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INVESTIGATION OF ANTIOXIDANT PROPERTY OF ETHANOLIC EXTRACT FROM RIND OF *NEPHELIUM LAPPACEUM* FRUIT IN NORMAL AND IMMUNESUPRESSED MICE

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ABSTRACT

One of the most conventional use of cyclophosphamide (CP) comprehends its immunosupressive effect. It has prooxidant character, and generation of oxidative stress after cyclophosphamide administration leads to decrease in the activities of antioxidant enzymes and increase in lipid peroxidation in liver, lung, spleen and serum of mice and rats. Attempts have been made in this research work**,** to evalutae the ethanolic extract of rind of Rambutan **(***Nephelium lappaceum***)**, for its protective effect against oxidative stress and cyclophosphamide induced oxidative stress. Sheep red blood cells have been used for antigen challenge and immunization**.** Cyclophosphamide at a dose of 50 mg/kg body weight, for three days per oral was administered to depress the antibody production. The assessment of antioxidant activity during normal and immune suppressive state was carried by estimation of lipid peroxidation (LPO) and superoxide dismutase (SOD). The findings demonstrated 100 mg /kg and 200 mg/kg of ethanolic extract administered along with cyclophosphamide evoked prominent decrease ($p<0.0001$) in the level of lipid peroxidation and increase **(**$p<0.0001$**)** in superoxide dismutase level respectively, when correlated to the group treated with cyclophosphamide only. In addition, 200 mg/kg ethanolic extract treated group had lower lipid peroxidation and higher superoxide dismutase in comparison to normal control group. The results propose that the ethanolic extract has the ability to counteract the oxidative

stress that occurs during normal antigen-antibody reaction and as well, cyclophosphamide induced immune suppression.

KEYWORDS: *Nephelium lappaceum*, Cyclophosphamide, immunosuppression, sheep red blood cells, lipid peroxidation, superoxide dismutase.

INTRODUCTION

The body is normally under a dynamic balance between free radical generation and quenching.[1] To protect the cells and organ systems of the body against reactive oxygen species (ROS), humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals.[2] Excessive production of ROS associated with inflammation may, however, lead to a condition of oxidative stress, loss of cell function, and ultimately apoptosis or necrosis.[3]

Relationship between antioxidants and immune system, and complication of oxidative stress on immune system: The relationship between oxidative stress and immune function of the body is well established. The immune defense mechanism uses the lethal effects of oxidants in a beneficial manner with ROS and reactive nitrogen species (RNS) playing a pivotal role in the killing of pathogens. The skilled phagocytic cells (macrophages, eosinophils), as well as B and T lymphocytes, contain an enzyme, the nicotinamide adenine dinucleotide phosphate(NADPH) oxidase, which is responsible for the production of ROS following an immune challenge.[4] Immune cells use ROS in order to support their functions and therefore need adequate levels of antioxidant defenses in order to avoid the harmful effect of an excessive production of ROS.[5] If the balance between free radical production and antioxidant defense is diminished, immune cells have an adverse effect on functions of body and suffer a senescent corrosion almost certainly linked to oxygen stress.[4] Oxidative stress is a major contributing aspect to the high mortality rates associated with dysregulation of immune and leads to several diseases and the immune system is mainly sensitive to oxidative stress.[1] All the factors responsible for the oxidative stress directly or indirectly participate in immune system defense mechanism.[4]

The immune status directly interplays with disease production process.[1] The major pathway of pathogensis for cell damage is via lipid peroxidation particularly in microsomes, mitochondria, and endoplasmic reticulum due to oxygen species and free radicals. The

oxidative modification of the proteins not only changes the antigenic profile of latter but also enhances the antigenicity as well. There exist several examples of autoimmune diseases resulting from oxidative modifications of self-proteins, namely, systemic lupus erythematosus, diabetes mellitus, and diffuse scleroderma. The production of free radicals involves macrophages and neutrophils to combat the invading microbes. The whole of the process is performed in host cells during the activation of phagocytes or the effect of bacteria, virus, parasites, and their cell products reactivity with specific receptors. The multicomponent flavoprotein NADPH oxidase plays vital role in inflammatory processes by catalysing the production of superoxide anion radical O_2- and excessive production of reactive oxygen species (ROS) leads to cellular damage. These cellular damages in general lead to altering immune response to microbes and ultimately altered susceptibility to bacterial, viral, and parasitic infections.[4]

While screening the immunomodulatory activity, most of the studies employ agents like cisplatin, cyclophosphamide, or corticosteroids in order to induce the immunosuppression in the experimental animals. These agents are known to generate free radicals in the biological system and thereby cause oxidative stress. Cyclophosphamide (CP) is one of the most widely used alkylating antineoplastic agents that damage normal cells while killing cancerous cell in vivo. It introduces alkyl radicals into DNA strands of cells and stops cancer cells from growing. It has also an immunosuppressive effect-suppress the body's natural immune response, and used to treat some autoimmune diseases.[6]

Cyclophosphamide and antioxidant: Several studies indicate that CP has a prooxidant character, and generation of oxidative stress after CP administration leads to decrease in the activities of antioxidant enzymes and increase in lipid peroxidation in liver, lung, spleen and serum of mice and rats. CP and its metabolite acrolein cause inactivation of microsomal enzymes and result in increased reactive oxygen species (ROS) generation and LPO.[5] CP metabolites can react with carboxyl (-C[O]OH), mercapto (-SH), amino (-NH2), phosphate (-PO3H2) and hydroxyl (-OH) groups, and can form cross-links with DNA and proteins. Numerous studies have shown that cyclophosphamide can disrupt the redox balance of the tissues and this result in biochemical and physiological changes from oxidative stress.[6]

Natural compounds from medicinal plants having antioxidant and immune modulatory activities so dietary supplementation with antioxidants can help in such situation. Supplementation with the antioxidant vitamins such as vitamins C, E, and A or beta carotene

protected immune responses in individuals exposed to definite environmental sources of free radicals. In addition these vitamins and selenium have an antioxidant effect due to their ability to transform ROS into constant and harmless compounds, or by scavenging both ROS and reactive nitrogen species (RNS) with a redox based mechanism. Thus, an adequate intake of vitamins and antioxidant elements are an essential for an efficient function of the immune system, cause of control of immune function affecting both innate T cell mediated immune reply and adaptive antibody reply, as a consequence altering the balanced host response.[1]

Rambutan (*Nephelium lappaceum*) is a medium sized evergreen tree found commonly in Malaysia. They have an open structure growing 12-15 m high, and produces ellipsoidal fruits in clusters of long thick, soft, hairs or spines on the surface; known as Rambutan (in Malay 'rambut' stands for hair). It is a tropical fruit belonging to *Sapindaceae* family. Rambutans are small, red colored fruits with spiky hair on the skin. They have sweet, juicy flesh that is slightly acidic and small brown colored seeds. The hairy outgrowth has eye catching red or yellow colour and it imparts a distinctive exotic appearance to its fruits.[7]

The rind of Rambutan, which is normally discarded have been reported to contain extremely high antioxidant activity,[8] anti-bacterial activity,[8] anti-viral,[9] anti-inflammatory,[4] anti-proliferative,[11] anti-hyperglycaemic activity.[12] The rind of Rambutan fruit was selected to investigate for its antioxidant property in immune suppressed and protective effect against CP induced oxidative stress based on the following evidences: Previous studies showed that the ethanolic extracts of rind were not cytotoxic to normal mouse fibroblast cells or splenocytes and that the ethanolic extract, either alone or in combination with other active principles, can be used in cosmetic, nutraceutical and pharmaceutical applications.[13] The rind of Rambutan had shown to contain chemical constituents exhibiting immunomodulatory activity such as tannins saponin, alkaloid, hydrocyanic acid, phenols, oxalate, tannins, volatile compounds, flavonoids and proteins.[14] Also it showed to contain substances exhibiting antioxidant activity such as ascorbic acid and phenolic compounds (anthocyanins, flavonoids, tannins, ellagic acid, corilagin, and geranin).[15]

MATERIALS AND METHODS

Chemicals and reagents

Cyclophosphamide tablets I.P. - 50 mg (Endoxan, Cadila Heath care Ltd.), Hydroxylamine hydrochloride (Qualigens Ltd.), Nitro blue Tetrazolium (High purity laboratory chemicals Ltd., Mumbai), Potassium chloride (Sisco Research Laboratories Pvt. Ltd., Mumbai), Sodium

carbonate (SD. Fine Chemicals Ltd., Mumbai), Sodium Citrate (Ranbaxy, Fine chemicals Ltd., Delhi), Trichloroacetic acid (Central Drug House (P) Ltd., Delhi), Trichloroacetic acid (Himedia Laboratory, Mumbai) and all other solvents used for experimental work were of analytical grade.

Collection and identification of the fruit

Nephelium lappaceum fruit was procured from the local market of Bangalore, India and was identified and authenticated by Dr. V Rama Rao (National Ayurveda Dietetics Research Institute, Bangalore).

Authentication Ref. number-**Authentication/S.M.P.U./N.A.D.R.I./BNG/2015-16/1267.**

Preparation of Extract

The fruit rind was shade dried for 2 days and then air dried in hot air oven for 4-5 days. The dried rind was cut into pieces and coarsely powdered using mixer grinder. About 237.77g of dried rind powder of *Nephelium lappaceum* were subjected to extraction with 70% v/v of ethanol by Soxhlet apparatus for about 72h. During the process of extraction, the alternate filling and emptying of the body of the extractor goes on continuously till the powder was exhausted and it was confirmed by discoloration of the solvent at the side tube of the extractor (Siphon). Then after, the residue was removed by filtration and transferred to petri plates for concentration. The concentrated extract was further dried in tray dryer at 45°C. The resultant reddish brown semisolid extract was transferred to clean bottle. The ethanolic extract of *Nephelium lappaceum* was kept in cool place and it was used for further study.

Dose selection

The acute toxicity studies on the extract were found to be carried out already as per our literature review and it was reported to be safe up to 2000 mg/kg b.w.[16] The dose of EENL for the pharmacological studies was selected based on the previous research work performed. For this study, the selected doses were 100 and 200 mg/kg b.w p.o of animal.[17] The solutions were freshly prepared on the day of experiment by dissolving in distilled water and administered orally.

Experimental Animals

Swiss albino mice weighing between 18 - 25 g were procured from authenticated supplier (Invivo Bioscience, Bangalore) and were maintained in the animal house of PES College of

Pharmacy, Bangalore. All the animals were acclimatized for seven d under standard husbandry conditions, i.e. room temperature of 25 ± 1°C; relative humidity 45-55% and a 12:12 h light/dark cycle. The animals had free access to standard rat pellet, with water supplied *ad libitum* under strict hygienic conditions. Animals were habituated to laboratory conditions for 48 h prior to experimental protocol to minimize if any of non-specific stress. The experimental protocols were approved by the Institutional Animal Ethical Committee (Ref. No. PESCP/IAEC/05/14 Date: 13-12-2014) and conducted according to CPCSEA guidelines (CPCSEA Reg no: 600/PO/Ere/S/02/CPCSEA), Govt. of India. All the protocols and the experiments were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India, New Delhi.

Antigen

Sheep blood was collected from the slaughter house in tubes containing the Alsever's solution in the ratio 1:2. SRBC's was washed thrice in pyrogen free phosphate buffer saline (PBS) and centrifuged at 2500-3000 rpm for 10 min. The supernatant was removed with pasture pipette and suspended in normal saline. The number of SRBC was then adjusted to a concentration of $1X10^8$ cells in 0.1 ml after the RBC count. RBC count was carried out using Neuber's chamber and RBC pipette. This RBC suspension was used for immunization and challenge.[18]

Experimental design

Mice were divided into 5 groups of five mice each. Drugs were administered in various groups: Group I - vehicle (normal saline; 7 days), Group II - extract at a dose of 100 mg/kg (7 days), Group III - cyclophosphamide 50 mg/kg p.o. (day 4, 5 and 6), Group IV and V- extract 100 and 200 m/kg (7 days), respectively along with cyclophosphamide (day 4, 5 and 6). The animals from all groups were immunized by injecting 0.1 ml of SRBCs suspension containing 1×10^8 cells intraperitoneally on day 0.

Table 1: Experimental design.[19,20]

Sr. No	Group (N = 5)	Treatment(dose & route)	Treatment schedule
1	I Normal control	SRBC (1×10^8) 0.1ml i.p.	Day 0
		Saline p.o.	Day 1 to 7
		SRBC (1×10^8) 0.1ml	Day 7
2	II EENL control	SRBC (1×10^8) 0.1ml i.p.	Day 0
		100 mg/kg p.o.	Day 1 to 7
		SRBC (1×10^8) 0.1ml	Day 7
3	III CP control	SRBC (1×10^8) 0.1ml i.p.	Day 0
		CP p.o. 50 mg/Kg, p.o.	Day 4 to Day 6
		SRBC (1×10^8) 0.1ml	Day 7
4	IV EENL (Low dose) + CP	EENL 100 mg/kg p.o.	Day 1 to 7
		SRBC (1×10^8) 0.1ml i.p.	Day 0
		CP p.o. 50 mg/Kg, p.o.	Day 4 to Day 6
		SRBC (1×10^8) 0.1ml	Day 7
5	V EENL (High dose) + CP	EENL 200 mg/kg p.o.	Day 1 to 7
		SRBC (1×10^8) 0.1ml i.p.	Day 0
		CP p.o. 50 mg/Kg, p.o.	Day 4 to Day 6
		SRBC (1×10^8) 0.1ml	Day 7

Antioxidant parameters: The spleen tissue was used to estimate the antioxidant parameters like LPO and SOD.

Preparation of spleen tissue homogenate: Spleen was washed superficially with isotonic saline to remove blood. Whole spleen was weighed and suspended in phosphate buffer individually. The tissue was homogenized (approximately 0.5mg of tissue per ml of phosphate buffer (pH 8.0, 0.1 M) using homogenizer tubes and a motor-driven Teflon pestle (Remi motors).

Estimation of lipid peroxidation (LPO)

Principle: Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. TBARS return to normal levels over time, depending upon the presence of anti-oxidants. Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acid lipid peroxide, serves as a convenient index for determining the extent of the peroxidation reaction. TBARS assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, with MDA i.e. the breakdown product at 25°C. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid to yield a chromophore with absorbance maximum at 532 nm.[21,22] The intensity of the color (pink) corresponds to the level of lipid peroxidation in the sample.

Reagents

Phosphate buffer (0.1 M, pH 8), Trichloroacetic acid (10% w/v), Thiobarbituric acid (8% w/v).

Procedure: Aliquots of 0.5 ml distilled water and 1.0 ml 10% TCA were added to a volume of 0.5 ml of spleen tissue homogenate, mixed well and centrifuged at 3000 rpm for 10 min. To 0.2 ml supernatant, 0.1 ml of 8% thiobarbituric acid (TBA) was added. The total solution was placed in a water bath at 80ºC for 40 min and then cooled at room temperature. The absorbance of the clear supernatant was measured at 532 nm in spectrophotometer.[23]

Calculation: The values were calculated using molar extinction coefficient of TBA ($1.56 \times 10^5 M^{-1}cm^{-1}$) as follows:[24]

Concentration of MDA = absorbance/extinction coefficient

Estimation of Superoxide Dismutase (SOD)

Principle: SODs are metallo enzymes that catalyze the dismutation of the superoxide radical (O_2•-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), providing an important defense against oxidative damage. Because of instability of its substrate O_2•- all available assays of SOD are indirect and depends on its ability to scavenge O_2•- from reaction mixture.

Auto oxidation of hydroxylamine results in generation of O_2•-**.** This assay involves reaction between O_2•- and NBT, where the tetra zolium salt gets converted to a form azan dye as shown below.

$O_2^{\cdot-} + NBT^{2+} \leftrightarrow NBT^{+} + O_2$ (1)

$2NBT^{+} \leftrightarrow MF^{+} + NBT^{2+}$ (2)

$O_2^{\cdot-}$ = superoxide anion

NBT = Nitro Blue tetra zolium

MF = Form azan dye measured at 560 nm

SOD diminishes the extent of reduction in this initial stage with little influence on the latter part.

Involvement of SOD in this reaction reduces superoxide ion levels, thereby lowering the rate of form azan dye formation. SOD activity in the experimental sample is measured as the percent inhibition of the rate of form azan dye formation, which is measured at 560 nm.[25,26]

Reagents

EDTA, Sodium carbonate, NBT, hydroxylamine, Phenylazinemethosulphate (PMS):

Procedure: SOD activity was assayed by the method of Kono *et.al*. The assay system consisted of EDTA 0.1mM, sodium carbonate 50 mM and 96 mM of nitro blue tetrazolium (NBT). 2 ml of the above mixture, 0.05 ml of 20mM hydroxylamine and 0.05 ml of 180µ M Phenylazinemethosulphate (PMS) were added, for the test sample 0.1ml of Post mitochondrial supernatant was added and mixture was allowed to stand for 10min. The color intensity of the chromogen was measured at 560 nm against blank.[27]

Calculation: The results were calculated as units/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 100%. Molar coefficient of NBT = 36×10^3 $M^{-1}cm^{-1}$.[24]

Statistical Analysis

The values were expressed as Mean ± SEM from 5 animals. The results were subjected to Statistical analysis by using one- way ANOVA followed by Bonferroni's multiple comparison test or Dunnett's test to calculate the significance. $P<0.05$ was considered as significant.

RESULTS

Assessment of antioxidant activity by estimation of LPO and SOD

Effect of EENL on LPO and SOD in cyclophosphamide induced immunosuppressed mice. (Table 2) The markers of oxidative stress such as lipid peroxidation (LPO) and superoxide dismutase (SOD) in spleen tissue were estimated (Fig. 1, Fig. 2).

Table 2: Effect of ethanolic extract of *Nephelium lappaceum*on; oxidative stress parameters in normal and CP treated mice.

Sr.No	Group N=5	Treatment	LPO µmolMDA/mg protein	SOD units/mg protein
1	**I**	Normal control (saline + SRBC 1×10^8)	7.856± 0.019	1.647±0.004
2	**II**	EENL control (EENL 100 mg/kg + SRBC 1×10^8)	6.928± 0.072	3.921±0.046
3	**III**	CP control (saline + SRBC 1×10^8 + CP)	11.47±0.038***[a]	1.141±0.004***[a]
4	**IV**	EENL (lower dose 100 mg/kg) + SRBC 1×10^8 + CP	7.994 ±0.055***[b]	1.741±0.009***[b]
5	**V**	EENL (higher dose 200 mg/kg) + SRBC 1×10^8 + CP	5.978±0.022***[ab]	3.015±0.008***[ab]

Datas are expressed as Mean ± S.E.M, n=5; using one way analysis variance (ANOVA) followed by Tukey comparison test. *** = p<0.0001, ** = p<0.001, * = p<0.05. [a] = when compared with normal control, [b] = when compared with CP control, [ab] = when compared with normal control and CP control.

There was a remarkable (p<0.0001) increase of LPO in CP treated mice [11.47±0.038] when compared with the normal mice [7.856±0.019]. There was significant (p<0.0001) decrease in LPO in mice treated with EENL & CP 100 mg/kg [7.994 ±0.055] & 200 mg/kg [5.978±0.022] when it was compared with CP treated mice[11.47±0.038]. The group treated with 200 mg/kg had prominently lower value for LOD in contrast to normal control group and CP control group.

There was notable (p<0.0001) decrease in the SOD in CP treated mice [1.141±0.004] when it was collated with normal mice [1.647±0.004]. There was significant (p<0.0001) increase in this antioxidant parameter in mice treated with CP & EENL treated (Group 4 [1.741±0.009] & 5 [3.015±0.008]) when it was compared with CP [1.141±0.004] treated mice. The group treated with 200 mg/kg had prominently higher value for SOD in contrast to normal control group and CP control group.

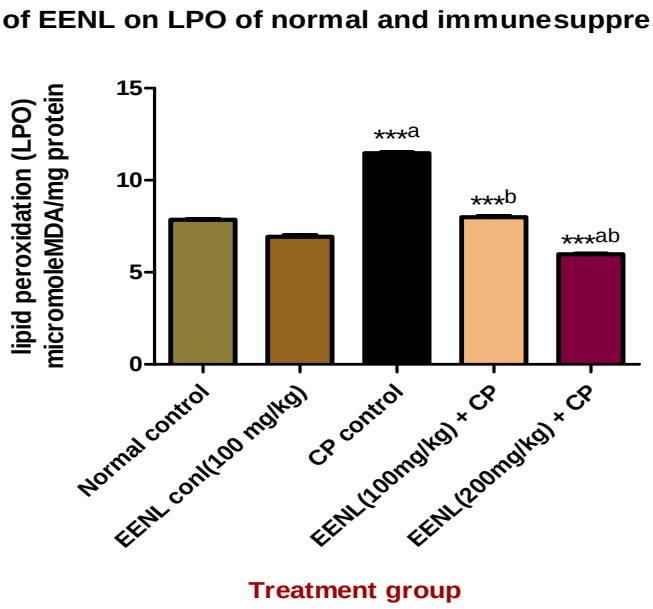

Fig. 1: Effect of EENL on LPO in normal, CP, EENL treated mice.

Values are expressed as Mean ± S.E.M. * = p<0.05, ** = p<0.01, *** = p<0.001 and [ns] = not significant. [a] = when compared with normal control, [b] = when compared with CP control. Statistically analyzed by one-way ANOVA followed by Tukey comparison test.

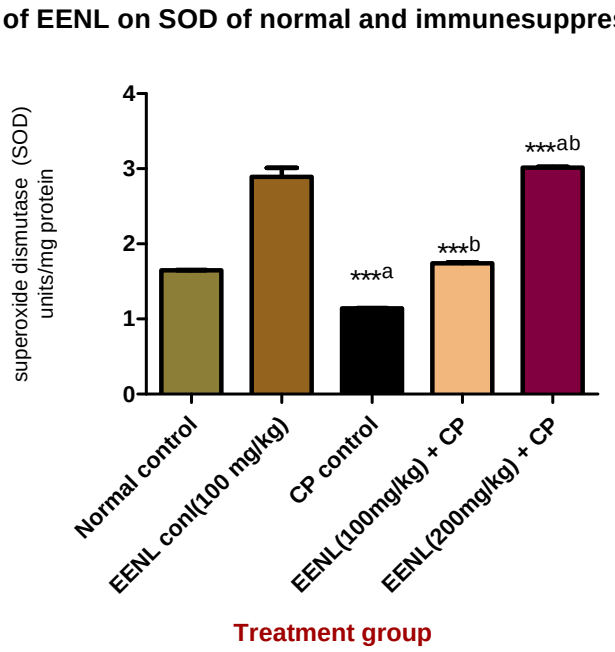

Fig. 2: Effect of EENL on SOD in normal, CP, EENL treated mice.

Values are expressed as Mean ± S.E.M. *** = p<0.0001, ** = p<0.001, * = p<0.05, [ns] = not significant. [a] = when compared with normal control, [b] = when compared with CP control, [ab] =when compared with normal control and CP control. Statistically analyzed by one-way ANOVA followed by Tukey comparison test.

DISCUSSION

Sheep red blood cells were used for antigen challenge and immunization. Antibody molecules, a product of B-lymphocytes and plasma cells, are central to humoral immune responses; IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins etc.[28] Immune cells use ROS in order to support their functions and therefore need adequate levels of antioxidant defenses in order to avoid the harmful effect of an excessive production of ROS.[5]

Cyclophosphamide was used as immunosuppressant as it exerts a depressive effect on antibody production, if given after antigenic stimulation. This has been attributed to its interference with helper T-cell activity.[29] Here in this study, cyclophosphamide at a dose of

50 mg/kg body weight, for three d p.o., showed a significant inhibition of antibody responses. Cyclophosphamide used while screening the immunomodulatory activity generates free radicals and therefore causes oxidative stress in tissues and cells.[30]

LDH is normally an important enzyme of energy metabolism in the body, and an increase of LDH content has been predominantly seen in malignant disease which can lead to serious damage to normal cells.[30] Malondialdehyde (MDA) is one secondary product of lipid peroxidation and has been extensively studied as a potential biomarker for oxidative stress. Induction of LPO has been reported in different tissues of experimental animals after CP administration.[31] CP and its metabolite acrolein cause inactivation of microsomal enzymes and result in increased reactive oxygen species (ROS) generation and LPO.[31] The present study shows that the administration of cyclophosphamide on 4^{th}, 5^{th} and 6^{th} day not only impaired the immune responses but also produced oxidative stress in mice. MDA level in the spleen tissue was significantly higher in mice treated with CP indicating lipid peroxide radical and oxidative stress generation. This increased MDA level was considerably reduced in immune suppressed mice treated with EENL at doses of 100 and 200 mg/kg (Table 2 and Fig. 1).

SOD is an enzyme present in all oxygen-metabolizing cells that plays an important role in maintaining the balance of oxidation and antioxidation and thus protecting cells from oxidative stress by removing superoxide anion free radical (O2-) of aerobic metabolism.[30,31] Cyclophosphamide also reduces the SOD activity.[31] Similarly the decreased level of SOD observed in cyclophosphamide control group was augmented by the treatment of immune suppressed mice with ethanolic extract of *Nephelium lappaceum* (Table 2 and Fig. 2). Also the group treated with 200 mg/kg had notable variance in the level of LPO and SOD when compared to normal control group. This can be attributed to the free radical scavenging capacity of the extract. In view of this, it appears that cyclophosphamide which is a strong generator of superoxide radicals might impair the immune response through oxidative stress.

To further note, the EENL treated mice have shown significant antioxidant activity against normal antigen-antibody reaction and CP induced oxidative stress by decreasing the level of LPO and increasing SOD level as compared with cyclophosphamide alone treated group. The extract hence manifests as a potent antioxidant hallmark.

CONCLUSION

The investigation validates that the rind of *Nephelium Lappaceum*, which is usually discarded, has protective effect against cyclophosphamide induced immunosuppression and oxidative stress. It possesses an antioxidant activity *in vivo*.; the extract prevented toxic effects of oxidative stress on immune system by decreasing the severity of lipid peroxidation (LPO) and increasing superoxide dismutase (SOD) levels in cyclophosphamide (CP) induced immunosuppressed mice.

However advance studies with regard to detailed pharmacological screening of each isolated component and their evaluations are necessary to elucidate the exact comprehensive mechanism of action.

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