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In Vivo & In Vitro Antioxidant Activity of Extracts of *Aegle Marmelos* Leaves.

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ABSTRACT

The present study was investigated the antioxidant activities of the various extracts of *Aegle Marmelos* (AM) belongs to family Rutaceae. The antioxidant activities of extracts have been evaluated by using a range of *in vitro* assays and *in vivo* hepatoprotective model. *In vitro* Antioxidant activity was evaluated by percentage inhibition in different assays including 2, 2'-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical scavenging assay hydrogen peroxide radical scavenging assays, reducing power capacity. The extract exhibited potent antioxidant activity compared to known antioxidant. The extracts of *A.Marmelos* were tested for *in vivo* efficacy by carbon tetrachloride (CCl4) induced liver damage rats in hepatoprotective model. The *in vitro* antioxidant activities of extracts showed significant activities on reducing power, DPPH, hydroxyl radical and hydrogen peroxide nearer to control group based on IC_{50} values. Oral administration of various extracts of *A.Marmelos* resulted in significant improvement on the levels of malondialdehyde (MDA) and superoxide dismutase (SOD), Catalse (CAT) in liver homogenate & Significant increase in level of MDA which was intoxicated by CCl4. CCl4 produced significant alteration of serum marker enzymes, total bilirubin, total protein and liver weight. The extracts significantly restored of these values towards normal compared to control group. Due to its natural origin and potent free radical scavenging ability *A.Marmelos* could be used as a potential preventive intervention for free radical mediated diseases.

Key words: DPPH, SOD, CAT, MDA, *In Vivo & In Vitro* Antioxidant assays

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INTRODUCTION

Free radicals are highly reactive molecules with an unpaired electron and are produced by radiation or as byproducts of metabolic processes. Free radicals are involved in the normal physiology of living organisms & also implicated in etiology of different degenerative diseases, such as heart diseases, stroke, rheumatoid arthritis, diabetes and cancer.[1] They initiate chain reactions which lead to disintegration of cell membranes and cell compounds, including lipids, proteins and nucleic acids[2] which ultimately affects the functional status of affected organs which leads to diseases ranging from serious illness to life threating conditions.[3] They are found to be responsible for the toxic effects of xenobiotics. In present study the plant *Aegle Marmelos* belongs to family Rutaceae related to citrus is used. *Aegle Marmelos* is a fruit-bearing tree indigenous to dry forest on hills and plains of central and southern India, SriLanka, Myanmar, Pakistan, Bangladesh, Nepal, Vietnam, Laos, Cambodia and Thiland. It has many Indian names, depending on the geographical region or the language, for example: Maredu (Andra Pradesh), Bel (Bengal), Bil (Gujarat), Kumbala (Karnataka), Vilwam (Kerala), Bilwa (Sanskrit), Kuvalum (Tamilnadu). Also in English there are different names: Bengal quince, Golden apple, Stone apple. Leaves, fruits, stem and roots of Aegle Marmelos have been used in ethno medicine for several uses.[4] Recent studies showed that a number of plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts exert potent antioxidant actions. A major defense mechanism is the antioxidant enzymes, which convert active oxygen molecules into non-toxic compounds. [5]

MATERIAL & METHODS

Plant material and extraction procedures

Collection of Plant Material: The fresh leaves of *Aegle Marmelos were* collected from Ayurvedic Botanical Garden, Gandhinagar. Leaves were identified and authenticated at Mehsana, Gujarat. Fresh leaves dried under shade. The coarsely powdered fresh leaves were stored in polythene bags at room temp until required.

Extract Procedures: The dried and coarsely powdered material of *Aegle Marmelos* (100g) was subjected to successive extraction in a Soxhlet apparatus with different solvents like petroleum ether, chloroform and methanol. After each extraction, the solvent was recovered using distillation assembly. In vacuum after evaporation of ethanol from the ethanolic extract, residues were obtained and were stored in desiccators. Fresh Juice was prepared by just mixing of fine powdered material of *Aegle Marmelos* with water to make juice.

Experimental Procedures

***In Vitro* Antioxidant Assays**

DPPH Free radical scavenging activity: The DPPH free radical scavenging activity of *A.Marmelos* extracts was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH (1, 1-diphenyl-2-picryl- hydrazyl)[6] For that, 4.3 mg of DPPH was dissolved in 3.3 ml of methanol in a test tube. Solution was protected from light. 150 µl of above solution was taken and diluted up to 3ml with methanol; the absorbance of solution was taken immediately at 517 nm on UV spectrophotometer using methanol as blank, served as control reading. Aliquots of different concentration ranging from 50 µg/ml to 500 µg/ml for the test and 10 µg/ml to 100 µg/ml for the standard were prepared. For the assay 150 µl of the test or std solution was added to 150 µl of DPPH solution and diluted up to 3ml with methanol, the absorbance of this solution was taken after 30 min at 517 nm on UV spectrophotometer using methanol as blank. The absorbance was taken in triplicate manner. Ascorbic acid was taken as reference. Percentage scavenging of DPPH free radical was calculated by comparing the absorbance of the control and test samples using the following equation showing in Figure 1.

$$\text{Scavenging Effects} = \frac{A\text{Control} - A\text{test}}{A\text{test}} * 100$$

Figure 1: An Equation for % Free Radical Scavenging Effects where $A_{Control}$ was the absorbance of the control reaction and A_{test} was the absorbance in the presence of the test sample

Hydroxyl Radical Scavenging Activity:

The ability of compound to scavenge OH^{o} was assessed by using the classic deoxyribose degradation assay.[5] For assay 2.0 ml of the assay mixture containing EDTA, FeCl3,H2O2 ,deoxyribose and sample extract (50 µg/ml to 500 µg/ml) was dissolved in distilled water with ascorbic acid. The mixture was incubated at 37°C for 1 hr. and 1 ml of the incubated mixture was mixed with 1 ml of 10% TCA (Trichloro Acetic Acid) and 1 ml of 0.4% TBA (Thiobarbituric Acid) to develop the pink chromagen. The absorbance was measured at 532nm against corresponding blank solution. Ascorbic acid was taken as reference.The hydroxyl radical scavenging activity of the extract was reported as % inhibition of deoxyribose degradation and was calculated as equation shown in Figure 1.

Hydrogen Peroxide Free Radical Scavenging Activity:

The ability of the compound to scavenge H_2O_2 was determined using Spectrophotometric

method by measuring the absorption with the extinction coefficient for H2O2 of 81 $M^{-1}cm^{-1}$. [7]For assay 40 mM H2O2 was prepared in phosphate buffer (pH 7.4) & Extracts at the different concentrations (50 µg/ml to 500 µg/ml) were added to a H2O2 solution and the absorbance of H2O2 was determined at 230 nm after 10 min incubation against a blank solution containing phosphate buffer without H2O2. Ascorbic acid was taken as reference. The percentage of scavenging of H2O2 was calculated by equation as shown figure 1.

Reducing Power Ability:

The capacity of extracts to reduce the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Prussian blue was determined by measuring the absorbance at 700 nm after incubation.[7] Different concentrations of extract(50 µg/ml to 500 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%) and the mixture was incubated at 50°C for 20 min. 2.5 ml of 10% TCA was added to the reaction mixture which was centrifuged at 5000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml), FeCl3 (0.5 ml, 0.1%) and the absorbance was measure at 700 nm. The reducing power of the extract was linearly proportional to the concentration of the sample. Ascorbic acid was taken as reference standard. Phosphate buffer (PH 6.6) was used as blank solution.

***In vivo* antioxidant Assays**

Female Wistar rats weighing 150-250gm procured from central animal facility of Institute. The animals were maintained in controlled temperature (24 ± 2^0C) as well as humidity (60-70%) in 12 –h light –dark cycles with standard diet and water will provide ad *libitium*. The care and the use of these animals were in accordance with the guidelines of the CPCSEA. An experimental protocol was approved by IAEC.

Experimental Design: The animals (Wistar female rats) weighing between 150-250 gm were divided into main four groups, six animals in each group. Animals in Group 1 were treated with Vehicle only twice a day P.O for 7 days served as Normal control Group. Group 2 animals were received CCl4: olive oil (1:1; 0.7ml/kg.i.p) at every 72 hr for 7 days served as Positive control Group. Others animals were pretreated twice daily with vehicle containing Petroleum Ether extract (AMPE), Chloroform extract (AMCL), Alcoholic Extract (AMAL), Fresh juice (AMAQ) of *Aegle Marmelos* 500mg/kg P.O for 7 days & Silymarin 100 mg/kg P.O for 7 days along with CCl4: olive oil (1:1; 0.7ml/kg.i.p) at every 72 hr. At the termination day, animals were anaesthetized using anesthetic ether and blood collected from retro orbital Plexuses. The blood samples were allowed to clot and the serum was separated by centrifugation at 2500 rpm for 15

min at 37° C and the serum was used for biochemical estimation. All the animals were then sacrificed and liver tissues were collected for the evaluation of in vivo antioxidant studies.

Determination of serum protein:

Lowry's method of protein estimation is the most widely accepted method for accurate determination of protein concentration. The method is a combination of biureat reaction and Folin-Ciocalteau reaction. In the first of reaction, protein binds to copper in alkaline medium and produces Cu2+ (Cupric ion). In the second step, Cu2+ catalyzes oxidation of aromatic amino acids by reducing phosphomolybdotungustate to heteropolymolybdenum blue. This reaction produces strong blue color, which predominantly depends upon tyrosine and tryptophan content of protein to a lesser extent cysteine and other residue of protein.

Determination of Superoxide dismutase (SOD):

The assay of SOD was based on the ability of SOD enzyme to inhibit spontaneous oxidation of adrenaline to adrenochrome. [8] The assay was carried out by adding 1ml tissue homogenate, 2 ml of carbonate buffer, 0.5ml EDTA. The reaction was initiated by addition of & 1.0 ml of epinephrine. The optical density of formed adrenochrome at pH 10.2 was read at 480nm for 3-min. at intervals of 30 sec. A negative control reading was taken by same method without addition of tissue homogenate. The results are expressed as units of SOD (mg/protein). One unit of SOD Activity induced approximately 50 % inhibition of Adrenaline. The results are expressed as nmol SOD U per mg of wet tissue.

Calculation

Change in absorbance/min (ΔA/min) = A2-A1/ 3 min

Where A2= Final absorbance after 3 min (180 seconds)

A1= Initial absorbance after 30 seconds

$$\% \text{ inhibition} = \frac{(\Delta A \text{ Sample/min})}{(\Delta A \text{ Blank/min})} * 100$$

Using the above calculations, a standard curve for SOD activity was plotted and the percentage SOD activity of each experimental group was deduced from the curve.

Determination of Catalase activity (CAT):

To estimate the CAT activity by the method [9] the reaction mixture consisted of 1.95 mL phosphate buffer (0.1 M, pH 7.4), 1.0 mL hydrogen peroxide (H2O2) (0.019 M), and 0.05 mL of supernatant in a final volume of 3 mL. Changes in absorbance were recorded at 240 nm for 2.5 min. Serum catalase activity is linear up to 100 kU/l. One unit catalase decomposes 1 pmol of

hydrogen peroxide/l min under these conditions. If the percentage inhibition of free radicals by CAT was calculated using the equation

$$\% \text{ inhibition} = \frac{A0 - A1}{A0} * 100$$

Where, A_0 is the absorbance of the control without extract and A_1 is the absorbance of the sample extract.

Determination of Lipid Peroxidation:

Lipid peroxidation was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA).[10] To 1 mL of supernatant of tissue Homogenate, 0.5 mL of 30% trichloroacetic acid (TCA) was added followed by ,0.5 mL of 0.8% TBA. The tubes were kept in a shaking water bath for 30 min at 80 °C. After 30 min of incubation the tubes were taken out and kept in ice-cold water for 10 min. These were then centrifuged at 800 g for 15 min. The amount of MDA was assessed by measuring the absorbance of supernatant at 540 nm at room temperature against an appropriate blank. The percentage inhibition of Lipid peroxidation was calculated using the equation:

$$\% \text{ inhibition} = \frac{A0 - A1}{A0} * 100$$

Where, A_0 is the absorbance of the control without extract and A_1 is the absorbance of the Sample extract.

Statistical Analysis

Experimental results were calculated by three Parallel measurements. Linear regression analysis was used to calculate IC_{50} values. Whenever needed. Differences among the tested antioxidants were analyzed by using one-way ANOVA. Values are expressed as the mean±SEM and differences between groups were considered to be significant if $p<0.05$.

RESULTS & DISCUSSION

***In Vitro* Antioxidant Activity**

DPPH Free Radical Scavenging Activity:

DPPH is a stable nitrogen centered free radical which produces violet color. It was reduced to a yellow colored product, diphenylpicryl hydrazine, with the addition of the extract in a concentration-dependent manner. Antioxidant molecules can be effectively quench DPPH free radicals and convert them to colorless, resulting in a decrease in absorbance with , the more potent the antioxidant activity of the extract [11] This assay is a commonly employed assay in

antioxidant studies of specific compounds or extracts across a short time scale. The DPPH radical scavenging activity was detected and compared with Ascorbic acid. Hence, it has been widely used for rapid evaluation of the antioxidant activity of plant extracts. [12] The results of the DPPH scavenging activity of *A.Marmelos* leaves extracts are shown in Figure 2. As Shown in Figure 2 that the maximum percentage scavenging of DPPH for Ascorbic Acid, petroleum ether, chloroform, alcohol, and aqueous extracts (fresh Juice) was 94.56 %, 62.56 %, 86.81 %, 76.78 % and 72.74 % respectively at highest concentration. The more potent activity observed in AMCL compare to Control group.

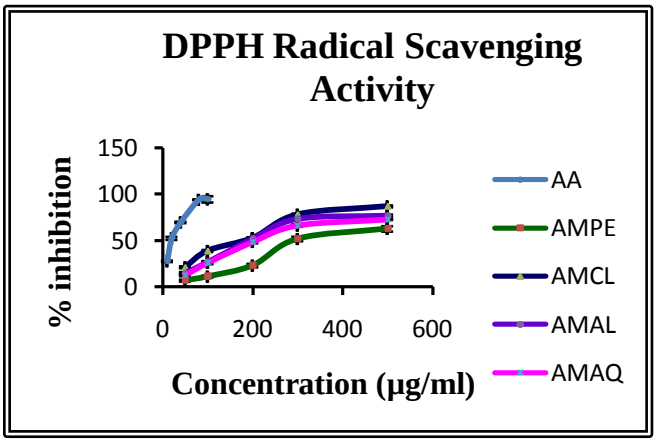

Figure 2: Comparison of % DPPH free radical scavenging activity of various leaves extracts of *A. Marmelos* & Ascorbic Acid. Values are expressed in mean ± S.E.M. Where n=3

Hydroxyl radical Scavenging Activity

Superoxide anion is a relatively weak oxidant, but it can generate more dangerous species, including singlet oxygen and hydroxyl radicals, which could cause the tissue damage.[13] The extract was examined for its ability to scavenge OH• radicals generated by the Fenton reaction. According to this method [1] the scavenging effect of any antioxidant on OH• radical is due to its inhibitory activity on the degradation of 2-deoxyribose-2-ribose. And therefore the scavenging effect of *A.Marmelos* leaves extract on OH• radicals may be by preventing degradation of 2-deoxyribose-2-ribose. The results of the Hydroxyl radical scavenging activity of *A.Marmelos* leaves extracts are shown in Figure 3.

Figure 3 shows that the maximum percentage scavenging of Hydroxyl Radical for Ascorbic Acid, petroleum ether, chloroform, alcohol, and aqueous extracts (fresh Juice) was 90.4 %, 67.51 %, 80.81 %, 77.96 % and 68.33 % respectively at highest concentration

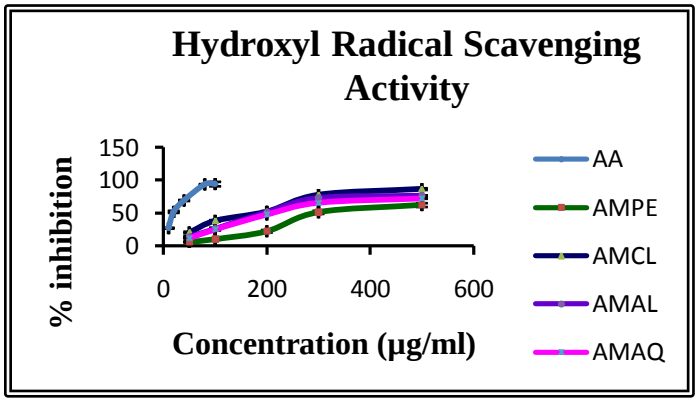

Figure 3: Comparison of % OH scavenging activity of various leaves extracts of *A. Marmelos* and ascorbic acid. Values are expressed in Mean ± S.E.M. Where n=3.

Hydrogen Peroxide Radical Scavenging Activity:

Scavenging activity of H2O2 on *A.Marmelos* leaves extract may be attributed to their phenolics, which can donate electrons to H2O2, thus neutralizing it to water. H2O2 can cross cell membranes rapidly and once inside the cell it can probably react with Fe2+ and possibly Cu2+ to form hydroxyl radicals and this may be the origin of many of its toxic effects.[1] It is therefore advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The H2O2 scavenging activity was detected and compared with Ascorbic acid. Figure 4 shows the maximum percentage scavenging of Hydroxyl Radical for Ascorbic Acid, petroleum ether, chloroform, alcohol, and aqueous extracts (fresh Juice) was 88.4 %, 64.78 %, 76.67 %, 79 % and 72.29 % respectively at highest concentration. The absorbance of the chromospheres is measured at 230 nm in the presence of the fractions. The extract capable of scavenging H2O2 in a concentration dependent manner.

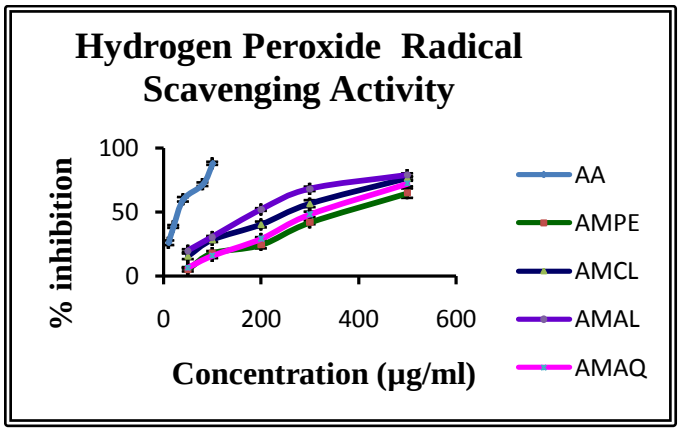

Figure 4: Comparison of % Hydrogen Peroxide Radical scavenging activity of various leaves extracts of *A. Marmelos* and ascorbic acid. Values are expressed in Mean ± S.E.M. Where n=3.

Reducing power ability

The reducing ability of a compound greatly depends on the presence of reductones, which have exhibit antioxidative potential by breaking the free radical chain by donating a hydrogen atom Figure. 5 shows the reductive capabilities of *A. Marmelos* extracts when compared to the standard Ascorbic acid. Like the antioxidant activity, the reducing power increased with increasing amount of the extract. For the measurement of the reductive ability, the Fe3+-Fe2+ transformation was investigated in presence of the extract. Presence of reductants causes the reduction of the Fe3+/ferricyanide complex to the Fe2+ form. This Fe2+ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Various leaves extracts of *A.Marmelos* showed higher absorbance in petroleum ether (0.59), chloroform (0.76), alcoholic (0.70), aqueous [fresh Juice (0.53)] when compared with the control was 1.299. It indicates reducing capacity of various extracts of *A.Marmelos* leaves which is an indicator of its antioxidant activity.

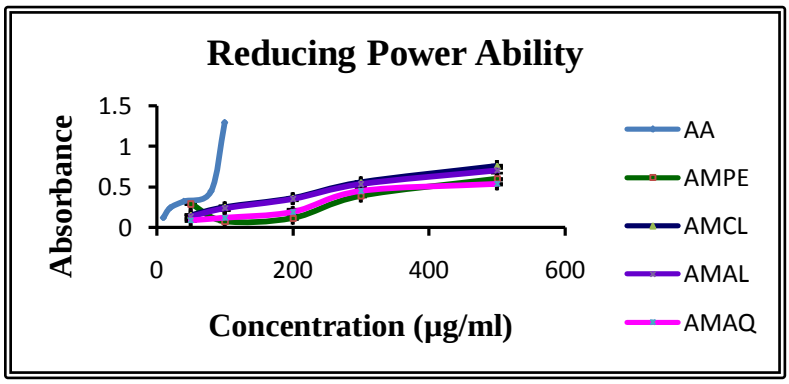

Figure 5: Comparison of Reducing Power ability of various leaves extracts of *A. Marmelos* and ascorbic acid. Values are expressed in Mean ± S.E.M. Where n=3

***In Vivo* Antioxidant Activity**

SOD has been touted as one of the most important enzymes in the enzymatic anti-oxidant defense system. SOD scavenges the superoxide anion to form hydrogen peroxide, hence diminishing the toxic effects caused by this radical. Catalase is an enzymatic antioxidant widely distributed in all animal tissues including RBC and liver. Catalase decomposes hydrogen peroxide and helps protect the tissues from highly reactive hydroxyl Radicals. The superoxide anion has been known to inactivate CAT, which is involved in the detoxification of hydrogen peroxide (2).MDA is the major oxidation product of peroxidized poly-unsaturated fatty acids and the increased MDA content is an important indicator of lipid peroxidation. Liver is the main detoxifying organ in the body and as such it possesses a high metabolic rate and it is subjected to

many insults potentially causing oxidative stress. Hence, a corrective measure to stabilize the hepatic antioxidant defense system is of paramount importance for the maintenance of health.

The results of SOD, CAT, and lipid peroxidation in the liver homogenate of control, hepatotoxic & experimental animals are shown in Figure 6, 7, 8. The level of hepatic antioxidant enzymes SOD, CAT, were significantly ($p<0.001$) lowered in CCl_4 intoxicated rats than the Normal control group whereas in MDA level significantly ($p<0.001$) increase in CCl4 intoxicated rats than the Normal control suggesting that generation of oxidative stress in liver by formation of Free radicals.

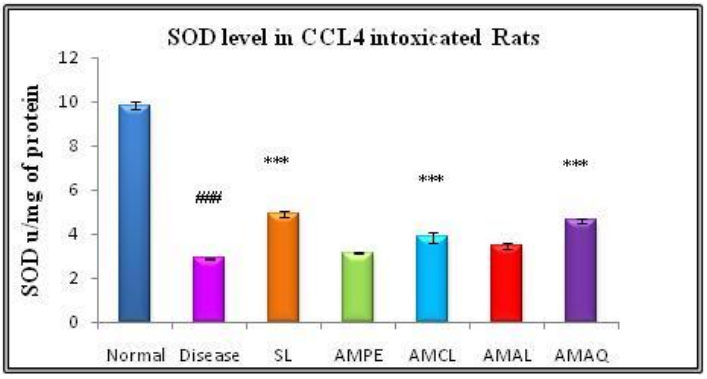

Figure 6: Effect of A.Marmelos Leaves extracts on SOD level in CCl4 intoxicated rats. Values are expressed in Mean ± S.E.M. Where n=6. Disease control=CCl4 (0.7ml/kg.i.p) only. ###P<0.001 designated as normal control versus disease control group. ***P<0.001 designated as disease control versus A.Marmelos extracts & Silymarin treated groups.

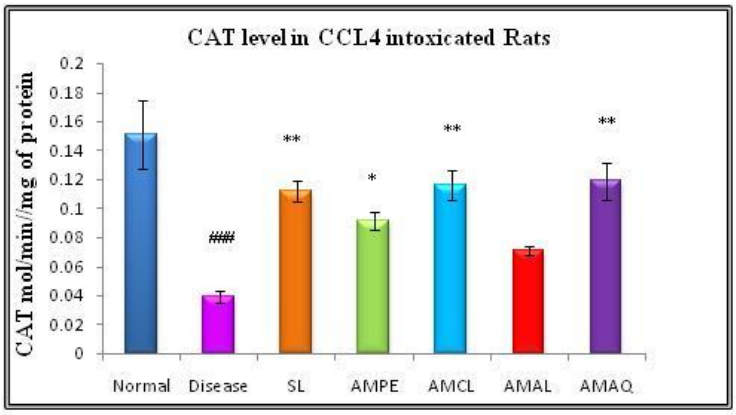

Figure 7: Effect of A.Marmelos Leaves extracts on CAT level in CCl4 intoxicated rats. Values are expressed in Mean ± S.E.M. Where n=6. Disease control=CCl4 (0.7ml/kg.i.p) only. ###P<0.001 designated as normal control versus disease control group. ***P<0.001, **P0.01, *P<0.05 designated as disease control versus A.Marmelos extracts & Silymarin treated groups.

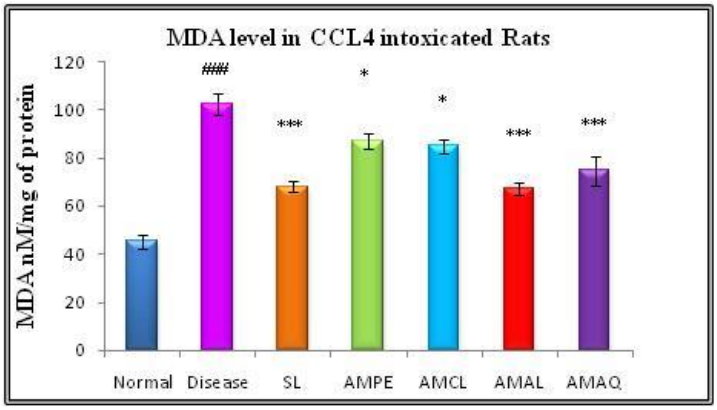

Figure 8: Effect of A.Marmelos Leaves extracts on MDA level in CCl4 intoxicated rats. Values are expressed in Mean ± S.E.M. Where n=6. Disease control=CCl4 (0.7ml/kg.i.p) only. ###P<0.001 designated as normal control versus disease control group. ***P<0.001, **P0.01, *P<0.05 designated as disease control versus A.Marmelos extracts & Silymarin treated groups.

The antioxidant activity or the inhibition of the generation of free radical is important in the protection against CCl4-induced liver lesion. Further study revealed that, the significant ($p<0.001$) reduced in level of SOD, CAT & increased in level of MDA. Reduced level of antioxidant enzymes (SOD and CAT) and high level of lipid peroxidation in liver tissue clearly indicating the hepatocytes necrosis due to production of reactive metabolites and reactive metabolites induced ROS production.

The increased level of SOD ($P< 0.001$) and CAT ($P< 0.01$) observed in AMCL & AMAQ treated rats suggesting the protective action against CCl_4 induced heaptotoxicity. The decreased lipid peroxidation ($P< 0.001$) in AMAL & AMAQ whereas in AMPE & AMCL it shows significant reduction in ($p<0.01$) level of MDA. The percentage inhibition of production of MDA, which is one of the end products of lipid peroxidation in liver tissue, was found to be low in CCl4 control group implying enhanced lipid peroxidation leading to tissue damage due to failure of antioxidant defense mechanisms against free radicals.

CONCLUSION

Free radicals are known to play a definite role in a wide variety of pathological manifestations of pain, inflammation, cancer, diabetes, Alzheimer, hepatic damage etc. Antioxidants fight free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms.

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