *et al.*, 2013a.

### DPPH radical scavenging activity

The stock solution was prepared by dissolving 24mg DPPH with 100mL methanol and stored at 20°C until required. The working solution was obtained by diluting DPPH solution with methanol. A 3mL aliquot of this solution was mixed with 100µL of the sample at various concentrations (1.25, 2.5, 5, 10, 20µg/mL). The reaction mixture was shaken well and incubated in the dark for 15min at room temperature. Then the absorbance was taken at 517nm. The control was prepared as above without any sample. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{Scavenging effect (\%)} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

### Super oxide free radical scavenging activity (SOSA)

The reaction mixture contained 1mL of NBT solution (312µM prepared in phosphate buffer, pH-7.4), 1mL of NADH solution (936µM prepared in phosphate buffer, pH-7.4)

different extracts of MR in different concentration (1.25, 2.5, 5, 10, 20 µg/mL) were added. Finally, reaction was accelerated by adding 100 µL PMS solution (120 µM prepared in phosphate buffer, pH-7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and absorbance at 560 nm was measured against methanol as a control. Percentage inhibition was calculated.

$$\text{Scavenging effect (\%)} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

#### Hydroxyl radical scavenging activity (HRSA)

The scavenging activity for hydroxyl radicals was measured with fenton reaction. Reaction mixture contained 60 µL of 1.0 mM FeCl<sub>2</sub>, 90 µL of 1 mM 1,10-phenanthroline, 2.4 mL of 0.2 M phosphate buffer (pH 7.8), 150 µL of 0.17 M H<sub>2</sub>O<sub>2</sub>, and 1.0 mL of extract at various concentrations (1.25, 2.5, 5, 10, 20 µg/mL). Adding H<sub>2</sub>O<sub>2</sub> started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm was measured with UV visible spectrometer. The percentage inhibition of hydroxyl scavenging activity was calculated using the following formula.

$$\text{Percentage inhibition (\%)} = 1 - \frac{\text{Absorbance}(\text{test})}{\text{Absorbance}(\text{blank})} \times 100$$

Where, Absorbance (Test): Absorbance of the test (With extract) and Absorbance (Blank): Absorbance of the control (Without extract).

#### Reducing Power Assay (RPA)

A serial dilution of the extracts were performed (1.25, 2.5, 5, 10 and 20 µg/mL) in 0.2 M phosphate buffer pH 6.6 containing 1% ferrocyanate. The mixture was incubated at 50°C for 20 min. 10% trichloroacetic acid (TCA, 2.5 mL) was added to a portion of this mixture (5 mL) and centrifuged at 3.000 r.p.m for 10 min. The supernatant was separated and mixed with distilled water (2.5 mL) containing 1% ferric chloride (0.5 mL). The absorbance of this mixture was measured at 700 nm. The intensity of change in colour could be the antioxidant

activity of the extracts. The percentage of inhibition of radical was calculated as:

$$\text{Percentage inhibition (\%)} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

#### Metal chelating activity (MCA)

Fifteen µL of 2 mM FeCl<sub>2</sub> was added to 1 mL of different concentrations of the extracts (1.25, 2.5, 5, 10, 20 µg/mL). The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. The percentage inhibition of ferrozine-Fe<sup>2+</sup> complex formation was calculated as

$$\text{Percentage inhibition (\%)} = \frac{(A_0 - A_s)}{A_s} \times 100$$

where A<sub>0</sub> was the absorbance of the control, and A<sub>s</sub> was the absorbance of the extract/standard. Na EDTA was used as positive control.

#### Acute toxicity study

Acute toxicity study was carried out on EEDB and AEDB following OECD guidelines (OECD 423).

#### Experimental design (*in vivo*)

##### Carbon tetrachloride (CCl<sub>4</sub>) induced hepatotoxicity

Based on the *in vitro* antioxidant reports ethanol extract of *Morinda reticulata* (EEMR) and aqueous extract of *Morinda reticulata* (AEMR) were chosen for *in vivo* antioxidant study. Total 42 Wistar albino rats were divided into seven groups of 6 rats (180-230 g) in each group (Periasamy *et al.*, 2012) Treatment protocol is as follows:

Group I: Vehicle control (1.0 mL, p.o.) for 7 days, Group II: Treated with vehicle (1.0 mL, p.o.) twice daily for 7 days and CCl<sub>4</sub> (1.5 mL/kg) on seventh day (1:1 of CCl<sub>4</sub> in olive oil), Group III: Treated with Silymarin (25 mg/kg, p.o.) twice daily for 7 days and CCl<sub>4</sub> (1.5 mL/kg, p.o.) on seventh day, Group IV: Treated with EEMR (200 mg/kg) twice daily for 7 days and CCl<sub>4</sub> (1.5 mL/kg, p.o.) on seventh

day, Group V: Treated with EEMR (400mg/kg, p.o.) twice daily for 7 days CCl<sub>4</sub> (1.5mL/kg, p.o.) on seventh day, Group VI: Treated with AEMR (400mg/kg, p.o.) twice daily for 7 days CCl<sub>4</sub> (1.5mL/kg, p.o.) on seventh day, Group VII: Treated with AEMR (200mg/kg, p.o.) twice daily for 7 days CCl<sub>4</sub> (1.5mL/kg, p.o.) on seventh day. On the 8<sup>th</sup> day, 18h after the last dose of CCl<sub>4</sub>, blood was withdrawn from the retro orbital plexus under light ether anaesthesia following which they were sacrificed by an excess of anaesthetic agent for the isolation of liver for preparation of tissue homogenates and histopathological studies.

#### Paracetamol induced Hepatotoxicity

Wistar Albino rats (180-230g) of both sexes were used. Animals were randomized and divided into seven groups of six animals in each group, silymarin (25mg/kg) is used as the standard drug (Deepak *et al.*, 2008). Group I: Vehicle control (1.0mL, p.o.) for 7 days, Group II: Treated with vehicle (1.0mL, p.o.) twice daily for 7 days and Paracetamol (2g/kg, p.o.) on seventh day, Group III: Treated with Silymarin (25mg/kg, p.o.) twice daily for 7 days and Paracetamol (2g/kg, p.o.) on seventh day, Group IV: Treated with EEMR (400mg/kg, p.o.) for 7 days and Paracetamol (2g/kg, p.o.) on seventh day, Group V: Treated with EEMR (200mg/kg, p.o.) twice daily for 7 days and Paracetamol (2g/kg, p.o.) on seventh day, Group VI: Treated with AEMR (400mg/kg, p.o.) twice daily for 7 days and Paracetamol (2g/kg, p.o.) on seventh day,

Group VII: Treated with AEMR (200mg/kg, p.o.) twice daily for 7 days and Paracetamol (2g/kg, p.o.) on the seventh day. The biochemical parameters were estimated after an overnight fast following the last dose. Blood and liver samples were collected for biochemical and histological studies.

#### Evaluation of antioxidant parameter Total protein (TP) (Biuret method)

The protein content of the tissue homogenates was measured by the method of Lowry *et al.*, 1951. 0.5mL of tissue homogenate was mixed with 0.5mL of 10% TCA and centrifuged for 10min. The precipitate obtained was dissolved in 1.0mL of 0.1N NaOH and 0.1mL is used for the estimation. A 0.1mL

aliquot was mixed with 5.0mL of alkaline copper reagent and allowed to stand at room temperature for 10min. 0.5mL of Folin's phenol reagent was added and the blue color developed was read at 640nm after 20min. TP was expressed as g/dL.

$$\text{Total protein in g/dl} = \frac{\text{Absorbance of sample} \times C}{\text{Absorbance of standard}}$$

where C refers to the protein concentration in standard protein solution in g/dL.

#### Reduced Glutathione (GSH)

Liver tissue samples were homogenized in 8.0mL of 0.02M EDTA in an ice bath (Ellman *et al.*, 1961). Aliquots of 5.0mL of the homogenates were mixed in 15.0mL test tubes with 4.0mL distilled water and 1.0mL of 50% TCA. The tubes were centrifuged for 15min at approximately 3000r.p.m, the 2.0mL of supernatant was mixed with 4.0mL of 0.4M Tris buffer, pH 8.9, 0.1mL Ellman's reagent [5,5-dithiobis(2-nitro-benzoic acid)] (DTNB) added and the sample shaken. The absorbance was read within 5min of the addition of DTNB at 412nm against a reagent blank with no homogenate. Results were expressed as µg/100mg tissue.

#### Malondialdehyde (MDA)

Liver tissues were homogenized with ice cold 1.5% thiobarbituric acid to make a 10% homogenate. Three milliliters of 1% phosphoric acid and 1mL of 0.6% thiobarbituric acid (TBA) aqueous solution were added to 0.5mL of 10% homogenate. The mixture was heated for 45min and after cooling; 4mL of n-butanol was added and mixed (Farida *et al.*, 2013). The absorbance of butanol phase was measured at 535 and 520nm. The difference between the two measurements was used as the MDA value (nmol/g protein).

#### Super oxide dismutase (SOD)

Liver tissues were homogenized with 0.05M carbonate buffer at pH 10.2 containing EDTA. The tissue homogenates were centrifuged at 4°C for 15min at 1500×g and supernatants were removed, kept on ice until the enzyme assay. 6-Hydroxydopamine hydrobromide was prepared in distilled water and added to the buffer containing tissue so that the final 6-OHDA concentration was 10-4M.

Table I. *In vitro* antioxidant effect of benzene, chloroform, ethanol and aqueous extracts of *M. reticulata* on various assay methods

Extracts	IC <sub>50</sub> values (mcg/mL)				
	DPPH	SOSA	HRSA	RPA	MCA
<b>BEMR</b>	34.25±2.89	25.68±3.2	28.99±0.70	48.56±3.68	23.75±1.95
<b>CEMR</b>	28.5±1.44	25.43±2.87	20.83±0.25	30.69±1.90	20.62±1.56
<b>EEMR</b>	20.52±1.87	17.65±1.77	12.99±0.25	19.17±1.44	15.47±0.49
<b>AEMR</b>	24.39±0.60	22.55±0.29	16.40±0.40	22.85±2.09	18.51±0.34
<b>Ascorbic acid (AA)</b>	18.23±1.22	19.23±0.37	15.01±0.32	16.76±0.43	21.15±0.95

BEMR- Benzene extract of *M. reticulata*, CEMR- Chloroform extract of *M. reticulata*, EEMR- Ethanol extract of *M. reticulata*, AEMR- Aqueous extract of *M. reticulata*.

The increase in absorbance was then measured for 15s at 490nm using V-600 UV-Vis Spectrophotometer. Activity was expressed as a percentage inhibition of autoxidation in the tested sample, and this percentage was then expressed as µg/100mg tissue (Raju *et al.*, 2013b).

#### HPTLC STUDY

Five µL of MR ethanol extract was spotted on TLC plate. The sample was spotted as sharp band of 6 mm width using spray on technique with a Camag 100µL sample syringe on pre-coated silica gel aluminium plate 60F<sub>254</sub>, (10x10cm) using a Camag Linomat V automatic sample applicator. The bands were applied at a distance of 10mm from the bottom edge of the plate and the distance between the two bands was 11.4mm.

Prior to chromatography procedure TLC plates were washed with ethanol and activated at 60°C for 5min. Respective mobile phases (chloroform: methanol, 9:1) were added in TLC twin trough developing chamber. TLC developing chambers were closed with lids and to allow for saturation of solvent vapour. The sample loaded plates were kept in their particular mobile phase up to 70mm. The developing chamber assemblies were kept aside for saturation and development of chromatogram for 30min at room temperature. After 30min, developed plates were allowed to dry by hot air. The plates were photo documented at UV 254 and 366nm using photo documentation chamber. Finally, the plates were fixed in scanner stage and scanning was done at 200-400nm. The plates were kept in photo documentation chamber and captured

the images under, UV light at 254 and 366nm (Hassan, 2012).

#### Statistical analysis

All the values are presented as Mean ± Standard Error of Mean (SEM). Statistical significance was calculated by one way ANOVA followed by Students-Newman-Keuls comparison test  $p < 0.001$  was considered to be statistically significant. *In vitro* antioxidant activity was presented as Mean ± SEM.

#### RESULTS AND DISCUSSION

Phytochemical screening revealed that presence of phytosterol and tannin in petroleum ether extract, carbohydrate, anthraquinone, amino acid and protein in benzene extract, carbohydrate, anthraquinone and saponin in chloroform extract, carbohydrate, anthraquinone, tannins, flavonoid, saponin and phenolic compound in ethanol extract, Carbohydrate, anthraquinone, tannins, flavonoid and phenolic compound present in aqueous extract.

Acute toxicity study results revealed that EEMR and AEMR were administered separately up to 2000mg/kg BW and since these extracts did not produce any toxic symptoms of mortality, they were considered safe for further pharmacological screening.

The free radicals were scavenged by the extracts of various concentrations (1.25, 2.5, 5, 10, 20µg/mL) of the benzene, chloroform, ethanol and aqueous extracts of *M. reticulata* were tested for their antioxidant potency in different *in vitro* models and IC<sub>50</sub> values (Table I).

Table II. Effect of EEMR and AEMR on antioxidant parameters in CCl<sub>4</sub> administered rats

Animal groups	Total protein (g/dL)	MDA (nmol/mg protein)	GSH (µg/100mg tissue)	SOD (µg/100mg tissue)
Normal	6.9±0.32	1.48±3.21	6.32±0.29	9.4±0.96
CCl <sub>4</sub> (1.5mL/kg)	4.3±0.312	3.27±2.18	2.03±0.02	5.6±0.46
Silymarin(25mg/kg) + CCl <sub>4</sub> (1.5mL/kg)	6.86±0.72 <sup>a</sup>	1.4±1.3 <sup>a</sup>	6.1±0.67 <sup>a</sup>	9.6±0.79 <sup>a</sup>
EEMR(200mg/kg) + CCl <sub>4</sub> (1.5mL/kg)	6.13±0.23 <sup>a</sup>	2.1±0.31 <sup>a</sup>	4.4±0.62 <sup>a</sup>	7.6±0.67 <sup>a</sup>
EEMR(400mg/kg) + CCl <sub>4</sub> (1.5mL/kg)	7.13±0.12 <sup>a</sup>	1.51±0.132 <sup>a</sup>	5.9±0.71 <sup>a</sup>	8.5±0.73 <sup>a</sup>
AEMR(200mg/kg) + CCl <sub>4</sub> (1.5mL/kg)	5.7±0.173 <sup>a</sup>	2.4±0.73 <sup>b</sup>	4.2±0.78 <sup>a</sup>	6.7±0.13 <sup>a</sup>
AEMR(400mg/kg) + CCl <sub>4</sub> (1.5mL/kg)	6.42±0.13 <sup>a</sup>	1.9±0.78 <sup>a</sup>	4.8±0.23 <sup>a</sup>	7.8±0.23 <sup>a</sup>

Data expressed as Mean ± SEM for six observations P: <0.001 Compared to respective CCl<sub>4</sub> induced group (Group 2). The data analysed by one way ANNOVA followed by Students Newman Keuls: a-p\*\*\*<0.001, b-p\*\*<0.01. EEMR- Ethanol Extract of *M. reticulata*; AEMR – Aqueous Extract of *M. reticulata*.

Table III. Effect of EEMR and AEMR on antioxidant parameters in Paracetamol administered rats

Treatment	Total protein (g/dl)	MDA (nmol/mg protein)	GSH (µg/100mg tissue)	SOD (µg/100mg tissue)
Normal	5.8±0.62 <sup>a</sup>	2.18±1.28 <sup>a</sup>	5.94±0.34 <sup>a</sup>	9.6±1.28 <sup>a</sup>
Paracetamol (2g/kg)	3.7±0.312	5.62±2.16	2.03±0.02	5.6±0.46
Silymarin(25mg/kg) + Paracetamol (2g/kg)	5.9±0.78 <sup>a</sup>	2.24±1.013 <sup>a</sup>	6.13±0.96 <sup>a</sup>	9.4±0.043 <sup>a</sup>
EEMR(200mg/kg) + Paracetamol (2g/kg)	5.1±0.26 <sup>a</sup>	3.18±0.94 <sup>a</sup>	4.94±0.062 <sup>a</sup>	8.0±0.06 <sup>a</sup>
EEMR(400mg/kg) + Paracetamol (2g/kg)	5.6±0.51 <sup>a</sup>	2.30±0.094 <sup>a</sup>	5.85±0.71 <sup>a</sup>	9.1±0.052 <sup>a</sup>
AEMR(200mg/kg) + Paracetamol (2g/kg)	4.6±0.17 <sup>a</sup>	3.92±0.078 <sup>b</sup>	4.12±0.032 <sup>a</sup>	7.8±0.02 <sup>a</sup>
AEMR(400mg/kg) + Paracetamol (2g/kg)	5.5±0.71 <sup>a</sup>	2.98±1.52 <sup>a</sup>	5.12±0.051 <sup>a</sup>	8.3±0.05 <sup>a</sup>

Data expressed as Mean ± SEM for six observations p<0.001 Compared to respective paracetamol induced group. The data analysed by One way ANOVA followed by Students-Newman-Keuls: a-p\*\*\*<0.001, b-p\*\*<0.01. EEMR- Ethanol Extract of *M. reticulata*; AEMR – Aqueous Extract of *M. reticulata*

The scavenging effect on DPPH radical increased with the raise in concentration of each extract. The percentage inhibition of BEMR was varying from 10% with 1.25mcg/mL of the extract to 29% with 20mcg/mL of extract. Similarly the percentage inhibition of CEMR was

varying from 12% with 1.25mcg/mL of the extract to 37% with 20mcg/mL of extract. The percentage inhibition of EEMR was varying from 18% with 1.25mcg/mL of the extract to 50% with 20mcg/mL of extract. Similarly the percentage inhibition of AEMR was varying from 16% with 1.25mcg/mL of the

extract to 41% with 20mcg/mL of extract. The  $IC_{50}$  value of the BEMR, CEMR, EEMR and AEMR were calculated to be  $34.25 \pm 2.89$ ,  $28.5 \pm 1.44$ ,  $20.52 \pm 1.87$  and  $24.39 \pm 0.6$  mcg/mL while the ascorbic acid was  $18.23 \pm 1.22$  mcg/mL.

The hydroxyl radical scavenging activity (HRSA) also increased with respect to increase in concentrations of the extracts. The  $IC_{50}$  values of BEMR, CEMR, EEMR and AEMR were found to be  $28.99 \pm 0.70$ ,  $20.83 \pm 0.25$ ,  $12.99 \pm 0.25$  and  $16.40 \pm 0.40$  mcg/mL respectively, while that of ascorbic acid was  $15.01 \pm 0.32$  mcg/mL. EEMR and AEMR extracts consumed lesser concentration than ascorbic acid in scavenging hydroxyl radical. Similarly in super oxide scavenging assay SOSA, EEMR extract consumed lesser concentration ( $17.65 \pm 1.77$ ) to scavenge the free radicals than the standard antioxidant ascorbic acid ( $19.23 \pm 0.37$ ). The percentage of inhibition in SOSA scavenging assay was maximum (58%) at 20mcg/mL of EEMR and 44% with AEMR. The  $IC_{50}$  value of the BEMR and CEMR was calculated to be  $25.68 \pm 3.2$  and  $25.43 \pm 2.87$  mcg/mL. In reducing power assay (RPA), in presence of EEMR and AEMR,  $Fe^{3+}$ /ferricyanide complex is reduced to the ferrous form.

The percentage inhibition of BEMR was varying from 11% with 1.25mcg/mL of the extract to 21% with 20 mcg/mL of extract. Similarly the percentage inhibition of CEMR was varying from 17% with 1.25mcg/mL of the extract to 33% with 20mcg/mL of extract. The percentage inhibition of EEMR was varying from 29% with 1.25mcg/mL of the extract to 77% with 20 mcg/mL of extract. Similarly the percentage inhibition of AEMR was varying from 30% with 1.25mcg/mL of the extract to 61% with 20mcg/mL of extract. The  $IC_{50}$  value of the BEMR, CEMR, EEMR and AEMR were calculated to be  $48.56 \pm 3.68$ ,  $30.69 \pm 1.90$ ,  $19.17 \pm 1.44$  and  $16.76 \pm 0.43$  mcg/mL while the ascorbic acid was  $16.76 \pm 0.43$  mcg/mL. The absorbance of ferrozine- $Fe^{2+}$  complex reduced with respect to the concentration of extracts. Upon the reduction of the redox potential, in such a way that chelating effect with a metal ion which is effective like a secondary antioxidant

and thereby stabilize the oxidized form of the metal ion.

In Metal chelating assay (MCA), the percentage inhibition of BEMR was varying from 17% with 1.25mcg/mL of the extract to 40% with 20mcg/mL of extract. Similarly the percentage inhibition of CEMR was varying from 19% with 1.25mcg/mL of the extract to 44% with 20mcg/mL of extract. The percentage inhibition of EEMR was varying from 24% with 1.25mcg/mL of the extract to 65% with 20mcg/mL of extract. Similarly the percentage inhibition of AEMR was varying from 25% with 1.25mcg/mL of the extract to 54% with 20mcg/mL of extract. The  $IC_{50}$  value of the BEMR, CEMR, EEMR and AEMR were calculated to be  $23.75 \pm 1.95$ ,  $20.62 \pm 1.56$ ,  $15.47 \pm 0.49$  and  $15.51 \pm 0.34$  mcg/mL while the ascorbic acid was  $21.15 \pm 0.95$  mcg/mL.

Antioxidant potential of EEMR and AEMR on continuous 7 days oral administration to  $CCl_4$  administered rats and the assessment parameters were summarized (Table II). The pre-treatment with extracts significantly ( $P < 0.001$ ) reversed the levels of total protein, GSH and SOD near to normal.  $CCl_4$  administration causes significant ( $P < 0.001$ ) decrease in total protein, GSH, SOD and increased level of MDA were found with the  $CCl_4$  control group. After 7 days treatment to group 4 to 7 with EEMR and AEMR, both doses restored the altered antioxidant parameters to the normal group.

Administration of paracetamol (2g/Kg) markedly reduced the levels of hepatic antioxidant enzymes and reduced glutathione in the positive control group (Group 2). The MDA levels were markedly increased in the positive control group. Hepatic levels of SOD decreased to  $5.6 \pm 0.46$ , reduced glutathione to  $2.03 \pm 0.02$  and total protein to  $3.7 \pm 0.312$  in positive control animals compared with the normal control rats. Higher doses of EEMR and AEMR exhibited significant ( $p < 0.001$ ) increase in the levels of SOD, reduced glutathione and total protein levels whereas a lower dose of AEMR was less significant ( $p < 0.01$ ) on rising of total protein in extract treatment group. There was a significant ( $p < 0.001$ ) decrease in MDA levels with all the

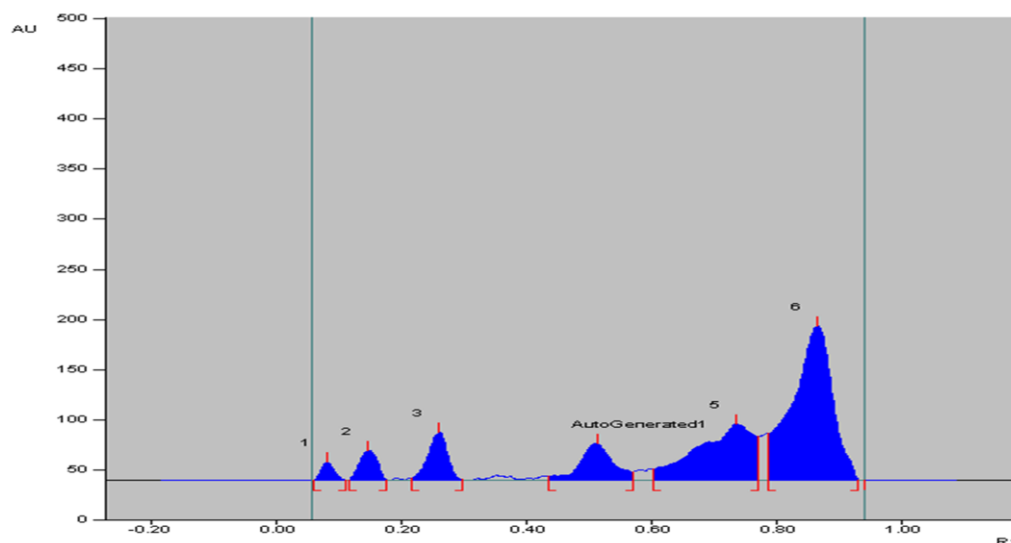


Figure I. HPTLC chromatogram of EEMR showing different peaks of phytoconstituents

Table IV. HPTLC profile of EEMR

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned Substance
1	0.06Rf	0.1AU	0.08Rf	18.2AU	5.33%	0.11Rf	0.2AU	240.0AU	1.95%	Unknow*
2	0.12Rf	0.3AU	0.15Rf	29.7AU	8.69%	0.18Rf	0.3AU	524.7AU	4.27%	Unknow*
3	0.22Rf	2.0AU	0.26Rf	48.0AU	14.03%	0.30Rf	0.6AU	908.6AU	7.39%	Unknow*
4	0.44Rf	3.9AU	0.51Rf	36.6AU	10.69%	0.57Rf	8.5AU	1224.0AU	9.95%	AutoGenerated1
5	0.60Rf	11.2AU	0.74Rf	55.7AU	16.28%	0.77Rf	43.4AU	3174.9AU	25.81%	Unknow*
6	0.79Rf	56.5AU	0.87Rf	153.8AU	44.98%	0.93Rf	0.2AU	6227.4AU	50.63%	Unknow*

doses of extracts other than the lower dose ( $p < 0.01$ ) of AEMR (Table III). The liver protein content also restored more or less to normal in extracts treated rats.

#### HPTLC study

The mobile phase selected for the HPTLC studies of EEMR was chloroform: methanol at the ratio of 9:1. By performing TLC with the mobile phase identified after trial and error method by trying different mobile Phase. Detection was performed under UV 254nm and 366nm and after spraying with Godin reagent (1% vanillin in EtOH containing 2%  $H_2SO_4$ ) and heating at 105°C. The result was depicted (Figure I and Table IV).

Antioxidant compounds may function as free radical scavengers, initiator of the complexes of pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation. Phenolic compounds and flavonoids

are major constituents of most of the plants reported possessing antioxidant and free radical scavenging activity (Satheesh *et al.*, 2010).

Polyphenols are the major plant compounds with a high level of antioxidant activity. This activity could be due to their ability to adsorb, neutralize and to quench free radicals. Their ability as free radical scavenger could also be attributed to their redox properties, presence of conjugated ring structures and carboxylic group which have been reported to inhibit lipid peroxidation (Oyedemi *et al.*, 2010). In this present study, phytochemical test of EEMR showed that presence of phenolic compounds and flavonoid and are responsible for antioxidant activity.

DPPH is widely used to evaluate the free radical scavenging effect of natural antioxidant. DPPH is a stable free radical at room temperature (Satheesh *et al.*, 2010). As the electron became paired in the presence of



free radical scavenging the absorption vanishes and the resulting discoloration stoichiometrically coincides with the number of electrons taken up. The bleaching of DPPH absorption is representative of the capacity of the test drugs to scavenge free radicals independently. The bleaching of DPPH molecules can be correlated with the number of available hydroxyl groups. We can infer that, the activity of the extract may be probably due to the presence of a substance with an available hydroxyl group. The extracts are able to reduce the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine (Kumaravel *et al.*, 2013).

Super oxides are produced from molecular oxygen due to oxidative enzymes of the body as well as by nonoxidative reactions by autocatecholamines (Bhaskar *et al.*, 2006). Overproduction of super oxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. Super oxide anion are generated in PMS-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan (Rajesh *et al.*, 2011). In cellular oxidation reactions, superoxide radicals have their initial effects magnified because they produce other kinds of cell-damaging free cells and oxidizing agents (Jayasri *et al.*, 2011).

Hydroxyl radical scavenging activity is measured as the percentage of inhibition of hydroxyl radical generated in the Fenton's reaction mixture (Bhaskar *et al.*, 2006). Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules such as protein, DNA and lipids; cause's lipids peroxidation [10]. The Fenton reaction generates hydroxyl radicals (OH) which degrade DNA deoxyribose, using  $\text{Fe}^{2+}$  salts as an important catalytic component. Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of the product (Kumaravel *et al.*, 2013).

In the reducing power assay, the more antioxidant compounds convert the oxidation form of iron ( $\text{Fe}^{3+}$ ) in ferric chloride to ferrous ( $\text{Fe}^{2+}$ ) (Mahmood Reza *et al.*, 2008). Compounds with reducing power indicate that they are electron donors and can reduce the

oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Jayanthi *et al.*, 2014). In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound.

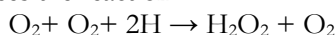
Direct reaction of a substance is not the only mechanism by which the antioxidants may display their activity. Secondary, preventive, or type 2, antioxidants act through numerous possible mechanisms. These antioxidants do not convert free radicals to more stable products but slow the rate of oxidation by several different mechanisms. One of the most important mechanisms of action of secondary antioxidants is chelation of pro-oxidant metals. Iron and other transition metals (copper, chromium, cobalt, vanadium, cadmium, arsenic, nickel) promote oxidation by acting as catalysts for free radical reactions. These redox-active transition metals transfer single electrons during changes in oxidation states. Chelation of metals by certain compounds decreases their prooxidant effect by reducing their redox potentials and stabilizing the oxidized form of the metal. Chelating compounds may also sterically hinder the formation of the metal hydroperoxide complex (Marijana *et al.*, 2011).

The total protein level reduced in  $\text{CCl}_4$  and paracetamol induced oxidation stress in rats and was due to the stabilization of endoplasmic reticulum leading to protein synthesis. The antioxidant effects exhibited by EEMR 200mg/kg and 400mg/kg were statistically similar to that observed with standard Silymarin at 25mg/kg dose level. Because of the partially reduced forms of oxygen, during oxidation in a living system, are cytotoxic, protective antioxidant enzymes (including superoxide dismutase (SODs), catalase, and glutathione peroxidases) usually exist to convert these reduced forms of oxygen to water. Superoxide dismutase is an enzyme which has an overall effect to lower the steady-state concentration of superoxide in the liver cells.

Lipid peroxidation (LPO) is a major consequence in  $\text{CCl}_4$  and paracetamol hepatotoxicity. The TBA reactive substance test showed an incidence of lipid peroxidation by

carbon tetrachloride treatment. Lipids undergo peroxidation in the presence of reactive oxygen species, which interfere with the structural appearance of these lipids and make them deleterious in the process. To regulate lipid peroxidation, there is a defence system involving antioxidant enzymes that play an imperative role in scavenging reactive oxygen species. The susceptibility of an organism to free radical stress and peroxidative damage is related to the balance between the free radical burden and the adequacy of antioxidant defences. Treatment with the extract however decreased the extent of lipid peroxidation caused by  $\text{CCl}_4$  as well as paracetamol and restored the antioxidant protection of the living cell. Lipid soluble antioxidants may therefore play a role in reducing lipid peroxidation.

Pre-treatment with MR could ameliorate the oxidative stress as demonstrated by the increase in SOD activity in the MR treated group similar to the normal group. SOD catalyses the reaction:



Using reduced glutathione as the reducing agent, glutathione peroxidases also convert hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) to water. Consequently, the oxidative stress is either reduced or eliminated. Hence, GSH constitutes the first direct line of defence against free radical activity in the cells and is a critical determinant of tissue susceptibility to oxidative damage.

The extract may therefore have precursors to or constitute some lipid soluble antioxidants as well as have significant regenerative or de novo synthesis effect on glutathione peroxidase (GPx) and reduced glutathione (GSH).

A potential of the antioxidant property underlying MR may be attributed to the antioxidative constituents (Donatus *et al.*, 1990).

HPTLC analyses were performed to identify chromatographic markers and to compare the chromatographic fingerprints of MR with authentic materials (Olivier *et al.*, 2007). HPTLC study confirmed that ethanol extracts *M. reticulata* possess six phytochemical constituents which were shown fingerprints with respective  $R_f$  values from 0.08 to 0.87. To justify the antioxidant potency report, there is a

need for isolation and identification of each constituent, is essential which confirm the respective pharmacological activity.

## CONCLUSION

The present study revealed antioxidant property may be due to the phytoconstituents such as a flavonoid and phenolic compound in MR. Ethanol extract was found more potent *in vitro* and *in vivo* antioxidant scavenging. So ethanol extract of MR may be used as natural antioxidant and can be used for curing various ailments caused by free radicals. It is recommended that phytoconstituents present in MR can be identified individually and its more activities can be revealed in future studies.

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