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Protective Effect of Silymarin on Human Erythrocyte Against Tert-butyl Hydroperoxide Induced Oxidative Stress *in vitro*

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ABSTRACT

Human erythrocytes are an excellent model system to study interaction of pro-oxidants with membranes. The present study, was carried out to evaluate the *in vitro* antioxidant properties and protective effects of silymarin (milk thistle) in human erythrocyte against tert-butyl hydroperoxide (t-BHP), a potent oxidative stress inducer chemical. Protective effect of silymarin was assessed *in vitro* by monitoring the antioxidant marker glutathione (GSH) and malondialdehyde (MDA) in three groups of erythrocytes: (I) vehicle control, (II) tert-butyl hydroperoxide incubated group and (III) t-BHP co-incubated with various concentrations of silymarin. The effects of silymarin on lipid peroxidation MDA and GSH content in erythrocytes were assessed. It was observed that activity of GSH was significantly decreased and the malondialdehyde levels were elevated when human erythrocytes were incubated with tert-butyl hydroperoxide in concentration as well as time dependent manner. The protective effect of silymarin is elucidated by the significant reversal of the GSH content and malondialdehyde reduction in human erythrocyte. Therefore the results of our present study suggested that silymarin possess substantial protective effect against *in-vitro* induced oxidative stress damages by t-BHP.

Key words: Oxidative stress, silymarin, tert-butyl hydroperoxide, malondialdehyde, GSH

INTRODUCTION

The plant products that are used in traditional medication (Ayurveda) may serve as a potential source of the new therapeutic agents that could be used in the management and prevention for the chronic diseases (Chikezie, 2011; Joseph and Jini, 2011; Butkhup and Samappito, 2011; Chanda *et al*., 2011; Jain *et al*., 2011; Gill *et al*., 2012). Therefore, the herbal antioxidants have become a vital area of research since past few years (Premanath *et al*., 2011; Yahaya *et al*., 2012). Silymarin (synonyms: silybin, silibinin, silliver), a plant derived flavonoid classified as benzopyranones (Fraschini *et al*., 2002) is isolated from the fruits and seeds of the milk thistle (*Silymarin marianum*), which is a mixture of three structural components: silibinin, silydianine and silychristine (Fig. 1). Milk thistle is a member of the Asteracae family (Kren and Walterov, 2005). Studies in various animal models have shown that silymarin regulates the dynamic balance of the activities of enzymes of phase I, phase II and antioxidant enzymes and counteracts against hepatotoxic reactions of various environmental toxins (Upadhyay *et al*., 2007). It has been reported

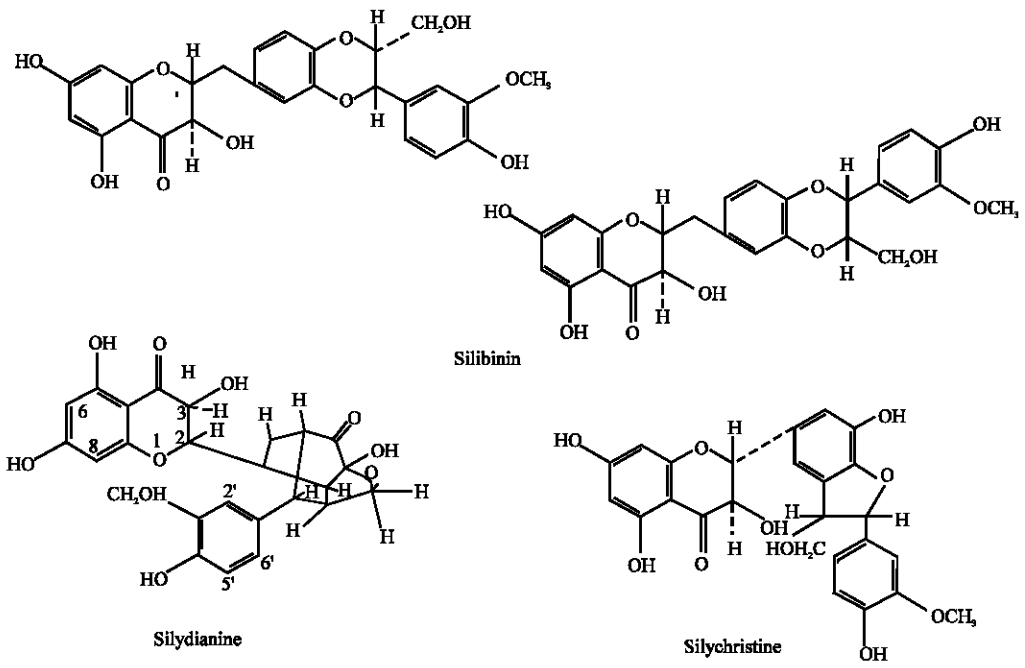

Fig. 1: Silymarin: mixture of three structural components: silibinin, silydianine and silychristine

as having multiple pharmacological activities including antioxidant, hepatoprotectant and anti-inflammatory agent, antibacterial, antiallergic, antimutagenic, antiviral, antineoplastic, antithrombotic agents and vasodilatory actions (Abascal and Yarnell, 2003).

Erythrocytes are a convenient model to understand oxidative damage to membranes from various pro-oxidants as they are particularly sensitive to oxidative stress (Mansour *et al*., 2009). This is because erythrocyte membranes contain high amounts of Polyunsaturated Fatty Acids (PUFA) have higher concentration of oxygen and heme (Nikolaidis and Jamurtas, 2009).

Red blood cells can be regarded as circulating antioxidant carriers, reflecting exposure to ROS. Indeed, they have been used as a model for the investigation of free-radical induced oxidant stress because of several reasons; they are continually exposed to high oxygen tensions, they are unable to replace damaged components, the membrane lipids are composed partly of polyunsaturated fatty acid side chains which are vulnerable to peroxidation and they have antioxidant enzyme systems (Konyalioglu and Karamenderes, 2005).

Under normal conditions, the continuous production of free radicals is compensated by the powerful action of protective enzymes like superoxide dismutase, catalase and glutathione peroxidase that are believed as major antioxidant enzymes present in the human body that protect against the oxygen toxicity. But large number of drugs and other xenobiotics can stimulate the generation of reactive oxygen species by redox cycling and it could be more vulnerable compared to normal condition (Abraham *et al*., 2005).

The aim of our study was to investigate protective antioxidant effect of silymarin against oxidative damage induced by in t-BHP *in vitro*.

MATERIALS AND METHODS

Chemicals: Sodium di-hydrogen phosphate (NaH_2PO_4), di-sodium-hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl), sodium chloride (NaCl), thiobarbituric acid (TBA) and

trichloroacetic acid (TCA) were purchased from Merck, Darmstadt, Germany, dimethylsulfoxide (DMSO) reduced glutathione (GSH), heparin and tert-butylhydroperoxide (t-BHP) were purchased from HIMEDIA Laboratories Pvt. Ltd. India. Silymarin, the commercially available plant flavonoid was purchased from Ranbaxy Laboratory Limited, India.

Collection of blood and isolation of erythrocytes: A volume of 3 mL heparinized human blood was collected by venipuncture from a group of 28 to 32 years old healthy subjects, not exposed to radiation, drugs or any antioxidant supplementation including Vitamins C and E. The blood was centrifuged at 1800x g for 10 min at 4°C. After the removal of plasma, buffy coat and upper 15% of the packed Red Blood Cells (RBCs), the RBCs were washed twice with cold PBS (0.9% NaCl, 10 mM Na_2HPO_4, pH 7.4).

Induction of oxidative stress: Oxidative stress was induced *in vitro* by incubating washed erythrocytes with 10 μM t-BHP for 60 min at 37°C, the effect of silymarin was evaluated by co-incubating erythrocytes with t-BHP and silymarin for 60 min at 37°C. The concentration and duration of t-BHP used in the present study to induce oxidative stress in erythrocytes was the same as described previously by Luqman and Rizvi (2006).

***In vitro* experiments with silymarin:** A stock solution of silymarin and t-BHP were prepared by dissolving it into 1% DMSO and 0.1% DMSO is used as vehicle control. In this experiment three groups of Erythrocytes-(I) vehicle control (II) t-BHP (10 μM) incubated group and (III) t-BHP co-incubated with various concentration(25, 50 and 100 μM) of silymarin.

Packed Red Blood Cells (RBCs) were suspended in 4 volumes of PBS containing 5 mmol L^{-1} glucose (pH 7.4). *In vitro* effects were evaluated by co-incubating the erythrocytes in the presence of silymarin at different concentrations at 37°C for 60 min along with t-BHP. After incubation the suspensions were centrifuged at 1800x g, the RBC were washed twice with at least 50 volumes of PBS and then subjected to assay for MDA level and GSH content.

Time dependent experiments were also done in which the coincubation time t-BHP with silymarin was varied between 15 to 120 min. Parallel control experiments were also performed.

Determination of erythrocyte GSH content: Erythrocyte GSH was measured following the method of Beutler (1984). This method was based on the ability of the -SH group to reduce 5, 5'-dithiobis, 2-nitrobenzoic acid (DTNB) and form a yellow colored anionic product whose OD is measured at 412 nm. The concentration of GSH is expressed in mg mL^{-1} packed RBCs and was determined from a standard curve.

Determination of MDA level: Lipid peroxidation was measured according to the method of Esterbauer and Cheesman (1990). Packed erythrocytes (0.2 mL) were suspended in phosphate buffer, pH 7.4. The lysate (1 mL) was added to 1 mL of 10% Trichloroacetic Acid (TCA) and mixture was centrifuged for 5 min at 3000x g. The supernatant (1 mL) was added to 1 mL of 0.67% thiobarbituric acid (TBA) in 0.05 mol L^{-1} NaOH and boiled for 30 min at 90°C cooled and the absorbance was monitored at 532 nm. The concentration of MDA was determined from a standard plot. MDA content is expressed in expressed as nmol mL^{-1} of packed erythrocytes.

Statistical analysis: Statistical analysis was performed using Graph Pad Prism version 5.01 for Windows Software, San Diego California USA. All values were expressed as Mean±SD deviation of 5 observations. Data were analyzed using one way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple pair wise comparisons between the various treated groups. Statistically significant levels are considered at $p<0.001$, $p<0.01$ and $p<0.05$.

RESULTS AND DISCUSSION

The data presented in Fig. 2 suggested that the treatment with silymarin exhibited significant protection of 32, 65 and 76% erythrocyte GSH content depletion against t-BHP induced stress at concentrations of 25, 50 and 100 μM, respectively with compared to control. Maximum GSH protection ($p<0.001$) was found at 100 μM silymarin concentration.

A time dependent effect of silymarin against tBHP induced oxidative stress is shown in Fig. 3. Maximum GSH protective effects of silymarin were found at 60 min at 100 μM concentration ($p<0.001$).

The result indicated in Fig. 4 that the treatment with silymarin offered significant protection against t-BHP induced oxidative stress and reduced the lipidperoxidation or MDA content by 27%, 52 and 64 at concentrations of 25, 50 and 100 μM, respectively. Maximum protection ($p<0.001$) was found at 100 μM silymarin concentration which significantly reduced the extent of lipid peroxidation.

A time dependent effect of silymarin against t-BHP induced oxidative stress has been shown in Fig. 5. Maximum protective effect of silymarin to inhibit lipid peroxidation was found significantly ($p<0.001$) at 60 min at 100 μM concentration.

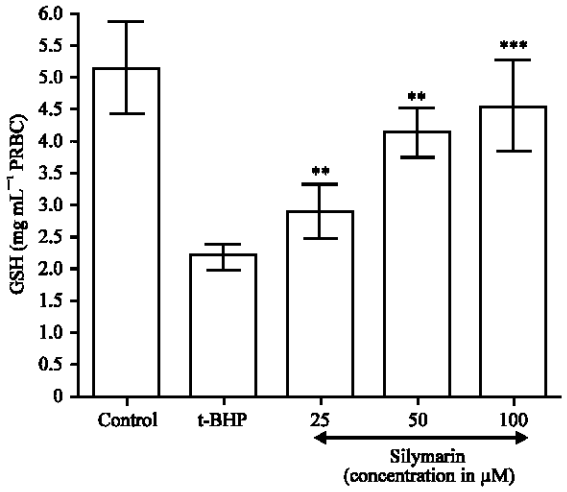

Fig. 2: Concentration dependent effect of silymarin on t-BHP induced changes in intracellular GSH content in human erythrocytes. Incubation with t-BHP caused a decrease in GSH content. Treatment with silymarin showed significant protection against t-BHP induced stress at concentrations of 25, 50 and 100 μM. GSH content is expressed in mg mL^{-1} PRBC. ***, ** and * shown as the mean value which are statistically significant at $p<0.001$, $p<0.01$ and $p<0.05$, respectively

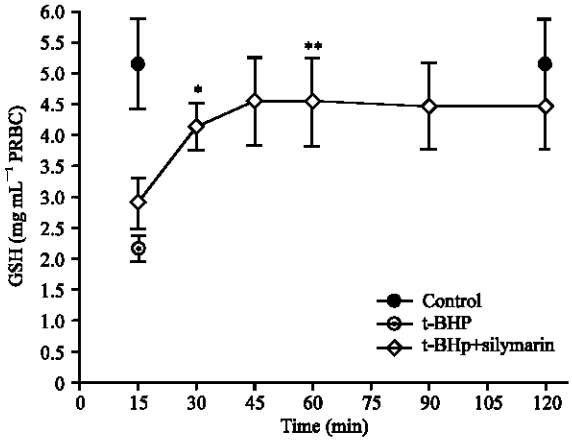

Fig. 3: Time dependent effect of silymarin on erythrocyte GSH content in t-BHP induced oxidative stressed erythrocytes (*p<0.05 and **p<0.01, in comparison with the effect at 15 min). Final concentrations of both silymarin and t-BHP were 100 and 10 μM, respectively

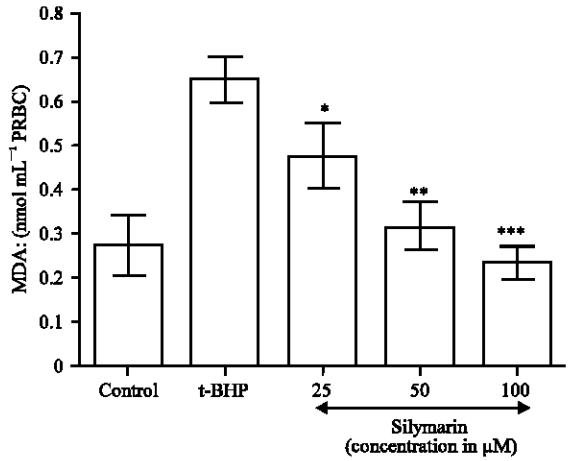

Fig. 4: Concentration dependent effect of silymarin on t-BHP induced changes in MDA content in human erythrocytes. Incubation with t-BHP caused a increase in MDA content. Treatment with silymarin showed significant protection against t-BHP induced stress at concentrations of 25 μM, 50 μM and 100 μM. MDA content is expressed in expressed as nmol mL^{-1} of packed erythrocytes. ***, ** and * shown as the mean value, which are statistically significant at p<0.001, p<0.01 and p<0.05, respectively

Erythrocytes are particularly vulnerable to oxidative damage due to the high degree of polyunsaturated fatty acids in their membranes leading to the formation of the lipid peroxidation product MDA (Mansour *et al.*, 2009). Oxidative free radicals resulting from cellular metabolism tend to disturb cellular membrane integrity and enzyme activities in red blood cells (Halliwell and Gutteridge, 1986, 2006).

GSH, an efficient antioxidant present in almost all living cells, is also considered as a biomarker of redox imbalance at cellular level (Ross, 1988). The results shown in Fig. 2 and 3 reflected

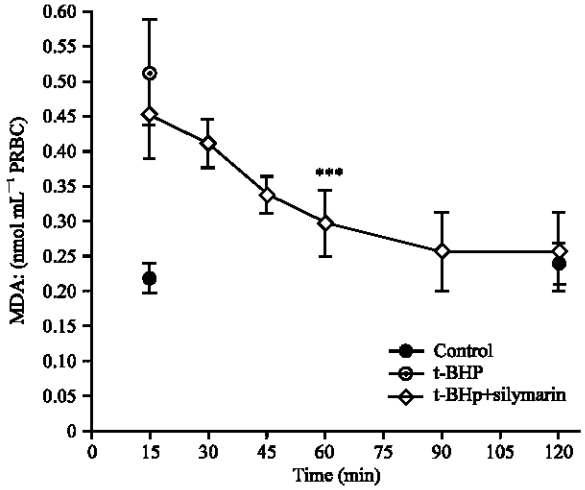

Fig. 5: Time dependent effect of silymarin on membrane MDA content in t-BHP induced oxidative stressed erythrocytes (***p<0.001, in comparison with the effect at 15 min). Final concentrations of both silymarin 100 μM and t-BHP were 10 μM

significant increase in erythrocyte GSH content in silymarin treated subjects, which highlights the role of silymarin in mitigation of t-BHP induced oxidative stress. These results indicated the dose as well as time dependent protective effect of silymarin.

Because of the potential deleterious effects of free radicals and hydroperoxides, perturbations that stimulate lipid peroxidation and weaken antioxidant defense capability may cause or increase cellular susceptibility to oxidative damage (Konyalioglu and Karamenderes, 2005). The results of our experiments also suggest that erythrocyte membrane MDA contents are may be protected by silymarin in concentration dependent manner as shown in Fig. 4 which emphasizes the role of silymarin as an antioxidant and its function against oxidative stress in red blood cells. Silymarin is also reported to increase the activity of superoxide dismutase in erythrocytes and other antioxidative enzymes by other workers (Singh and Agarwal, 2002; Kiruthiga *et al.*, 2007; Upadhyay *et al.*, 2007).

CONCLUSION

Our results allow us to draw a conclusion that silymarin possess substantial protective effect against t-BHP induced oxidative stress *in-vitro* and acts as a potent free radical scavenger. These finding provide a basis for the development of novel therapeutic strategies, such as antioxidant supplementation like a flavonoid silymarin for protection against the damages caused by environmental contaminants and several chronic diseases. Further, these findings may also help understand some of the beneficial effects of other plant products to be used for better human health management.

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REFERENCES

Abascal, K. and E. Yarnell, 2003. The many faces of *Silybum marianum* (milk thistle): Part 2-Clinical uses, safety and types of preparations. Altern. Complement Ther., 9: 251-256.

Abraham, W.M., A.J. Bourdelais, A. Ahmed, I. Serebriakov and D.G. Baden, 2005. Effects of inhaled brevetoxins in allergic airways: Toxinallergen interactions and pharmacologic intervention. Environ. Health Perspect., 113: 632-637.

Beutler, E., 1984. A Manual of Biochemical Methods. Grunne and Stratton, New York, USA.

Butkhup, L. and S. Samappito, 2011. *In vitro* free radical scavenging and antimicrobial activity of some selected Thai medicinal plants. Res. J. Med. Plant, 5: 254-265.

Chanda, S., R. Dave and M. Kaneria, 2011. *In vitro* antioxidant property of some Indian medicinal plants. Res. J. Med. Plant, 5: 169-179.

Chikezie, P.C., 2011. Oxidative stress indicator of human sickel erythrocytes incubated in aqueous extracts of three medicinal plants. Asian J. Biochem., 6: 373-383.

Esterbauer, H. and K.H. Cheesman, 1990. Determination of aldehyde lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. Methods Enzymol., 186: 407-410.

Fraschini, F., G. Demartini and D. Espostii, 2002. Pharmacology of silymarin. Clin. Drug Invest., 22: 51-65.

Gill, N.S., S. Dhawan, A. Jain, R. Arora and M. Bali, 2012. Antioxidant and anti-ulcerogenic activity of wild *Punica granatum* ethanolic seed extract. Res. J. Med. Plant, 6: 47-55.

Halliwell, B. and J.M.C. Gutteridge, 1986. Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. Arch. Biochem. Biophys., 246: 501-514.

Halliwell, B. and J.M.C. Gutteridge, 2006. Free Radicals in Biology and Medicine. 4th Edn., Clarendon Press, Oxford.

Jain, V., S.K. Verma, S.S. Katewa, S. Anandjiwala and B. Singh, 2011. Free radical scavenging property of *Bombax ceiba* Linn. root. Res. J. Med. Plant, 5: 462-470.

Joseph, B. and D. Jini, 2011. Insight into the hypoglycaemic effect of traditional indian herbs used in the treatment of diabetes. Res. J. Med. Plant, 5: 352-376.

Kiruthiga, P.V., R.B. Shafreen, S.K. Pandian, S. Arun, S. Govindu and K.P. Devi, 2007. Protective effect of silymarin on erythrocyte haemolysate against benzo(a)pyrene and exogenous reactive oxygen species (H_2O_2) induced oxidative stress. Chemosphere, 68: 1511-1518.

Konyalioglu, S. and C. Karamenderes, 2005. The protective effects of *Achillea* L. species native in Turkey against H_2O_2-induced oxidatived damage in human erythrocytes and leucocytes. J. Ethnopharmacol., 102: 221-227.

Kren, V. and D. Walterov, 2005. Silybin and silymarin-new effects and applications. Biomed. Papers, 149: 29-41.

Luqman, S. and S.I. Rizvi, 2006. Protection of lipid peroxidation and carbonyl formation in proteins by capsaicin in human erythrocytes subjected to oxidative stress. Phytother. Res., 20: 303-306.

Mansour, S.A., A.T. Mossa and T.M. Heikal, 2009. Effects of methomyl on lipid peroxidation and antioxidant enzymes in rat erythrocytes: *In vitro* studies. Toxicol. Ind. Health, 25: 557-563.

Nikolaidis, M.G. and A.Z. Jamurtas, 2009. Blood as a reactive species generator and redox status regulator during exercise. Arch. Biochem. Biophys., 490: 77-84.

Premanath, R., J. Sudisha, N.L. Devi and S.M. Aradhya, 2011. Antibacterial and anti-oxidant activities of fenugreek (*Trigonella foenum graecum* L.) leaves. Res. J. Med. Plant, 5: 695-705.

Ross, D., 1988. Glutathione, free radicals and chemotherapeutic agents. Mechanisms of free-radical induced toxicity and glutathione-dependent protection. Pharmacol. Ther., 37: 231-249.

Singh, R.P. and R. Agarwal, 2002. Flavonoid antioxidant silymarin and skin cancer. Antioxid. Redox Signal, 4: 655-663.

Upadhyay, G., A. Kumar and M.P. Singh, 2007. Effect of silymarin on pyrogallol and rifampicin-induced hepatotoxicity in mouse. Eur. J. Pharmacol., 565: 190-201.

Yahaya, T., J. Okpuzor and T. Ajayi, 2012. The prophylactic efficacy of roselle (*H. sabdariffa*), moringa (*Moringa oleifera*), ginger (*Z. officinale*) and ugwu (*T. occidentalis*) on the hematology and serum protein of albino rats (*Rattus norvegicus*) exposed to cement dust. Res. J. Med. Plant, 6: 189-196.