



PROTECTIVE ROLE OF URSOLIC ACID IN DEN INDUCED OXIDATIVE STRESS MEDIATED HEPATOCELLULAR CARCINOMA - A FOCUS ON THIOL STATUS

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ABSTRACT

Hepatocellular carcinoma (HCC) is usually associated with long term inflammation and fibrosis and often occurs in cirrhotic livers. Liver is a self protective organ, has a dynamic regenerating capacity and can withstand the assault with its efficient detoxification system. In spite of this, poor prognosis of HCC has limited the treatment options, greatly. Laboratory studies indicate that chronic inflammation and hyperplasia induced by viral or nitroso compounds are closely associated with the development of HCC. In our current study, chemical carcinogens were used to induce liver cancer in male wistar rats. Chemical carcinogens cause free radical generation and consequently oxidative stress. The antioxidant nature of ursolic acid was confirmed, as it restored the activities of enzymes - catalase, superoxide dismutase and glutathione peroxidase. GSH redox couple contributed by GSH/GSSG seems to be the major redox component in a cell and also sensitive and susceptible to alterations during oxidative stress mediated changes. Decrease in GSH levels and increase in GSSG levels were observed in cancer bearing animals. This reflected a decrease in ratio of GSH/GSSG followed by a decreased redox status. Above said alterations were reversed in ursolic acid treated animals, suggesting that it is capable of acting at the cellular level, in restoring the reduction environment and cellular functions. Decreased expression of Ki 67 suggests the protective nature of Ursolic acid, thus establishing it as an effective chemopreventive and chemotherapeutic compound for treatment of Hepatocellular carcinoma.

Keywords: Ki 67, Hepatocellular carcinoma, Diethylnitrosamine, Ursolic acid, Phenobarbital

INTRODUCTION

Carcinogenesis in human and laboratory animals is a complex, multistep process and may result from the action of any one or a combination of chemical, physical, biological and genetic insult to cells¹. Carcinogenesis by small molecular weight chemicals involves either a direct action of the chemical on cellular DNA or metabolism of the parent chemical to produce a permanent chemical change in the DNA structure². Epidemiological and laboratory studies indicate that chronic inflammation and hyperplasia induced by viral or nitroso compounds are closely associated with the development of HCC^{3,4}. Experimental hepatocarcinogenesis can be induced by various chemical carcinogens such as diethylnitrosamine (DEN), Aflatoxin B1 etc. Diethylnitrosamine is a potent hepatocarcinogen in rats influencing the initiation stage of carcinogenesis and induces DNA base modifications, DNA strand breaks and in turn hepatocellular carcinomas without cirrhosis, through the development of putative preneoplastic focal lesions⁵. Administration of DEN to animals has been shown to cause cancer in liver and at lower incidences, in other organs⁶. Enhancement of DEN initiated carcinogenesis could be done by promoters such as Phenobarbital (PB) which transforms DEN initiated cells to foci and to hepatocellular carcinoma⁷. Phenobarbital has been found to be one of the most efficacious tumor promoting agents in the liver⁸. The DEN model of experimental hepatocarcinogenesis provides a unique tool for studying cellular changes resulting from the administration of carcinogen to the development of premalignant phenotype of the cell, mechanism of cell growth, differentiation and cell death⁹.

Diets rich in fruits and vegetables have been associated with reduced risk of developing chronic diseases such as cardiovascular disease, cancer and diabetes¹⁰. Terpenes are the class of compounds widely distributed in plants and are widely present in food consumed by humans¹¹. Triterpenoids like the steroids are formed in nature by the cyclisation of squalene with the retention of all 30 carbon atoms in the molecule¹². Ursolic acid, a pentacyclic triterpenoid exists in natural plants in the form of free acid or aglycones¹³. Ursolic acid (UA) (3 β hydroxyl-urs-12-en-28oic acid) is present in berries, leaves, flowers and many kinds of medicinal herbs such as *Perilla frutescens*¹⁴, *Rosmarinus officianalis* and *Eriobotrya japonica*¹⁵. It has wide range of pharmacological effects such as antitumor, antioxidant, anti-inflammatory, antihypertensive, hepatoprotective, antiulcer, antimicrobial, antihyperlipidemic and antiviral¹⁶. Oxidative stress can be linked to pathological conditions

such as cellular senescence, initiation and progression of cancer¹⁷. Regulated changes in ROS concentration are critical for functions including respiration, control of enzymatic activation, transcriptional regulation, modulation of signal transduction pathways, cell cycle progression and apoptosis¹⁸. The deleterious effects of ROS are overcome by normal cells which have enzymatic and non-enzymatic methods that help to maintain redox balance and prevent the damage on cellular structures¹⁹. The present study was hence aimed to carry out a systemic investigation of the protective role of ursolic acid on liver by estimating enzymic and non enzymic antioxidants along with assessment of redox status, as these parameters are considered for maintenance of biochemical homeostasis within the cell. Ki 67 expression by immunohistochemistry was also done to confirm the cancer occurrence.

MATERIALS AND METHODS

Animals

Male rats of Wistar strain, weighing 100-150 g were used for the study. The animals were divided into five groups of six animals each. All rats were housed in clean polypropylene cage and were maintained in a diurnal 12 hr light/dark cycle with constant temperature and humidity. The animals were fed a commercial diet and clean drinking water was made available ad libitum. This work was sanctioned and approved by the Institutional animal ethical committee vide No. IAEC NO: 02/078/07. The rats were divided into five groups of six animals each.

Group 1: Control animals treated with 0.2% gum acacia.

Group 2: N-nitrosodiethylamine in saline as single ip dose (200 mg/kg body wt). After a recovery period of 2 weeks, phenobarbital @ the dose of 0.6 % was finely ground with 0.2% gum acacia and was given in drinking water for 26 weeks.

Group 3: Ursolic acid administered at a dose of 20 mg/kg body wt. in 0.2% gum acacia p.o. 15 days before DEN injection as in group 2.

Group 4: Cancer bearing animals treated with Ursolic acid at 20 mg / kg body wt. in gum acacia p.o for 6 weeks after 26 weeks of DEN administration.

Group 5: Ursolic acid alone treated at 20 mg/kg body wt. in gum acacia for 6 weeks.

After the experimental period the animals were killed by cervical decapitation and liver was excised immediately, washed in ice cold saline to remove any extraneous matter, cleaned and blotted to dryness in filter paper.

A portion of the liver tissue was immediately utilized for immunohistochemistry. Remaining liver tissue was stored in -80°C and used for further studies. A 10% homogenate of liver tissue was prepared in appropriate buffers as necessitated by the protocols. Dilutions were decided based on the protein concentration.

PROTOCOLS

Immuno histochemical analysis of cancer antigen - Ki 67

Immunohistochemical analysis of Ki 67 expression was carried out on paraffin sections of liver tissue using the B-SAP universal staining kit (Span diagnostic ltd, Surat, India). Initially, the sections were deparaffinised in xylene and dehydrated in ethanol. The sections were then washed with phosphate buffered saline (PBS) and incubated with 3% H₂O₂ at room temperature for 15 min to quench exogenous peroxidase activity. After antigen retrieval (15 min of autoclaving in 10 mM citrate buffer pH 6.0), the slides were incubated for 5 min with blocking solution (10% normal goat serum) at room temperature. Then the sections were incubated for 4 hrs with monoclonal anti-Ki 67 antibody (Sigma chemicals). After washing with PBS, the sections were subsequently incubated with biotinylated secondary antibody for 30 min at room temperature. Later, the sections were treated with Diaminobenzidine chromogen for 15 min. Finally, the sections were washed with deionised water, counter stained with hematoxylin and mounted. Photographs were taken using Nikon microscope ECLIPSE E 400, model 115, Japan.

Enzymic and nonenzymic antioxidants

Catalase (E.C. 1.11.1.6) (CAT)

The activity of catalase (CAT) was estimated by the method of Sinha²⁰. CAT causes rapid decomposition of H₂O₂ to H₂O. The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H₂O₂ with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus formed was measured at 610 nm. The activity of CAT was expressed at μ moles of H₂O₂ consumed/min/mg protein.

Superoxide dismutase (E.C. 1.15.1.1) (SOD)

The activity of SOD was estimated according to the method of Marklund and Marklund²¹. The degree of inhibition of auto-oxidation of pyrogallol, in alkaline pH by SOD was used as a measure of the enzyme activity. The enzyme activity is defined as units /mg protein, where one unit is equal to the amount of enzyme required to inhibit auto oxidation of pyrogallol by 50%.

Glutathione Peroxidase (E.C. 1.11.1.9) (GPX)

Activity of GPX was assayed by the method described by Rotruck et al.²². The remaining GSH after the enzyme catalyzed reaction was complexed with 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) that absorbs at maximum wavelength of 412 nm. Enzyme activity was expressed as μ g of GSH consumed /min/mg protein.

Reduced glutathione (GSH)

GSH was determined by the method of Moron et al.²³ which is based on the reaction of GSH with DTNB to give a compound that absorbs at 412 nm. The amount of GSH was expressed as n mole / mg protein. The GSSG level was measured after samples were derivatised with 1% 1-flouro-2, 4-dinitrobenzene according to Asensi et al.²⁴.

GSH/GSSG Ratio

Redox state was determined by the redox index: $(\text{GSH} + 2 \times \text{GSSG}) / (2 \times \text{GSSG} \times 100)$

Estimation of total thiols (TSH)

The levels of TSH were estimated by the method of Sedlack and Lindsay²⁵. This method is based on the reaction of tissue homogenate with DTNB wherein DTNB is reduced by the thiol (SH) group to form 1 mole of -nitro 5-mercaptobenzoic acid per mole -SH. The absorbance of the supernatant was read at 412 nm. The concentration of TSH was expressed as μ g/mg protein.

Non protein thiol (NPSH)

NPSH was estimated as similar to TSH with slight modifications. The tissue homogenate was mixed with 10% TCA to precipitate the proteins and centrifuged. DTNB reacts with the supernatant and the color intensity was measured at 412 nm. The concentration of NPSH was expressed as μ g/mg protein.

Statistical analysis

Data are presented as the mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was used to detect the significant changes between the groups. The student least significant difference (LSD) method was used to compare the means of different groups and the significance was denoted by 'P' value. Commercial software program (Sigma STAT, Version 7.5) was used for statistical analysis.

RESULTS

The results of both qualitative and quantitative entities are compared and discussed to understand both the condition of the disease and the efficacy of the candidate drug selected, to ameliorate the severity of the disease.

Immunohistochemical expression of Ki 67 [Figure 1-3]

There was an increased expression of Ki 67 in carcinogen induced (DEN +PB) rats which decreased on administration of ursolic acid, possibly due to apoptosis of damaged cells.

Sections of liver tissue of animals continuously treated with Ursolic acid exhibited no staining of Ki 67 (Fig.1). Sections of liver tissue of animals treated with carcinogen (DEN and PB) for 26 weeks showing intense staining due to over expression of Ki 67 (Fig.2). Sections of liver tissue of animals treated with ursolic acid for 6 weeks after carcinogen induction showing weak staining of Ki 67 (1+) due to increased apoptosis of liver cells (Fig.3).



Fig. 1: Group 3 (UA treated before DEN)

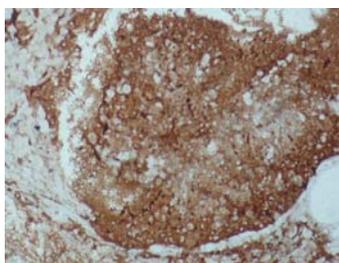


Fig. 2: Group 2 (DEN + PB 26 weeks)



Fig. 3: Group IV (UA treated after DEN)

Fig. 1-3: Ki 67 expression in liver tissues

Changes in antioxidant status [Figure 4]

Enzymic antioxidant status alters due to oxidative stress mediated hepatocarcinogenesis. Alterations in the levels reflect the severity of the damage. Significant ($p < 0.001$) decrease in the levels of all three

enzymes, emphasizes the generation of free radicals and oxidative stress due to toxicity induced by DEN and phenobarbital. Restoration of levels of these antioxidant enzymes were seen after administration of UA, which significantly ($p < 0.001$) increased in liver of Group 4 animals.

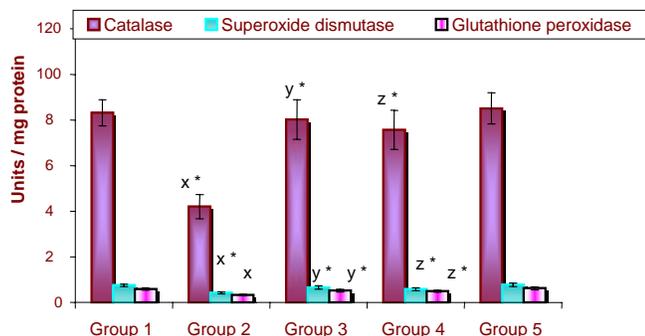


Fig. 4: Effect of Ursolic acid on enzymic antioxidant status in liver of control and experimental rats

Values are expressed as mean \pm SD for six rats in each experimental group

CAT- μ moles of H_2O_2 utilized / min/ mg protein; SOD - Units / mg protein; GPX - μ moles of GSH oxidized / min/ mg protein

x - as compared with Group 1; y - as compared with Group 2; z - as compared with Group 2

Statistical significance: * $p < 0.001$; # $p < 0.01$; \$ $p < 0.05$

Changes in the thiol (Non-enzymic antioxidants) status [Figure 5]

Non-enzymic antioxidant status during experimental condition has been well understood by the levels of total thiols (TSH), non-protein thiols (NPSH) and protein thiols (PSH). A significant ($p < 0.001$)

decrease in the levels of TSH and NPSH were recorded in cancer bearing animals (Group 2) reflecting a severe oxidative stress due to free radical generation UA being a potent antioxidant, restored the levels of TSH ($P < 0.001$; $p < 0.01$), PSH ($p < 0.001$; $p < 0.01$) and NPSH ($P < 0.001$; $p < 0.05$) in Group 3 and Group 4 animals, respectively.

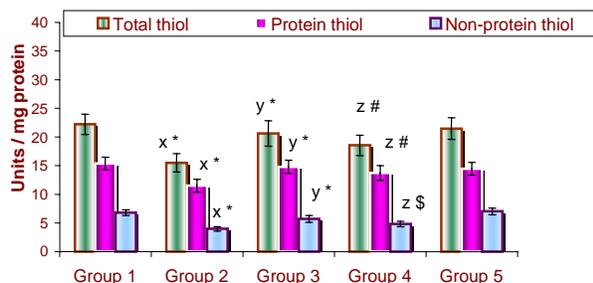


Fig. 5: Effect of Ursolic acid on Thiol (Non-enzymic antioxidant) status in liver of control and experimental rats

Values are expressed as mean \pm SD for six rats in each experimental group

TSH, PSH and NPSH - μ g/ mg protein

x - as compared with Group 1; y - as compared with Group 2; z - as compared with Group 2

Statistical significance: * $p < 0.001$; # $p < 0.01$; \$ $p < 0.05$

Changes in the redox status of the cell [Table 1]

The alteration of GSH redox couple is reflected in by the levels of GSH, GSSG and GSH / GSSG ratio. The redox state is calculated from the levels of these compounds. There was a significant reduction ($p < 0.001$) in the levels of GSH accompanied by an increase ($p < 0.001$) in GSSG levels, in cancer bearing animals. The ratio of GSH / GSSG was further calculated from the levels of GSH and GSSG. The

ratio significantly decreased in cancer bearing animals compared to control animals.

The redox state also decreased in cancer bearing animals. Ursolic acid administration restored the redox state ($p < 0.001$) to normal in Group 3 and Group 4 animals, respectively. This was reflected by a significant increase ($p < 0.001$; $p < 0.01$) in the levels of GSH and GSSG ($p < 0.001$) in Group 3 and Group 4 animals, respectively.

Table 1: Effect of Ursolic acid on Redox status of liver cell in control and experimental rats

| Groups | GSH (n moles) | GSSG (n moles) | GSH/GSSG | Redox |
|----------------------|-----------------|----------------|----------------|----------------|
| Control | 32.46 ± 2.54 | 1.78 ± 0.14 | 18.18 ± 0.04 | 0.16 ± 0.01 |
| DEN + PB (26 weeks) | 22.58 ± 2.32 *x | 3.09 ± 0.29 *x | 7.97 ± 0.02 *x | 0.11 ± 0.01 *x |
| UA teated before DEN | 29.96 ± 3.3*y | 1.9 ± 0.13*y | 15.24 ± 0.66*y | 0.15 ± 0.01*y |
| UA treated after DEN | 27.39 ± 2.65#z | 2.46 ± 0.24*z | 11.13 ± 0.02*z | 0.14 ± 0.01#z |
| UA alone treated | 31.72 ± 2.76 | 1.7 ± 0.16 | 18.6 ± 0.19 | 0.16 ± 0.01 |

(GSH) Glutathione Reduced; (GSSG) Glutathione Oxidised

Values are expressed as mean ± SD for six rats in each experimental group; GSH - n moles / mg protein; GSSG- n moles / mg protein

Redox = $GSH + 2 \times GSSG / 2 \times GSSG \times 100$

x - as compared with Group 1; y - as compared with Group 2; z - as compared with Group 2

Statistical significance: *p<0.001; # p<0.01; \$ p<0.05

DISCUSSION

Diethylnitrosamine (DEN) is one of the most important environmental carcinogens that primarily induce tumors of the liver, because of its relatively simple metabolic pathway and potent carcinogenic activity²⁶. In experimental liver carcinogenesis, early preneoplastic foci appear which are induced by an initiating carcinogen DEN and they exhibit moderately elevated rate of proliferation^{27,28}. These complex phenotypic foci appear to be hyperproliferative and are precursors of the benign neoplastic nodules²⁹.

Among the various methods available, two important methods of studying cancer cell proliferation are measuring the rate of DNA synthesis and the immunohistochemical reaction for proliferative cell marker³⁰. Ki67 protein is associated with active cell proliferation. It is expressed in all phases of cell cycle except G0, with highest expression seen in G2/M.

Proliferation correlates with its accumulation from G1-S-G2 phase to mitosis, where it is found at its highest content in nuclei of proliferating cells, but not in nuclei of quiescent or resting cells³¹. Ki-67 positive nuclei depicting its focal expression as well as plenty of immunopositive cells was observed in DEN-PB model, which increased over a period from 16 weeks to 32 weeks.

In our current study, Ki-67 was used to evaluate liver cell proliferation activity. The tissue sections of cancer bearing animals (group 2) showed strong positive nuclear staining, indicating that there was high rate of proliferation in these cells confirming malignant transformation.

Following carcinogen administration, altered hepatocyte foci (AHF) develop under conditions of initiator-promoter administration with an increase in focal cell proliferation. Administration of promoting agents such as phenobarbital causes expansion and selective enhancement of proliferation of the initiated cell populations over non-initiated cells in the target tissue resulting in the appearance of putative preneoplastic AHF³².

The intense staining observed in our current study corroborates well with above discussed, established facts which made us conclude that malignant transformation had occurred and that mainly in part due to the effect of DEN and promoter phenobarbital.

There was a weak staining graded as 1+ in UA treated groups. It can be stated that UA could have accelerated the repair mechanism of DNA to bring down the proliferation of neoplastic cells. Thiols are found to be essential in maintaining the functional integrity of enzymes preferably DNA repair enzymes such as DNA alkyl transferase, DNA glycosylase and DNA ligase. They are rich in thiols and also maintain genomic integrity of the cell. In conditions associated with oxidative stress, preferably carcinogenesis, alterations in the thiol status could deactivate these enzymes, thus accelerating the rate of cell proliferation.

Though, we have not gone in to the exact molecular mechanism underlying the protective and preventive role of ursolic acid, we assume that the decrease in Ki 67 labeling in treated tissues must have been due to the increased death of cancer cells and subsequent regeneration of normal cells, which the liver tries to compensate. This compensatory mechanism could have been induced by the protective effect of ursolic acid which is more prominently reported to be on liver.

Cells have developed a number of enzymic and non enzymic defense mechanism which provides protection against free-radical mediated reactions and thus maintains their homeostasis. Thus as long as the homeostasis is maintained between radical generation and radical dissipation, the cell functioning is not disturbed.

Antioxidant defense systems are therefore important in restoring the intracellular redox balance and their effects upon gene transcription. Catalase is a peroxisomal enzyme catalyzing the breakdown of hydrogen peroxide into oxygen and water. It plays a central role in organismal oxidant defense along with glutathione peroxidase and superoxide dismutase^{33,34}.

There was a significant decrease in the activities of catalase in DEN administered animals. Catalase activity in the liver of some transplantable hepatoma cells and cultured hepatoma cell lines have found to be decreased³⁵, where they have explained the reason for decrease is due to the depression of enzyme biosynthesis³⁶, which in turn is because of depression of catalase gene expression³⁷.

Similarly there was a significant decrease in the activities of superoxide dismutase and glutathione peroxidase in carcinogen treated animals. Administration of UA has significantly increased the activities of these enzymes in Group 3 and Group 5 animals.

Earlier, we have published³⁸ that UA has decreased the levels of MDA, thus bringing down lipid peroxidation and in turn oxidative stress. The effect of UA on these enzymes can be due to its antioxidative property of quenching free radicals. It has been reported that UA increases the levels of catalase and its mRNA expression in rats.

Thus, we interpret that UA due to its effect on increasing the mRNA expression of catalase³⁹ might tend to increase the protein synthesis, which in turn leads to increase in the activity of the enzyme. Since catalase preferably quenches H₂O₂, UA might elicit its antioxidative potential at the level of catalase.

Further, since catalase is synthesized by microbodies and hepatocytes contain large number of it, administration of UA might have increased the proliferation rate of microbodies, thus increasing their synthesis. Thus, though all three enzymes contribute to the antioxidant defense system, it can be stated that the mechanism of action of UA is mainly mediated or targeted towards increasing the levels of catalase.

Since, quenching of H₂O₂ can decrease the levels of radicals and oxidative stress, subsequent elevation of GPX and SOD would have occurred in the cell. Ursolic acid treatment at 60 mg / kg body wt had decreased the levels of GPX and SOD by 22% and 25% respectively, compared to normotensive Dahl-sensitive rats ⁴⁰.

Thiols are a class of organic compounds that contain a sulfhydryl group (-SH), also known as thiol group, that is composed of a sulfur atom and a hydrogen atom attached to a carbon atom. Among all the antioxidants that are available in the body, thiols constitute the major portion of the total body antioxidants and plays significant role in defense against reactive oxygen species ⁴¹.

The total thiol status of the body especially thiol (-SH) groups present on protein are considered as major plasma antioxidants in vivo and most of them are present over albumin ⁴² ultimately behaving as major reducing groups, present in our body fluids ⁴³. Under conditions of stress, oxidation of cysteine residues can lead to the formation of mixed disulphides between protein thiol groups and low molecular weight mass thiols, preferably with glutathione.

In our current study, decrease in the levels of total thiols, non protein thiols and protein thiols confirms the occurrence of oxidative stress formed due to the electrophiles generated by the toxicity of DEN and the promoter phenobarbital. This decrease may also be due to decrease in thiol antioxidant GSH. GSH has diverse important functions such as storage and transport of cysteine, maintaining the reduced state of proteins and thiols, and protecting cells from toxic compounds such as reactive oxygen species ^{44,45}.

The decrease of GSH levels in the cancer tissue, in the current study confirms that GSH homeostasis at the cellular level is disturbed which is maintained by a balance between biosynthesis, uptake, oxidation and export. Reversal on the levels of thiols, were observed on treatment with UA. The protective effect of UA on cells seems that, it acts to restore the redox state of cells by increasing the levels of GSH and protein thiols.

Thus, UA might act as an antioxidant in restoring the levels of thiol status, which in turn, is reflected in restoring the biological activity of the cell. Since, many enzymes have essential thiols and are thus inactivated during oxidative stress; restoration of thiol status induced by UA may be partly responsible for the favorable alterations in homeostasis of the cell.

Among various intracellular redox couples, glutathione couple is most important since glutathione concentration is about 500 - 1000 fold higher than TRX and NADPH ⁴⁶. Redox state of the system is estimated by taking the ratio of GSH / GSSG. Upon the effects of oxidative stimuli GSH / GSSG ratio tends to decrease by raising the concentration of glutathione disulphide or decreasing the reduced glutathione amount. As explained above, decrease in the GSH / GSSG ratio was observed due to decreased levels of reduced glutathione and increased levels of GSSG.

Primary target of redox regulation are found to be the sulphhydryl group of cysteine residue ⁴⁷. Oxidative alteration of a reactive cysteine usually leads to the formation of sulphenic derivative that in the presence of GSH is efficiently transformed in a mixed disulphide bond (GS-R). Therefore, glutathione-s-thiolation represents a first line defense against oxidative stress.

Since, there are no earlier reports of UA being tested for alterations in the redox status, we assume that ursolic acid could have executed the protective mechanism by increasing the GSH and PSH content, thus restoring the redox environment which is conducive for the biological process to proceed normally, thus accelerating DNA repair process and ultimately apoptosis of cancer cells. Earlier studies also states that UA blocked cancer cell growth in mammalian cells at the primary step of DNA replication by acting on DNA polymerases ⁴⁸.

Thus, from the current study we put forward that UA exhibits antiproliferative effect on DEN induced experimental hepatocarcinogenesis in animal model. by restoring the levels of

glutathione and that of GSH / GSSG ratio by inactivating ROS via restoration of antioxidant enzymes such as catalase.

Further, it can also be assumed that ursolic acid might execute a normalization process by maintaining adequate levels of GSH which is essential for detoxification of xenobiotics. Current observation of restoration of GSH / GSSG ratio was similar to that observed by Martin Aragon ⁴⁹ where ursolic acid restored the activity of GR and GSH / GSSG ratio in CCl₄ treated rats.

Thus, the above discussed facts, suggest that ursolic acid participates in restoring the reduction environment of a cell which might also be a contributing factor in restoration of cellular functions.

With this note we conclude that use of such compounds isolated from plants could be a better option to meet the objective of finding a suitable treatment for hepatocellular carcinoma. Since, liver as such is an organ with regenerating capacity and withstands maximum assault; holistic way of living can protect the liver through out our life.

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