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Evaluation of *In vivo* antioxidant activity of a triterpene isolated from *Madhuca Longifolia* L leaves

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Abstract: *Free radical stress leads to tissue injury and progression of disease conditions such as arthritis, hemorrhagic shock, atherosclerosis, diabetes, hepatic injury, aging and ischemia, reperfusion injury of many tissues, gastritis, tumor promotion, neurodegenerative diseases and carcinogenesis. Safer antioxidants suitable for long term use are needed to prevent or stop the progression of free radical mediated disorders. Many plants possess antioxidant ingredients that provide efficacy by additive or synergistic activities. Madhuca longifolia L leaves are used as strong astringent, used for the treatment of liver and spleen diseases, rheumatism and tumors. Antioxidant activity of the crude extracts of leaves of Madhuca longifolia L were assessed using reducing power assay, super oxide radical scavenging activity and hydroxyl radical scavenging activity. The potent fraction was tested for in vivo efficacy. Methanolic extract and isolated triterpene (derivative of Madhucic acid) exhibited potent antioxidant activity compared to the standard. In vivo studies on potent fraction demonstrated a reduction in hepatic malondialdehyde with simultaneous improvement in hepatic glutathione and LPO levels respectively, because of its natural origin and potent free-radical scavenging ability Madhuca longifolia L leaves can be used as a potential preventive intervention for free radical-mediated diseases.*

Keywords: *Madhuca longifolia L, antioxidant, Triterpene, GSH and LPO*

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I. Introduction

A common theme which underlies etiology of several degenerative disorders is free radical stress. The production of free radicals is inextricably linked to the inflammatory process. Free radicals prime the immune response, recruit inflammatory cells and are innately bactericidal.[1, 2]Some of these free radicals play a positive role *in vivo* such as energy production, phagocytosis, regulation of cell growth and intercellular signalling, or synthesis of biologically important compounds. [3]However, free radicals are very detrimental in attacking lipids in cell membranes and also DNA inducing oxidation that cause membrane damage such as membrane lipid peroxidation and decrease in membrane fluidity and also cause DNA mutation leading to cancer.[4]Free radicals and oxidants activate nuclear factor-κB, a nuclear transcription factor, resulting in an up regulation of pro-inflammatory mediators such as interleukin-1, interleukin-8 and tumor necrosis factor-α .[5] This in turn stimulates the immune response; increases oxidant production and can lead to further tissue damage. A potent scavenger of these free radical species may serve as a possible preventive intervention for free radical mediated diseases.[6] Recent studies have shown that a number of plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts exert potent antioxidant actions.[7]*Madhuca longifolia L* is a folklore medicinal plant; it is commonly used for the treatment of snakebite as antidote in Southern part of Tamilnadu and India .The seed oil is used for cooking purpose. Its flowers are widely used for making local liquor and leaves are used in headache. New oleanene, triterpenoid and saponins in addition alkaloids were found to strongly inhibit lipid peroxidation induced in isolated tissues via antioxidant activity.[8] The objective of present study was to isolate a bioactive from *M.longifolia* leaves and to study its anti-oxidant potential.

II. Materials And Methods

Plant material

The leaves of *M.longifolia* were collected in Nov 2009 from Konchavaram forest Gulbarga, Karnataka, India and authentication was done by Prof Y.N. Seetharam, Dept of Botany, Gulbarga University Gulbarga, Karnataka India where a voucher specimen has been deposited in the herbarium (HGUG no: 723).

Extraction and isolation

Air dried leaves (500 g) of *M. longifolia* were reduced to a fine powder, which was subjected to hot continuous extraction in a soxhlet extractor, successively with petroleum ether (40-60°C). Each time before extracting with the next solvent, the powder material was dried in hot air oven below 50°C. Each extract was concentrated by distilling off the solvent followed by evaporation to dryness on a water bath. All extracts were kept in a descicator and stored in a refrigerator for phytochemical and pharmacological studies. The methanolic extract was subjected for column chromatography on silica gel (60-120 mesh) and eluted with following solvent systems: chloroform : methanol (8:2) and (1:9).The chloroform : methanol (1:9) fraction was repeatedly chromatograph on column and the collected fraction was checked on TLC until it gave a single spot of bright red colour (100 mg) (R_f value : 0.89). The isolated compound was subjected for spectral analysis and the compound was identified as 10-(carboxyoxy)-1,2,2,6a,9,9,hexamethyldocosahydropicene-4a-carboxylic acid which showed m.p at 310°C, λ_{max} 254nm, the IR (KBr) V_{max} cm^{-1} 3468.32 (OH) stretching, 2922-2808(C-H) stretching, 1709(C=O),1612(COOH), 1213 C-O-C); ^{1}H-NMR (DMSO) suggesting the structural similarities with Madhucic Acid [9]which was identified and confirmed by LCMS,IR, ^{1}H-NMR (Figure 1).

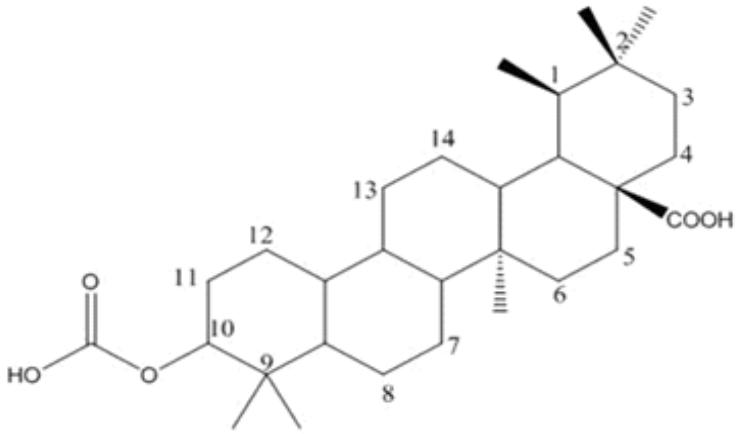

Figure 1: Chemical structure of compound isolated from *Madhuca longifolia L* leaves10-(Carboxyoxy)-1, 2, 2, 6a, 9, 9-hexamethyldocosahydropicene-4a-carboxylic acid.

Animals

Swiss albino male rats weighing 150-200g were maintained under standard environmental conditions and had access to standard diet and water ad libitum. Animals were housed in polythene cages with 12:12 h light and dark cycle. All experiments were performed in accordance with the guidelines for the care and use of laboratory, as adopted and promulgated by the institutional ethical animal committee, CPCSEA, India (Reg. no 34800/2009/CPCSEA).

Drugs and chemicals

Nitro blue tetrazolium, and all the solvents used in the study were of analytical grade and were procured from SD Fine Chemicals Limited, Mumbai, India. Thiobarbituric acid, malondialdehyde and other chemicals were obtained from Sigma Chemical Company.

Acute toxicity studies

In vivo toxicity was carried out according to the method of [10]. Albino male mice were divided into four groups consisting of six animals each. Graded doses of *Madhuca longifolia* methanolic extract (MLME), derivative of Madhucic acid (dMA) were administered orally. One group serving as control was treated with normal saline. The animals were monitored for 24 h after drug administration for gross behavioural changes and mortality. Dose at 50% mortality in a group was observed and considered as lethal dose (LD_{50}). Based on the results of preliminary toxicity studies, the doses for the further studies were fixed at 50 mg/kg for MLME and 5 mg/kg for dMA.

***In vivo* Antioxidant activity**

The method of [11] was used in the study. Wister albino rats were divided into 5 groups of 6 animals each. Group-I Negative control received vehicle (1 ml/kg po) Group-II Positive control CCl_4 (1.0 ml/kg body weight), Group-III Standard silymarin (100 mg/kg po), Group-IV- MLME (50 mg/kg po) and Group-VI-dMA (5 mg/kg po).

On 5th day, 30 min after the administration of Gum acacia, silymarin (100 mg/kg), MLME (50 mg/kg) and dMA (5 mg/kg) was given to Group-III, IV, V and VI respectively, CCl_4 (1.0 ml/kg body weight) was given orally. After 48 hours of CCl_4 induction, rats were sacrificed under mild ether anesthesia by dislocation and liver tissue was collected for the estimation of tissue GSH and LPO levels.

Reduced Glutathione (GSH) assay

GSH is an intra-cellular reductant and plays major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds.[12]Deficiency of GSH in the lens leads to cataract formation. Glutathione also plays an important role in the kidney and takes part in a transport system involved in the reabsorption of amino acids.[13] Liver tissue samples were homogenized in ice cold trichloroacetic acid (1gm tissue in 10 ml 10% TCA) in an ultra trux tissue homogenizer. The mixture was centrifuged at 3000 rpm for 10 min. Then 0.5 ml of supernatant was added to 2ml of (0.3M) disodium hydrogen phosphate solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4mg/ml in 1% sodium acetate) was added and absorbance was taken at 412 nm.

Lipid Peroxidation (LPO) assay

LPO is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotics and aging. Malondialdehyde (MDA) is one of the end products in the lipid peroxidation process. Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals, which is accepted as an indicator of lipid peroxidation. 1.0 ml of test samples (0.1-2.0 mg of membrane protein or 0.1-2.0 µmol of lipid phosphate) with 2.0 ml of TCA-TBA-HCL mixed thoroughly. Solution was heated for 15 m and cooled. Then precipitate was removed by centrifugation at 1000 rpm for 10 m and absorbance of each test sample was determined at 535 nm against a blank that contained all the reagents except lipid.

Statistical analysis

The data of the current experiment are presented as mean ± SEM (standard error mean).The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnet's t-test and the results were regarded as significant at $P < 0.05$.

***In vivo* Antioxidant activity**

There was marked depletion of GSH level in CCl_4 treated groups. Silymarin (100 mg/kg) increased tissue GSH by 94.87%.Test samples of 50 and 5 mg/kg have shown lesser increase in GSH levels by 35.20 and 58.97% respectively than standard silymarin in Table 1.

Table 1: Effect of MLME and dMA on tissue GSH levels in CCl_4 induced hepatotoxicity in rats

Treatment	Absorbance Mean S.E.M	percentage Increase
Negative Control (1ml vehicle)	0.91 ± 0.01	--
Positive Control CCl_4(1 ml/kg)	0.35 ± 0.02	--
CCl_4+ Silymarin (1ml/kg p.o +100 mg/kg p.o)	0.70 ± 0.03	94.87
CCl_4 + MLME (1ml/kg p.o + 50 mg/kg p.o)	0.53 ± 0.03***	35.20
CCl_4 + dMA (1 ml/kg p.o + 5 mg/kg p.o)	0.60± 0.03***	58.97

Values are the mean S.E.M. of six observations each.
Significance ** P<0.001, compared to CCl_4 induced rats.

In vivo lipid peroxidation assay of CCl_4 enhanced the lipid peroxidation. The treatment of rats with MLME, and dMA (50 and 5 mg/kg respectively) significantly reduced the lipid peroxidation. Silymarin (100 mg/kg) showed 68.62% inhibition, whereas MLME (50 mg/kg) showed 32.41% and dMA (5 mg/kg) showed 58.56% inhibition in Table 2.

Table 2: Effect of MLME and dMA on tissue LPO in CCl_4inducedhepatotoxicity in rats

Treatment	Absorbance Mean S.E.M	percentage inhibition
Negative Control (1ml vehicle)	0.21 ± 0.01	--
Positive Control CCl_4(1 ml/kg)	0.52 ± 0.01	--
CCl_4 + Silymarin (1 ml/kg p.o +100 mg/kg p.o)	0.23 ± 0.02***	68.62
CCl_4+ MLME (1 ml/kg p.o + 50 mg/kg p.o)	0.27 ± 0.04**	32.41
CCl_4 + dMA (1 ml/kg p.o + 5 mg/kg p.o)	0.24 ± 0.01***	58.56

Values are the mean S.E.M. of six observations each.
Significance ***P<0.001, compared to CCl_4induced rats.

III. Result And Discussion

Carbon tetrachloride is known to produce oxidative damage in the liver by enhancing peroxidation of membrane lipids, a deleterious process solely carried out by free radicals.[14]The free radical attacks the cell membrane, thus leading to destabilization and disintegration of the cell membrane as a result of lipid peroxidation. It prevents the loss of lipophilic antioxidant a-Tocopherol, by repairing tocopheryl radicals and protection of the hydrophilic antioxidant ascorbate[15] and increasing intracellular concentration of glutathione. Therefore, it may decrease the concentration of lipid free radicals and terminate initiation and propagation of lipid peroxidation.[16]

Glutathione reductase (GR) is the enzyme responsible for the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH). Glutathione S-tranferases (GSTs) are the group of multifunctional isoenzymes located both in the cytosol and the endoplasmic reticulum of the liver plays an important role in the detoxification of toxic electrophiles with glutathione to form more water soluble compounds. [17] GST is considered as first line of defence against oxidative injury along with other antioxidant enzymes, decomposing O_2 and H_2O_2 before interacting to form the more reactive hydroxyl radicals.[18, 19] In the present investigation the level of reduced glutathione and the activities if glutathione-dependent enzymes (GR and GST) were found to be reduced significantly in rats administered with CCl_4. The depression in GSH content and GST make the cell more susceptible to toxic electrophilic compounds. The over production of ROS as indicated by the elevated level of TBARS by the CCl_4, in the present study may be associated with depletion of GSH level and GST activity. Oral administration of along with glutathione and glutathione-metabolizing enzymes, contributed significantly to the intracellular antioxidant defence system by acting as a powerful consumer of singlet oxygen and hydroxyl radicals.MDA is the major oxidation product of peroxidised poly-unsaturated fatty acids and the increased MDA content is an important indicator of lipid peroxidation.[20] Liver is the main detoxifying organ in the body and as such it possesses a high metabolic rate and it is subjected to many potentially causing oxidative stress. Hence, a corrective measure to stabilize the hepatic antioxidant defence system is of paramount importance for the maintenance of health. Present study was undertaken to assess the effect of MLME and dMA on the *in vivo* antioxidant status through the estimation of MDA concentration in the liver of rats. The hepatic MDA content of animals subjected to induced oxidative stress was found to be significantly increased. This enhanced oxidative stress however was significantly reduced ($p<0.05$) in both the test groups. MLME and dMA showed statistically significant inhibition of lipid peroxidation as shown by the reduction in hepatic MDA level and the efficacy was found to be better than that of CCl_4. Glutathione, a major non-protein thiol in living organisms, plays a central role in coordinating the body's antioxidant defence processes. Excessive peroxidation causes increased glutathione consumption. The tissue glutathione levels, however, were significantly elevated in the groups supplemented with MLME and dMA. Treatment with MLME and dMA resulted in significant increase at $p<0.05$ in hepatic glutathione levels compared to that of standard silymarin-induced oxidative stress. Therefore, it clearly demonstrates that MLME and dMA have protective role against oxidative damage in the liver tissue. Hepatic glutathione status exhibited by MLME and dMA was better than that obtained by CCl_4.

IV. Conclusion

As observed from the data the components present in the methanolic extract of *Madhuca longifolia L* leaves have antioxidant activity. Naturally occurring triterpene and phenolic compounds are reported to possess free radical scavenging properties, due to their hydroxyl group. The components present in the leaves not only scavengers off the free radical but also inhibit the generation of free radicals. It may be thus concluded that the fraction obtained from methanolic extract of *Madhuca Longifolia L* leaves possess significant antioxidant activity.

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