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ASSESSMENT OF FREE RADICAL SCAVENGING ACTIVITY AND ANTIOXIDANT MEDIATED HEPATOPROTECTIVE EFFECTS OF *MALLOTUS ROXBURGHIANUS* MUELL. IN DOXORUBICIN INDUCED OXIDATIVE STRESS IN SWISS ALBINO MICE

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Keywords:

Mallotus roxburghianus, Hepatoprotection, Free radical scavenging, Antioxidants, Lipid peroxidation

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ABSTRACT: The aim of this study was to investigate the phytochemical constituents and therapeutic potential of various solvent extracts of *Mallotus roxburghianus* both *in-vitro* and *in-vivo.* Free radicals scavenging activities of various extracts were determined in a cell free system and the hepatoprotective effects was investigated in doxorubicin induced oxidative stress in Swiss Albino mice by estimating various antioxidant activities using standard methodology. Different extracts of *M. roxburghianus* inhibited the generation of ABTS (2, 2'-azino-bis-(3- ethylbenzothiazoline- 6- sulfonic acid), DPPH (1, 1-diphenyl-2-picryl-hydrazyl) and superoxide anions in a concentration dependent manner and the aqueous extract showed the highest scavenging activities with IC_{50} of 87.38 ± 0.69 µg/ml, 3.24 ± 0.21 µg/ml and 92.65 ± 1.18 µg/ml for ABTS, DPPH and superoxide radicals, respectively. Phytochemical analysis revealed that aqueous extract possessed the highest phenolic (347.6 ± 2.9 mg GAE/g dry extract) and flavonoid (68.80 ± 0.11 mg quercetin/g dry extract) contents. The free radicals scavenging activity was significantly correlated with phenolic (r^2 = 1.00; $p<0.001$) and flavonoid (r^2 = 1.00; $p<0.001$) contents for various extracts. Aqueous extract was found to exhibit the highest reducing power and its anti-hemolytic activity was also observed to be the most potent among various extracts. Treatment of mice with aqueous extract of *M. roxburghianus* prior to doxorubicin administration significantly elevated the liver glutathione contents, and activities of glutathione-s-transferase, catalase and superoxide dismutase followed by a decline in the doxorubicin-induced lipid peroxidation. Our study indicates that *M. roxburghianus* extracts scavenge different free radicals and the most potent aqueous extract possesses antihemolytic and hepatoprotective activities.

INTRODUCTION: Reactive oxygen species (ROS) including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) are produced from molecular oxygen during normal cellular metabolism, especially in the organisms that are using oxygen for energy production.

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At low to moderate concentrations, ROS are required in modulating various physiological functions such as gene expression, cellular growth and defense against infection [1]. Apart from indigenous sources the exogenous factors including cigarette smoke, air pollutants, ozone exposure, hyperoxia, radiation, ultraviolet light, certain drugs, pesticides and industrial solvents also generate free radicals [2]. ROS exceeding the ability of the organism to mount an antioxidant defence against them result in oxidative stress ensuing tissue damage, which may be involved in several diseases including coronary heart disease, neuro-degenerative disorders, diabetes, arthritis, inflam-

mation, lung damage and cancer [3]. Although cells are equipped with an impressive repertoire of antioxidant enzymes as well as small antioxidant molecules, which are able to take care of the normal ROS produced during various metabolic processes however, these agents may not be sufficient enough to normalize the redox status during increased oxidative stress [4]. Therefore, to maintain optimal body function, exogenous antioxidants supplementation may be required to restore the redox homeostasis in cells.

Many modern drugs used for the treatment of several diseases have been isolated from plants before they were synthesized chemically and the secondary metabolites of plants have gained importance as natural antioxidants [5]. It has also been reported that consumption of natural antioxidants reduced the risk of cancer and many chronic diseases [6]. Epidemiological studies also indicated that dietary intake of antioxidant substances from plant is inversely associated with mortality from coronary heart disease [7]. Doxorubicin (DOX) is a chemo-therapeutic agent extensively used for the treatment of solid and hematopoietic tumors. However, cancer therapy with DOX results in cardiotoxicity and hepatotoxicity [8]. Many evidences indicate that DOX-induced toxicity is mainly caused by increased oxidant production, stimulation of lipid peroxidation, and subsequent alteration of cellular membrane integrity [9]. Therefore, we speculated that enhancement of antioxidants may be able to largely prevent DOX-triggered toxicity.

Mallotus roxburghianus (MR) is a shrub to small tree belonging to the family Euphorbiaceae. It is locally called as Zawngtenawhlung and found widely in Mizoram, India, typically in the tropical evergreen forests and mixed bamboo forests. It is also found to be distributed within the Chittagong Hill tracts of Bangladesh and Myanmar. *Mallotus roxburghianus* is traditionally used by the local people of Mizoram for the treatment of various ailments including fever, hypertension, inflammation and diabetes [10]. From the genus *Mallotus, M. philippenensis* has been reported to possess antioxidant and anti-plasmodial [11], wound healing [12] and anti-inflammatory activities [13]. The anti-diabetic and antioxidant property of *M. oppositifolius* have also been reported [14].

The leaf extract of *M. repandus* has been also reported to exert analgesic and anti-inflammatory effects [15]. Methanol extract of *M. roxburghianus* has also been reported to accelerate testicular recovery from the damaging influence of hyperthermia and elevated the levels of antioxidant enzymes in alloxan diabetic rats [16, 17]. Antioxidant capacity is widely used as a parameter to assess medicinal potential of natural products or their bioactive components. *In vitro* screening methods have been used for further in-depth chemical elucidation and pharmacological investigations of medicinal plants [18]. Thus, the present study aimed to investigate the free radical scavenging activity of *M. roxburghianus in-vitro* and its antioxidant potential in mouse liver.

MATERIALS AND METHODS:

Chemicals: Gallic acid, Quercetin, nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), phenazine metho sulfate (PMS), 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH), 2-deoxyribose, and 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were obtained from HiMedia Laboratories Pvt., Ltd., (Mumbai, India). Thiobarbituric acid (TBA) and 5, 5' dithio 2-nitrobenzoic acid (DTNB) were obtained from Merck Specialities Pvt., Ltd. (Mumbai, India). Doxorubicin (Getwell Oncology Pvt., Ltd., Haryana, India) was purchased from local pharmacy.

Collection of Plant Material and Preparation of Extracts: *M. roxburghianus* was collected from Lengpui, Mizoram, India. It was identified and authenticated by the Department of Horticulture and Aromatic Medicinal Plants, Mizoram University, Aizawl. The leaves were then shade dried at room temperature for 30 days and pulverized by using mixer grinder and kept in an air tight plastic container prior to the extraction. The dried powdered leaves were then sequentially extracted with petroleum ether, chloroform, methanol and distilled water according to the increasing polarity using Soxhlet apparatus at their respective boiling points until the solvent becomes colorless. The liquid extracts were filtered using Whatman no. 1 filter paper and concentrated using a rotary evaporator (Buchi, Germany) under reduced pressure at 40 °C for 5 h. The extracts obtained were then collected and stored at -20 °C until use.

Determination of Free Radical Scavenging Activity: The ability of different extracts of *Mallotus roxburghianus* to inhibit the generation of various free radicals *in-vitro* was carried out as described below.

DPPH Radical Scavenging Activity: DPPH radical scavenging activity was carried out according to Leong and Shui [19] with minor modifications. To different concentrations of various extracts of *M. roxburghianus* (0.5 ml, 1 - 1000 µg/ml), 1 ml of methanol solution of 0.1 M DPPH was added and the mixture was allowed to stand in the dark for 30 min. The absorbance was measured at 523 nm using UV-visible spectrophotometer (SW 3.5.1.0. Bio-spectrometer, Eppendorf India Ltd., Chennai). Methanol was utilized as blank. The results were compared with that of the control prepared as above without sample. The antioxidant activity of the extract was expressed as IC_{50}, which was defined as the concentration (µg/ml) of extract that inhibited the formation of DPPH radicals by 50%. Ascorbic acid was used as the positive control and each study was performed in triplicate. The scavenging activity was then estimated based on the percentage of DPPH radicals scavenged using the formula:

$$\text{Scavenging (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound.

Determination of Superoxide Radical Scavenging Activity: Superoxide scavenging activity was determined by the nitrobluetetrazolium (NBT) reduction method [20] with minor modifications. The reaction mixture consisted of 0.5 ml of NBT solution (1 M NBT in 100 mM phosphate buffer, pH-7.4), 0.5 ml NADH solution (1 M NADH in 100 mM phosphate buffer, pH-7.4) and 0.1 ml of different extracts of *M. roxburghianus* (dissolved in respective solvent) and ascorbic acid (dissolved in 50 mM phosphate buffer, pH-7.4). The reaction was started by adding 100 µl of PMS solution (60 µM PMS in 100 mM phosphate buffer, pH-7.4) to the mixture. The tubes were then uniformly illuminated with an incandescent visible light for 15 min after which optical density was measured at 530 nm. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of control and the experimental tubes. The ability to scavenge the superoxide radical was calculated using the following formula:

$$\text{Scavenging (\%)} = (1 - A_e / A_o) \times 100$$

Where A_o is absorbance without sample and A_e is absorbance with sample.

ABTS Radical Scavenging Activity: ABTS assay was performed according to the method of Re *et al*., [21]. A stock solution was prepared by mixing equal volumes of 7 mM ABTS and 2.45 mM potassium per sulphate followed by incubation for 12 h at room temperature in the dark to yield a dark-colored solution containing $ABTS^{\bullet+}$ radicals. A working solution was prepared freshly before each assay by diluting stock solution with 50% methanol for an initial absorbance of about 0.70 (±0.02) at 745 nm. Free radical scavenging activity was then assessed by mixing 150 µl of different fractions of various extracts of *M. roxburghianus* (1 - 1000 µg/ml, dissolved in their respective solvents) with 1.5 ml of ABTS working standard. The decrease in absorbance was measured exactly 1 min after mixing the solution up to 3 min. Data for each assay was recorded in triplicate. Ascorbic acid was used as positive control. The scavenging activity was estimated based on the percentage of ABTS radicals scavenged using the formula:

$$\text{Scavenging (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound.

Reducing Power: The reducing power of various extracts was determined using a method described earlier [22] with minor modifications. Different extracts of *Mallotus roxburghianus* dissolved in their respective solvent were mixed with 2.5 ml of 0.2 M phosphate buffer (pH- 6.6) and 2.5 ml of 1% potassium ferricyanide solution. The mixture was incubated at 50 °C for 20 min after which 2.5 ml of 10% TCA was added to the mixture.

The mixture was then centrifuged at 3000 rpm for 10 min after which 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 1% ferric chloride solution. Absorbance was

measured at 700 nm. Increasing absorbance of the reaction mixture indicates increase in reducing power.

Phytochemical Analysis:

Determination of Total Phenolic Content: The total phenolic content was determined using the method described earlier [23]. 1 ml of *Mallotus roxburghianus* extracts dissolved in their respective solvent (0.25 - 4.0 mg/ml) was mixed with 5 ml Folin-Ciocalteu's reagent (diluted ten-fold). The mixture was then incubated for 5 min before addition of sodium carbonate (4 ml, 0.115 mg/ml). After 2 h of incubation in the dark at room temperature absorbance was measured at 765 nm. Calibration curve was also prepared by mixing methanol solution of gallic acid (1 ml, 0.25- 4.0 mg/ml) with the reagents above and absorbance was recorded at 765 nm. All determinations were carried out in triplicate. The total content of phenolic compounds in the extract was expressed as gallic acid equivalents (GAE) mg/g of the dry extract.

Determination of the Total Flavonoid: Total flavonoid content was determined by the method described earlier [24]. Briefly, 0.25 ml of different fractions of the extracts (0.25 - 4.0 mg/ml; dissolved in respective solvent) and quercetin standard solution was mixed with 1.25 ml of distilled water in a test tube, followed by the addition of 75 µl of 5% (w/v) sodium nitrite solution. After 6 min, 150µl of 10% (w/v) aluminium chloride solution was added and allowed to stand for next 5 min before the addition of 0.5 ml of 1 M NaOH.

The mixture was then made up to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm. The results were expressed as mg of quercetin equivalent of total extractable compounds. All estimations were performed in triplicate.

***Ex-vivo* Antioxidant Assay:**

Anti-hemolytic Activity: The antioxidant activity of different extracts of *Mallotus roxburghianus* was measured according to the inhibition of erythrocyte hemolysis [25]. Blood was collected from the heart of Swiss albino mice of same age group (10 - 12 w) and body weights (25 - 27 g) by means of heart puncture in a heparinized tube. The mice erythrocyte hemolysis was performed with H_2O_2 as free radical initiator. To 0.5 ml of 5% (v/v) suspension of RBC in PBS, 0.4 ml (0.5 mg/ml) of different extracts and 100 µl of 1 mol/L H_2O_2 was added. The reaction mixtures were shaken gently while being incubated at 37 °C for 3 h. After incubation the reaction mixture was again diluted with 4 ml of PBS and centrifuged at 2000 rpm for 10 min. The supernatant was collected and optical density was measured at 540 nm. The rate of inhibition was calculated using the formula:

$$\text{Inhibition rate (\%)} = [1 - (A_1 - A_2)/ A_0] \times 100$$

Where, A_0 is the absorbance of control (without sample), A_1 is the absorbance in the presence of the extract and A_2 is the absorbance without sample (RBC).

Effects of MRAE on Antioxidants Activities and Lipid Peroxidation: Since aqueous extract (MRAE) showed the best results in *in-vitro* assays, it was desired to determine various antioxidants and lipid peroxidation in the mouse liver.

Animals: Swiss Albino mice were procured from the Department of Zoology, North Eastern Hills University, Shillong, India. The animal care and handling was carried out according to the guidelines issued by world health organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). The animals were acclimatized to standard environmental conditions of temperature (22 ºC ± 5ºC) for 12 h light-dark cycles (Frontier Euro Digital Timer, Taiwan) at animal care facility at the Department of Zoology, Mizoram University, Aizawl, India. Four animals were kept in a sterile polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The animals were fed with commercially available food pellets and water *ad libitum*. The study was approved by the Institutional Animal Ethical Committee, Mizoram University, India (MZUIAEC17-18-15).

Experimental Design: The Swiss albino mice were randomized into six groups containing six animals each. The experimental groups were organized as follows:

Group I: The animals of this group consisted of the normal untreated control kept on a normal diet.

Group II (DOX Group): The animals of this group were treated intraperitoneally (i.p.) with freshly prepared doxorubicin (DOX) at a dosage of 5 mg/kg (dissolved in d. H_2O). Doxorubicin has been employed by several researchers to induce oxidative stress in animals [26].

Group III, IV and V (DOX + MR Group): The animals of these groups received aqueous extract of *M. roxburghianus* (MRAE) at a dose of 100, 150 and 200 mg/kg b. wt., respectively followed by a single dose of DOX (5 mg/kg, ip).

Group VI: The animals of this group were treated with MRAE at a dose of 200 mg/kg/day. Treatments were carried out orally for 7 consecutive days and on the 8th day all animals were sacrificed by cervical dislocation.

Processing of Liver Tissues for Biochemical Analysis: Liver tissues were immediately excised and homogenized with ice cold buffer (5 mM EDTA, 0.15 M NaCl, pH 7.4) in a glass homogenizer to produce 5% (w/v) homogenate. The homogenates were centrifuged for 30 min at 10,000 rpm at 4 °C and the supernatant were frozen at -80 °C in aliquots until used for biochemical assays. The protein content of the supernatant was determined using the standard method [27]. The following biochemical estimations were carried out.

Glutathione Assay: Glutathione (GSH) contents were measured using the method of Moron *et al*., [28] GSH was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm. The absorbance of the sample was read against blank at 412 nm. GSH concentration has been calculated from the standard graph and expressed in µmol/mg protein.

Glutathione-s-transferase Assay: Glutathione-s-transferase (GST) was measured by the method described earlier [29]. Briefly 50 µl of 20 mM CDNB was added to 850µl of 0.1 M phosphate buffer (pH 6.5) and incubated for 10 min at 37 °C. Then, 50 µl each of 20 mM GSH and tissue homogenate were added to the mixture. For blank distilled water was added instead of tissue homogenate. The absorbance of the sample was measured at 1 min interval for 6 min at 340 nm. GST activity was measured as:

GST activity = (OD of test – OD of blank/9.6 × vol. of test sample) × 1000

Where, 9.6 is the molar extinction coefficient for GST.

Catalase Assay: Activity of catalase (CAT) in the liver homogenate was measured by following the rate of disappearance of H_2O_2 at 240 nm [30]. Decomposition of H_2O_2 can be followed directly by the decrease in absorbance. The enzyme activity has been expressed in unit/mg protein. The catalytic activity of CAT at a time interval of 15 s was calculated by the following formula:

$$K = 0.153(\log A_0/A_1)$$

Where, A_0 is the absorbance at 0 sec and A_1 is the absorbance at 15 sec.

Superoxide Dismutase Assay: Superoxide dismutase (SOD) activity was determined by the nitrobluetetrazolium reduction method [31]. Briefly, 100 µl of homogenate and 186 µM PMS were mixed with 300 µl of 3 mM NBT and 200 µl of 780 µM NADH. The mixture was incubated for 90 s at 30 °C and 1 ml of acetic acid and 4 ml of n-butanol were added to stop the reaction. The blank consisted of all the reagents, except the liver homogenate. The absorbance of test and blank were measured at 560 nm and the enzyme activity has been expressed in unit (1 unit = 50% inhibition of NBT reduction) / mg protein.

Lipid Peroxidation Assay: Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids, serve as a convenient index for determining the extent of peroxidation reaction of lipids. MDA has been identified as the product of lipid peroxidation (LOO) that reacts with TBA to give a red species absorbing at 535 nm. LOO was estimated by the method described earlier [32].

Briefly, tissue homogenate was added to a mixture of 10% TCA, 0.8% TBA and 0.02N HCl in 1:2 ratio. The mixture was boiled for 10 min in a boiling water bath, cooled immediately at room temperature and centrifuged at 1000 rpm for 10 min. The supernatant was collected and its

absorbance was read at 535 nm against blank. The blank contained all the reagents minus the cell homogenate substituted with distilled water. The MDA concentration of the sample was calculated using the extinction coefficient of $1.56 \times 10^6\ M^{-1}cm^{-1}$.

Statistical Analyses: Data are expressed as mean ± standard error of mean. The IC_{50} values were calculated using the formula $Y = 100 \times A_1/(X+A_1)$ where $A_1 = IC_{50}$, Y = response (Y = 100% when X = 0), X = inhibitory concentration. One-way analysis of variance (ANOVA) was performed to test significant variations on phytochemical contents of various extracts and *in-vivo* antioxidants activity of treatment groups followed by Tukey multiple comparisons of means. Experimental results were further analyzed for Pearson correlation coefficient between phenolics, flavonoids and different antioxidant assays. SPSS ver. 21.0 software (SPSS Inc, Chicago, Illinois, USA) and Graphpad prism software ver. 6.0 were used for the statistical analyses. A $p<0.05$ was considered statistically significant.

RESULTS:

DPPH Radical Scavenging Activity: *In-vitro* antioxidant assay of *Mallotus roxburghianus* extracts revealed the presence of antioxidant potential. Various extracts of *Mallotus roxburghianus* showed a concentration dependent increase in the scavenging of DPPH radicals as indicated by the increasing discoloration of DPPH. Maximum scavenging effect was seen at a concentration of 50 µg/ml for aqueous extract (MRAE) and 100 µg/ml for methanolic extract (MRME) that plateaued thereafter. The chloroform extract (MRCE) required higher concentrations of 800 µg/ml to exert the highest scavenging activity at **Fig. 1**. The comparison between different extracts revealed that the MRAE was most effective as its IC_{50} was lowest (3.24 ± 0.21 µg/ml) followed by MRME (IC_{50}; 8.52 ± 0.42 µg/ml) and MRCE (IC_{50}; 321.22 ± 10.91 µg/ml) **Table 1**. The DPPH scavenging activity of various extracts of *M. roxburghianus* showed a significant positive correlation with phenolic ($r^2 = 0.95$; $p<0.001$) and flavonoid contents ($r^2 = 0.99$; $p<0.001$).

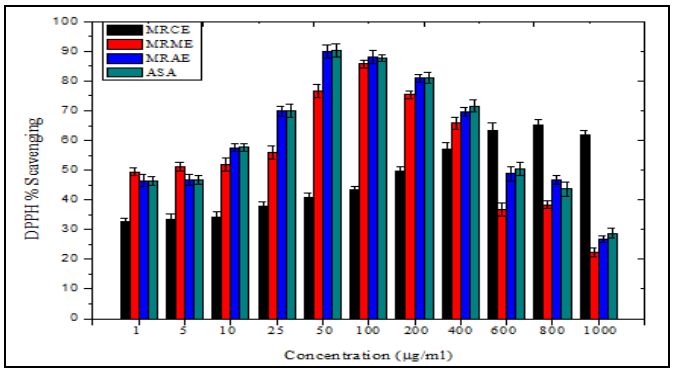

FIG. 1: DPPH SCAVENGING ACTIVITY OF VARIOUS EXTRACTS OF *M. ROXBURGHIANUS* AND THE STANDARD ASCORBIC ACID. Values are expressed as Mean ± SEM, n = 3, $p<0.05$

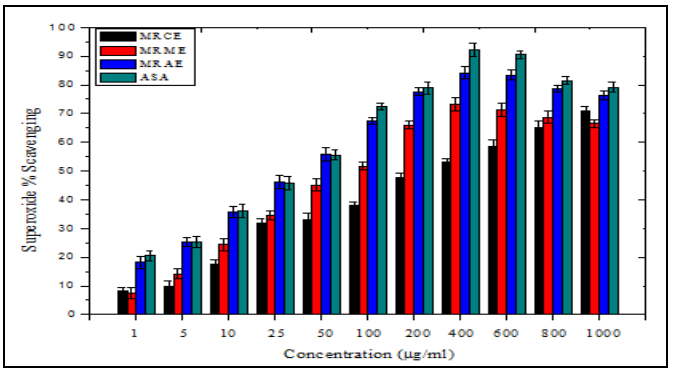

FIG. 2: SUPEROXIDE RADICAL SCAVENGING ACTIVITY OF *M. ROXBURGHIANUS* AND THE STANDARD ASCORBIC ACID Values are expressed as Mean ± SEM, n = 3, $p<0.05$

Superoxide Radical Scavenging Activity: Superoxide radical scavenging activity of various extracts of *Mallotus roxburghianus* showed a concentration dependent inhibition of superoxide radical generation. Maximum $O_2^{\bullet-}$ scavenging activity was observed at a concentration of 400 µg/ml for MRAE and MRME that declined with a further increase in their concentration. The scavenging activity of MRCE increased up to 1000 µg/ml the highest concentration evaluated **Fig. 2**. The MRAE possessed the highest superoxide radical scavenging activity (IC_{50}; 92.65 ± 1.18 µg/ml) followed by MRME (174.42 ± 2.41 µg/ml) and MRCE (483.19 ± 1.42 µg/ml) **Table 1**. A significant positive correlation of phenolic ($r^2 = 0.99$; $p<0.001$) and flavonoid contents ($r^2 = 0.98$; $p<0.001$) was found for superoxide radical scavenging activity for different extracts.

TABLE 1: IC_{50} (µg/ml) OF VARIOUS EXTRACTS OF *M. ROXBURGHIANUS* AND THE STANDARD ASCORBIC ACID (ASA)

Extracts	ABTS	DPPH	Superoxide
MRAE	87.38 ± 0.69^b	3.24 ± 0.21^a	92.65 ± 1.18^a
MRME	108.58 ± 0.77^c	8.52 ± 0.42^c	174.42 ± 2.42^b
MRCE	-	321.22 ± 10.91^d	483.19 ± 1.42^c
ASA	65.98 ± 1.05^a	4.98 ± 0.66^b	87.40 ± 4.55^a

Values are expressed as Mean ± SEM, n=3; Means not sharing the same letter are significantly different at $p < 0.05$.

ABTS Radical Scavenging Activity: $ABTS^{\bullet+}$ radical scavenging activity of *Mallotus roxburghianus* extracts increased in a concentration dependent manner as indicated by discoloration of the $ABTS^{\bullet+}$ with the rise in the concentrations of different extracts of *Mallotus roxburghianus*, which was measured spectrophotometrically at 745 nm. The percentage inhibition of absorbance was calculated and plotted as a function of concentration of the extract and of standard ascorbic acid **Fig. 3**.

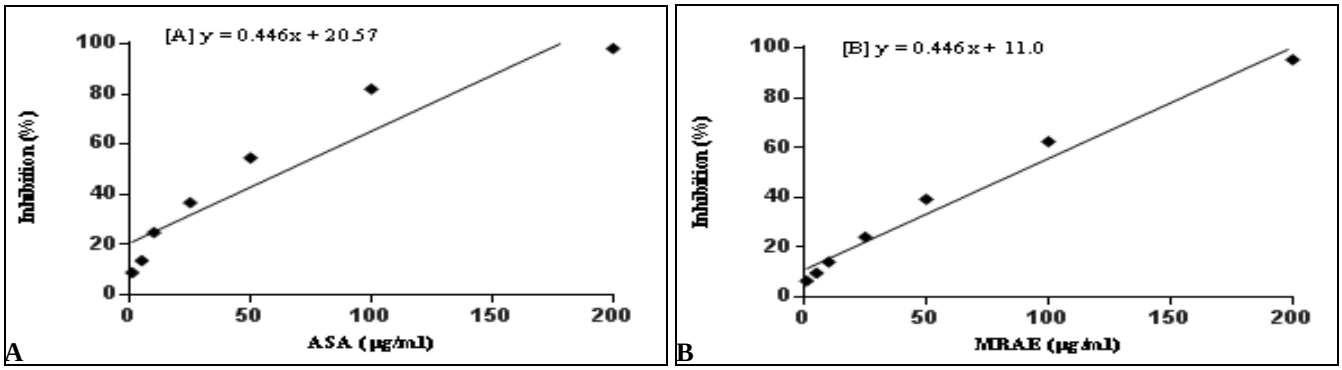

FIG. 3: EFFECTS OF [A] STANDARD ASCORBIC ACID (ASA) [B] AQUEOUS EXTRACT OF *MALLOTUS ROXBURGHIANUS* (MRAE) ON ABTS RADICAL CATIONDECOLORIZATION ASSAY. THE PERCENTAGE OF INHIBITION IS PLOTTED AGAINST CONCENTRATION OF SAMPLE

Maximum $ABTS^{\bullet+}$ scavenging activity was recorded at a concentration of 200 µg/ml for MRAE and MRME that declined thereafter, however, the greatest $ABTS^{\bullet+}$ scavenging activity of MRCE was found at 1000 µg/ml **Fig. 4**. Among all the three extracts evaluated the MRAE exhibited the highest $ABTS^{\bullet+}$ scavenging activity with a lowest IC_{50} of 87.38 ± 0.69 µg/ml when compared to MRME (IC_{50}; 108.58 ± 0.77 µg/ml) while the IC_{50} of MRCE was indeterminable within the given concentration. Significant positive correlation between phenol (r^2 = 1.00; p<0.001) and flavonoid (r^2 = 1.00; p<0.001) contents for $ABTS^{\bullet+}$ scavenging activity was obtained for various extracts.

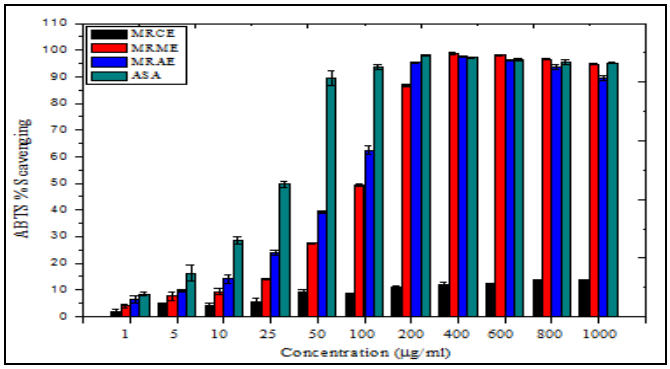

FIG. 4: ABTS RADICAL SCAVENGING ACTIVITY OF *M. ROXBURGHIANUS* AND THE STANDARD OF ASCORBIC ACID. Values are expressed as Mean ± SEM, n=3, p < 0.05.

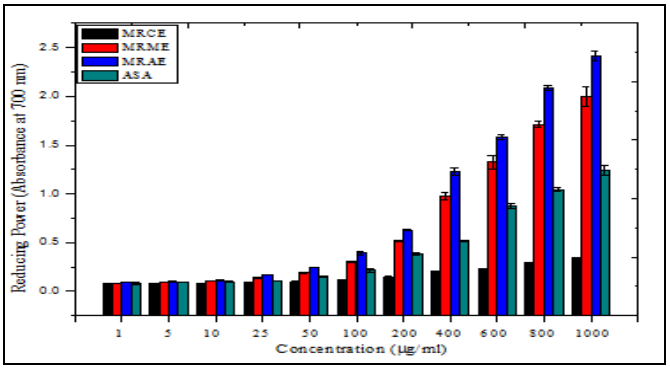

FIG. 5: REDUCING POWER OF VARIOUS EXTRACTS *M. ROXBURGHIANUS* AND THE STANDARD ASCORBIC ACID (ASA). Values are expressed as Mean ± SEM, n=3.

Reducing Power: The reducing power of various extracts of *Mallotus roxburghianus* was determined by measuring the transformation of Fe^{3+} into Fe^{2+}. The reducing activity of *Mallotus roxburghianus* extracts increased in a concentration dependent manner **Fig. 5**. The highest reducing activity was recorded for MRAE (2.413 ± 0.02) followed by MRME (1.990 ± 0.05 µg/ml) and MRCE (0.333 ± 0.05) at 1 mg/ml. The reducing activity of MRAE and MRME were found to be higher than ascorbic acid (1.24 ± 0.05), which was used as a standard.

Total Phenolic Contents: The total phenolic contents of *Mallotus roxburghianus* extracts increased in a concentration dependent manner **Fig. 6**, MRAE has a significantly higher (p<0.001)

amount of total phenols (347.6 ± 2.9 mg gallic acid equivalent/g dry extract) than that of MRME (304.3 ± 0.33 mg gallic acid equivalent/g dry extract) and MRCE (216.3 ± 1.45 mg gallic acid equivalent/g dry extract).

Total Flavonoids: The amount of total flavonoids also increased in a concentration dependent manner **Fig. 7**. The highest amount ($p<0.001$) of total flavonoids were recorded for MRAE (68.80 ± 0.11 mg quercetin equivalent/g dry extract) followed by MRME extracts (66.66 ± 0.08 mg quercetin equivalent/g dry extract). The flavonoids were least for MRCE, where the amount was only 30.30 ± 0.05 mg quercetin/ g dry extract.

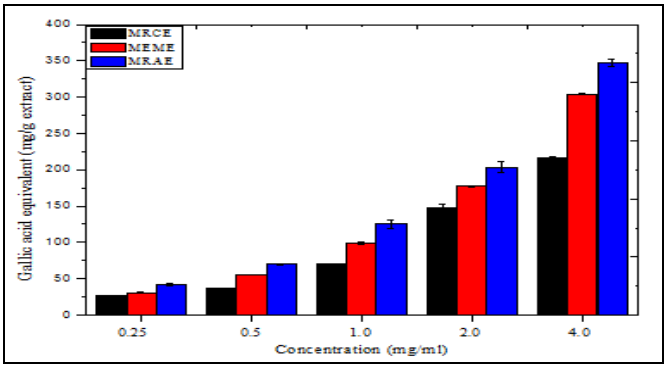

FIG. 6: TOTAL PHENOLIC CONTENTS OF VARIOUS EXTRACTS OF *M. ROXBURGHIANUS* DETERMINED AS GALLIC ACID EQUIVALENT. Values are expressed as Mean ± SEM, n=3

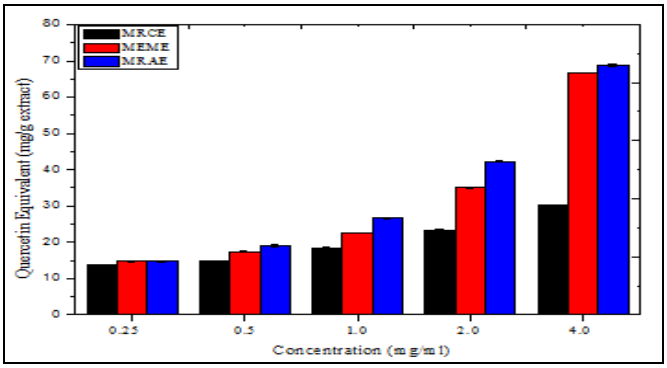

FIG. 7: FLAVONOID CONTENTS OF VARIOUS EXTRACTS OF *M. ROXBURGHIANUS* DETERMINED AS QUERCETIN EQUIVALENT. Values are expressed as Mean ± SEM, n=3

Anti-hemolytic Activity: The anti-hemolytic activity of various extracts of *M. roxburghianus* was determined and a maximum anti-hemolytic activity was observed at a concentration of 0.5 mg/ml for all the extracts that declined thereafter **Fig. 8**. Despite the non-significant variations among various extracts, at a concentration of 0.5 mg/ml, MRAE showed the highest inhibition activity against erythrocyte hemolysis with an inhibition rate of 95.39% followed by MRME and MRCE with the inhibition rate of 90.66% and 85.66% respectively **Fig. 8**.

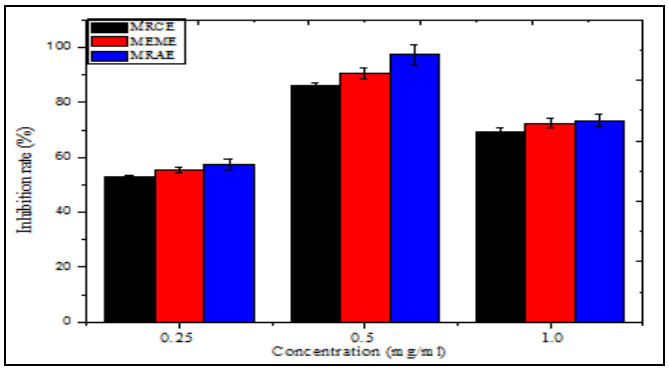

FIG. 8: THE ANTI-HEMOLYTIC ACTIVITY OF VARIOUS EXTRACTS OF *M. ROXBURGHIANUS* IN *EX VIVO* ANALYSIS. Values are expressed as Mean ± SEM, n=3

Effects of MRAE on Antioxidants Activities and Lipid Peroxidation: Intraperitoneal administration of DOX reduced the GSH contents (52.95%) and GST (55.58%), SOD (40.58%) and CAT (82.30%) activities in the liver of Swiss albino mice in comparison with the normal control mice **Fig. 9A-D**. The treatment of mice with 100, 150 and 200 mg/kg MRAE for consecutive 7 days before DOX administration resulted in a significant rise in the GSH concentration and activities of GST, SOD and CAT enzymes in the liver of mice **Fig. 9A-D**.

The DOX administration led to a significant ($p<0.001$) rise in the MDA levels in mouse liver when compared to the normal untreated control. Treatment of mice with 100, 150 and 200 mg/kg) MRAE prior to DOX administration results in significant alleviation of MDA level ($p<0.001$) as compared to DOX treatment alone **Fig. 9E** and maximum reduction of MDA (61.97%) was observed at a dose of 200 mg/kg b.wt. Treatment of mice with 200 mg/kg MRAE alone did not induce any significant change in the antioxidants activities and MDA level in the liver of Swiss albino mice.

DISCUSSION: The reduction of methanolic DPPH solution to non-radical form DPPH-H has been extensively used to evaluate antioxidative property of certain compounds. Various extracts of *M. roxburghianus* effectively reduced the stable radical DPPH to the yellow-colored diphenyl-picrylhydrazine. The cysteine, glutathione, ascorbic

acid, tocopherol, polyhydroxyl aromatic compound have been reported to reduce DPPH due to their hydrogen donating ability [33]. The scavenging activities of different *Mallotus roxburghianus* extracts increased in a concentration dependent manner and MRAE has shown significantly higher scavenging activity than the standard ascorbic acid used. In an earlier study the methanol extract of *Mallotus roxburghianus* has been reported to scavenge DPPH free radicals *in-vitro* [34]. Several plant extracts including *Agele marmelos, Croton caudatus, Oroxylum indicum* and *Syzygium cumini* have been reported to effectively scavenge the DPPH free radicals *in-vitro* [35, 36, 37].

Superoxide ($O_2^{\bullet-}$) radical produced as result of incomplete metabolism of oxygen serves as a precursor for more reactive oxygen species, which contributes to the tissue damage and various diseases. Superoxide can decompose to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, which are more reactive than $O_2^{\bullet-}$ and initiate lipid peroxidation into cellular components. Thus, neutralization of superoxide radical will inhibit the chain of ROS generation and protect the cells from the oxidative stress. Many flavonoids have been reported to scavenge superoxide anion radical earlier [38]. Various extracts of *Mallotus roxburghianus* have been found to inhibit the production of superoxide radical in a concentration dependent manner and the $O_2^{\bullet-}$ scavenging effect of MRAE was found to be similar to that of the standard ascorbic acid. The plant extracts of *Agele marmelos, Croton caudatus, Oroxylum indicium, Syzygium cumini, Schima wallichi* have been reported to inhibit the generation of $O_2^{\bullet-}$ radicals earlier [35, 36, 37].

The interaction of ABTS and potassium ferricyanide results in the production of a blue colored $ABTS^{\bullet+}$. The conversion of this preformed $ABTS^{\bullet+}$ to ABTS by various extracts of *Mallotus roxburghianus* occurred in a concentration dependent manner. The effectiveness of quenching $ABTS^{\bullet+}$ depends on the molecular weight of phenolic compounds, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups [39]. The $ABTS^{\bullet+}$ scavenging activity of *Mallotus roxburghianus* extracts might be due to the presence of high molecular weight phenolics such as catechin, and rutin derivatives. Although the scavenging activity of ascorbic acid was more pronounced as compared to *Mallotus roxburghianus* extracts, the study however, demonstrates the extracts as potent antioxidants. The reducing power of *Mallotus roxburghianus* extracts were evaluated by measuring the transformation of Fe^{3+}into Fe^{2+}. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. As shown in **Fig. 5**, the reducing power of *Mallotus roxburghianus* extracts was compared with the standard ascorbic acid, MRAE and MRME were found to be superior indicating their potential as good antioxidants. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition - metal ion catalysts, decomposition of peroxides, and prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [40].

The phytochemical analysis conducted on various extracts of *M. roxburghianus* revealed the presence of significant amounts of flavonoid and phenolic compounds. Because of their conjugated ring structures and presence of hydroxyl groups; many phenolic compounds have the potential to function as antioxidants by scavenging or stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species [41]. The protective effect of *Mallotus roxburghianus* extracts was evaluated on hemolysis caused due to oxidative damage of erythrocytes membrane. Erythrocytes were considered to be the major target of free radicals, leading to membrane damage and consequently to hemolysis [42].

As shown in **Fig. 8**, various extracts of *Mallotus roxburghianus* exhibited potent anti-hemolytic activity and MRAE possessed the highest inhibitory activity against mice erythrocytes hemolysis. The anti-hemolytic activity of *Mallotus roxburghianus* extracts could be due to the presence of significant amounts of phenolic and flavonoids compounds. Certain phenolic compounds have been reported to partition cell membrane, hindering diffusion of free radicals and consequently decreased the kinetics of free radicals reactions.

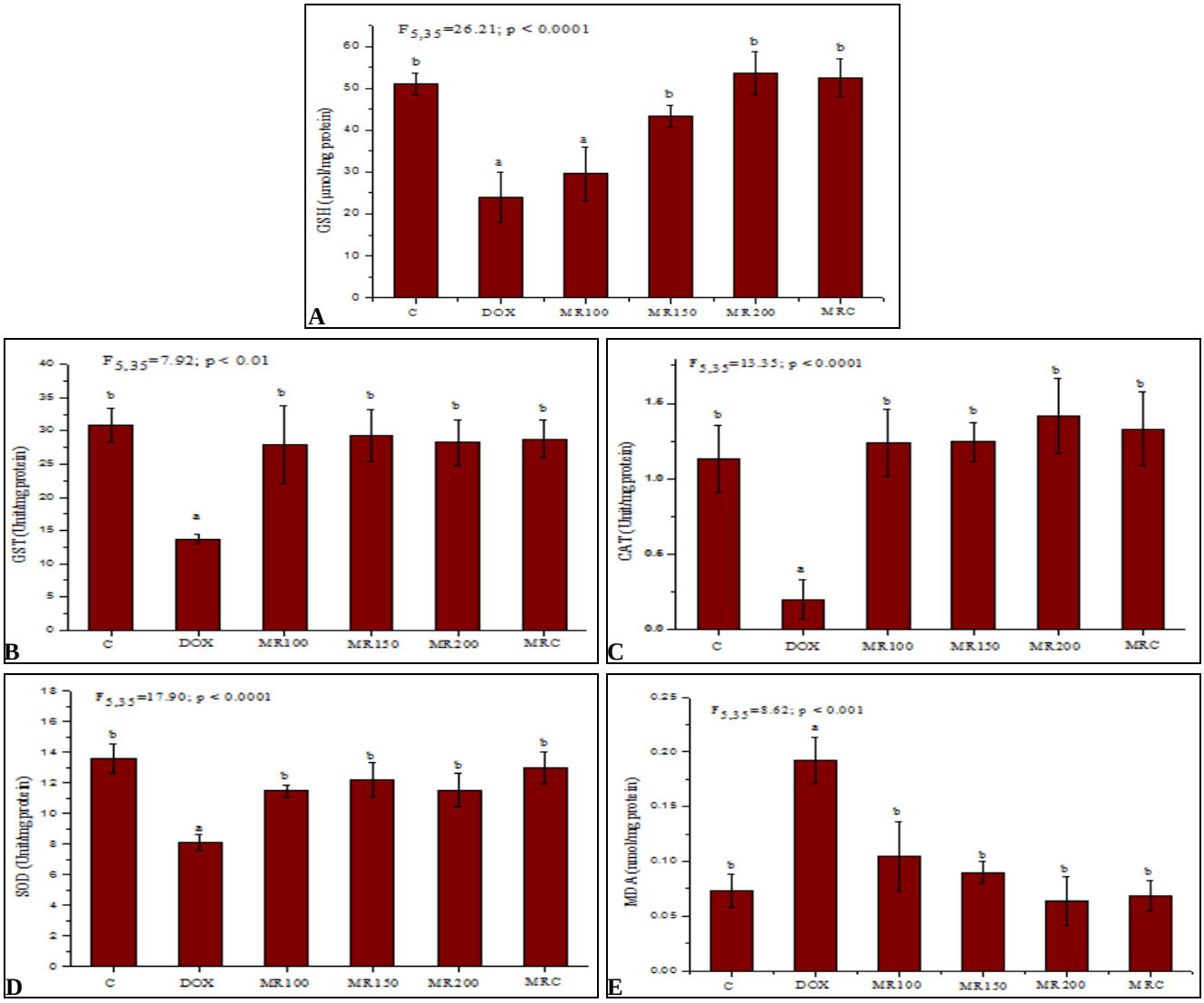

FIG. 9: EFFECTS OF AQUEOUS EXTRACT OF *M. ROXBURGHIANUS* ON ANTIOXIDANTS LEVELS OF DOX TREATED MICE IN LIVER TISSUE. (A) REDUCED GLUTATHIONE LEVEL (µmol/mg OF PROTEIN); (B) GLUTATHIONE-S-TRANSFERASE ACTIVITY (U/mg PROTEIN); (C) CATALASE ACTIVITY (U/mg PROTEIN); (D) SUPEROXIDE DISMUTASE LEVELS (U/mg PROTEIN); (E) QUANTIFICATION OF MALONALDEHYDE LEVELS (nmol OF MDA/mg PROTEIN) TO ASSESS LIPID PEROXIDATION. MEANS NOT SHARING THE SAME LETTER ARE SIGNIFICANTLY DIFFERENT. C–UNTREATED CONTROL GROUP; DOX–DOXORUBICIN TREATED GROUP; MR100, MR150 AND MR200- ORAL ADMINISTRATION OF AQUEOUS EXTRACT OF *M. ROXBURGHIANUS* AT THE DOSE OF 100 mg/kg, 150 mg/kg and 200 mg/kg b.wt RESPECTIVELY, FOLLOWED BY DOX TREATMENT. MRC–*M. ROXBURGHIANUS* CONTROL GROUP (200 mg/kg)

Flavonoids have also been observed to inhibit lipid peroxidation in the erythrocytes membrane and improved their integrity against lyses by binding to the membrane [43]. The study indicated that *Mallotus roxburghianus* extracts contains some molecules which interacted with lipids present in the erythrocyte membrane showing protective effect against hemolysis.

Doxorubicin is considered to be the most toxic anthracycline on hepatocyte [44]. Effort has been expended to understand the mechanisms of doxorubicin toxicity and to identify remedies that reduce its adverse effect such as antioxidants [45]. Free radicals play important role in doxorubicin toxicity and are in part generated by its redox-cycling [46]. Polyphenols as antioxidant molecules have been used to reduce doxorubicin toxicity [45]. The alleviation in antioxidants such as glutathione (GSH), and activities of glutathione-s-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) may be regarded as an indicator of increased oxidative stress [47].

The single injection (i.p) of DOX to mice results in significant depletion of antioxidants activity, which

is in agreement with earlier reports from this laboratory in rat and mice liver [48, 49]. Alleviation in GSH level might be due to its increased utilization by the hepatocytes in scavenging doxorubicin metabolites. It has been reported that most covalent binding of toxicant to hepatic protein occurs only after depletion of GSH [50]. Treatment of mice with MRAE resulted in significant increase in GSH level.

The plant extract may help in maintaining the cellular GSH by direct neutralization of free-radicals, or induced GSH synthesis by its bioactive compounds. The decrease in GST, SOD and CAT antioxidant enzymes activities in liver cells of mice could be due to mitochondrial over production of superoxide anion following DOX administration. The increase in these enzymatic activities in MRAE treated mice can be the consequence of up regulation of GST, SOD and CAT genes expression. Several studies have reported the effects of antioxidants against DOX toxicity.

Naringin a citrus bioflavonoid has been reported to increase GSH and activities of GST, SOD and CAT in the rat and mice liver earlier [48, 49]. Since lipid peroxidation (LOO) is a free radical oxidation product of polysaturated fatty acids, detection and measurement of LOO is the evidence which is frequently cited to support the involvement of free-radicals reactions in toxicity and disease progression [51]. Its occurrence in biological membranes causes impaired membrane function, impaired structural integrity, decrease in fluidity, and inactivation of a number of membrane bound enzymes and protein receptors [52]. The damage inflicted by free radicals is measured by the generation of MDA levels, the aldehyde product of lipid peroxidation [53].

In the present study, a significant increase (61.97%) in MDA levels in liver cells was observed in DOX-treated animals. Similarly, DOX has been reported to increase lipid peroxidation in rat and mouse liver [48, 49]. The increased LOO could be attributed to the overproduction of superoxide anion and reduction in detoxifying hyperperoxides. MRAE treatment inhibited DOX-induced cellular lipid peroxidation which may be due to scavenging of MDA molecules or inhibition of cytosolic lipid peroxidation chain reactions by the plant extract.

CONCLUSION: Our study demonstrates that various solvent extracts of *M. roxburghianus* exhibit a concentration dependent inhibition of free radicals, ferric reducing power and anti-hemolytic activity. The phytochemical analysis also revealed the presence of significant amounts of flavonoid and phenolic compounds which might be responsible for free radical scavenging and antioxidant activities. Significant positive correlation was observed between phytochemical contents and the free radicals scavenging activity of various extracts.

Aqueous extract of *Mallotus roxburghianus* possesses high antioxidant activity and it elevated the doxorubicin-induced decline in various antioxidants and reduced lipid peroxidation. Therefore, *Mallotus roxburghianus* may be a potential source of antioxidant adjuvant therapy for cancer treatment. However, efforts to understand the mechanism(s) through which *Mallotus roxburghianus* exerts antioxidant activities and the synergic effect between doxorubicin need to be investigated further.

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