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A Study on the efficacy of Withaferin-A in reducing oxidative stress against DEN induced hepatocellular carcinoma

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Abstract: *To evaluate the modulating efficacy of Withaferin-A (WFA) against N-nitrosodiethylamine (DEN)-induced hepatocellular carcinoma in male rats at the biochemical, oxidative stress and antioxidants levels. The animals were divided into five groups 6 rats each. Group I was served as control. Group II was treated with dose of DEN. The group III and IV were served as pre- and post-treated animals respectively. Groups V was serve drug control treated with Withaferin-A alone. Administration of DEN has brought down the levels of membrane bound enzymes and antioxidants; whereas increased level of ROS, free radicals and LPO, which were later found to be reversed by the administration of Withaferin-A. The findings from the above study suggest the effectiveness of Withaferin-A in reducing the oxidative stress mediated changes in the liver and improving the functions, antioxidant capacities of rats.*

Key words: *DEN, Hepatocellular Cellular Carcinoma, Withaferin-A, Oxidative Stress*

I. Background

Cancer is a major health problem and arises from the uncontrolled proliferation and spread of clones of transformed cell. Particularly, hepatocellular carcinoma (HCC) is the third most frequent cause of death in worldwide. Most HCC occur in cirrhotic livers, and the common chronic inflammation associated with severe oxidative stress (1). Over production of Reactive Oxygen Species (ROS) through either endogenous or exogenous insults is harmful to living organisms and is termed oxidative stress (2). Oxidative stress can damage cellular macromolecules, increase free radicals, leading to DNA and protein modification and lipid peroxidation associated with the development of several diseases (3,4,5,6), but the precise mechanisms are still remaining to be elucidated. It is now assumed that ROS are involved in both the initiation and progression of cancer (7, 8, 9). Damage to DNA by ROS has been widely accepted as a major cause of cancer (10). The ROS are involved in a diversity of important phenomena in medicine, such as chemotherapeutic effects, mutagenesis, carcinogenesis and aging (11, 12). In this regard understanding the role for ROS in the biology of malignant neoplasia, especially hepatocarcinoma is important for prognosis and treatment. There is a large body of evidence indicating that ROS play a pathogenic role in carcinogenesis (13). Still the metabolism of ROS in cancer cells is a research area that has not been intensively pursued. Among the targets of ROS, DNA damage appears most important in tumor biology since it is firmly established that cancer is a genetic disease (14, 1). Oxidative stress can increase DNA mutation rate and induce DNA damage, genomic instability, DNA-protein cross-linkage and cell proliferation (15). Particularly, the hydroxyl radical has been proved that it is responsible for a number of base modifications, including the formation of thymine and thymidine glycol, 8-hydroxy-2-deoxyguanosine (8-OHdG) and 5-hydroxylmethyluracil (13). The 8-Hydroxy-2'-deoxyguanosine is one of the major oxidatively modified DNA base products *in vivo* (16, 17, 18). It is reasonable to suppose that membrane lipids near the ROS generation site are the most severely damaged. Food plant components that interfere with these aspects of the cancer process are potential candidates to improve many cancer related deficits.

Based on the literature survey of Indian Association of Cancer Research (IACR) in India there is a higher prevalence of Hepatocellular carcinoma (19). However, many therapies have been implemented for treating HCC, until date none of them are found to increase the survival rate due to various reasons like systemic toxicity, advanced stage of the tumor, chronic infections. Currently available synthetic drugs do not prevent the progression of disease without causing serious side effects. So the most of the people depend on herbal drugs for combating the disease process, as there are practically no toxic side effects in plant based drugs. Nutritional therapy is a key component for the treatment of cancer cachexia and help in controlling malignant disease in some situations (20).

Therefore, developing more effective and less toxic anticancer agents, including natural products, is necessary to prevent or retard the process of hepatocarcinogenesis (21). Administration of herbal antioxidants has been shown to be preventive agents against DEN induced hepatocarcinoma (22). Withania somnifera is known to alter the oxidative stress markers of the body. The root extract has found to significantly reduce the lipid peroxidation (23) and increase the superoxide dismutase (SOD) and catalase activities, thus carrying free radical scavenging property (12). It is been proved to have hepatoprotective effect against radiation induced (24) and iron induced toxicity (25). However, protective activity of Withaferin-A has not been scientifically investigated against DEN induced hepatotoxicity. Hence, an attempt was made to investigate the protective effect of Withaferin-A against hepatocarcinogenesis.

II. MATERIALS AND METHODS

Chemicals and Reagents: Withaferin-A, DEN and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used (analytical grade) were obtained from Sisco Research Laboratories Pvt., Ltd. (Mumbai, India) and Glaxo Laboratories (CDH Division, Mumbai, India).

Animal care and Housing:

Male Wistar Albino rats, weighing 150-180g, were used. The animals were procured from Central Animal House Block, Dr. ALM PG IBMS, University of Madras, Taramani, Chennai-113 and maintained in a controlled environmental condition of temperature and humidity on alternatively 12 h light/dark cycles. All animals were fed standard pellet diet (Gold Mohor rat feed, Ms.Hindustan Lever Ltd., Mumbai) and water *ad libitum*. This research work on wistar albino male rats was sanctioned and approved by the Institutional Animal Ethical Committee (IAEC NO. 02/016/08).

Experimental procedure

Group I served as control animals. Group II animals were treated with N-Nitrosodiethylamine (DEN) (0.01% dissolved in water) daily and kept for 12 weeks to induce liver cancer. Group III comprised of control animals treated orally with Withaferin-A (50mg/kg b.wt/day) for 21 days to first dose of the carcinogen and treated continuously until the 12th week and sacrificed (Pre-treatment group). Group IV were DEN treated animals as in group II, treated with Withaferin-A from the 12th week to the 15th week (Post- treatment group) as in group III animals. Group V is drug control animals treated with Withaferin-A alone as in group III.

After the experimental period, the animals were killed by cervical dislocation and blood and liver tissues were used for the further analyses. The tissues were excised immediately and was washed in ice-cold saline to remove any extraneous matter, cleaned, blotted to dryness in filter paper. A 10% homogenate of liver tissues were prepared in was prepared in Tris-HCl buffer 0.1M (pH-7.4) using a Potter Elvejhem glass homogenizer as necessitated by the protocol. Dilutions were decided based on the protein concentration. The method of Lowry et al (26) was adopted for the estimation of protein content in the serum and tissue homogenates.

Blood collected with 5% EDTA was used for erythrocyte isolation according to the method of Dodge et al (27) and modified by Quist (28). The level of lipid peroxidation was assayed by the method of Ohkawa et al (29). Na^+ K^+ ATPase activity was assayed by the method of Bonting (30). Ca^{++} ATPase was estimated by the method of Hjerten and Pan (31). Mg^{++} ATPase was assayed according to the method of Ohinishi et al (32). Liberated inorganic phosphorus was estimated by the method of Fiske and Subbarow (33). All enzyme activities were expressed as μmoles of inorganic phosphorus liberated/min/mg protein. Superoxide dismutase (SOD) was estimated by the method of Marklund and Marklund (34). The enzyme activity is defined as Units /mg protein. The activity of catalase (CAT) was estimated by the method of Sinha (35) and expressed as nmoles of H_2O_2 consumed/min/ mg protein. Activity of glutathione peroxidase (GPX) was assayed by the method of Rotruck et al (36), expressed as μmoles of GSH oxidised/min/mg protein. Generation of ROS assayed by according to LeBel et al (37) as modified by Kim et al (38). The free radicals were estimated by standard protocols (39, 40, 41). The protein carbonyl, DNA- protein cross links content was quantified by the method of Levine et al (42), Zhitkovich and Costa (43). The 8-OHdG was resolved by HPLC and quantified by UV detection by the method of Kaur and Halliwell (44).

III. Results

Effect of withaferin-A on Oxidative Stress

Figure: 1 depicts the effect of Withaferin-A on the level of reactive oxygen species (ROS) in control and experimental rats. The level of ROS was found to be significantly ($p<0.001$) increased in tumour-bearing rats (group II) when compared to control (Group I) rats. Withaferin-A (Group III and IV) treatment significantly ($p<0.001$) decreased the level of ROS when compared with untreated tumour-bearing rats (Group II). The levels

were restored to near normal on treatment with withaferin-A. There was no significant change in the levels of ROS in Group V rats when compared with control (Group I) rats.

Table: 1 shows the levels of free radicals in control and experimental rats. The radicals such as superoxide radicals, hydroxyl radical and hydrogen peroxide were found to be increased significantly ($p<0.001$) in Group II rats when compared with control (Group I) rats. The level of these radicals was restored to near normal in Withaferin-A treated (Group III and IV) rats. The radicals were significantly ($p<0.001$) decreased in group III and Group IV rats. Group V showed no significant change in the level of the radicals when compared with control (Group I) rats.

Effect of withaferin-A on Macromolecular Damage

Variations in the levels of lipid peroxidation products, reflects the extent of oxidative stress and damage to lipid composition of plasma membrane. **Figure: 2** shows the levels of lipid Peroxidation (LPO) in liver of control and experimental rats in each group. In tumour induced rats (Group-II), the lipid Peroxidation levels in liver were significantly ($P < 0.001$) increased when compared to control rats (Group-I), whereas pre-treated and post-treated rats (Group-III &IV) showed a statistically ($P < 0.001$) significant decrease when compared to tumour induced rats (Group II). These levels were brought back to near to normal levels in withaferin-A alone treated rats (Group-V).

Table: 2 represents the effect of withaferin-A on the levels of protein carbonyls and DNA- protein cross links in the liver of control and experimental rats. There was a significant ($p<0.001$) increase in the protein carbonyls and DNA protein cross link levels in group II cancer bearing animals when compared with group I controls. On treatment with withaferin-A, these levels were significantly decreased in group III ($p<0.001$) and group IV ($p<0.05$) animals when compared with group II cancer bearing animals. Protein carbonyls and DNA protein cross link showed no significant difference between withaferin-A alone treated group V animals and control group I animals.

Figure: 3 depicts the effect of withaferin-A on the 8-hydoxy 2 deoxyguanosine (8-OH dG) content in the liver tissue of various experimental animals. Group II cancer bearing animals showed an increase in the levels of 8-OH dG by 2.1 fold when compared with group I control animals. Withaferin-A treatment showed a considerable decrease (1.3 fold) in the 8-OHdG content. Group IV animals treated with withaferin-A also showed a significant decrease by 1.4 fold. Likewise, group V animals treated with withaferin-A decreased the 8-OHdG levels by 1.9 fold when compared with HCC induced rats (Group II)

Activities of enzymic and non-enzymic antioxidants in liver

Table: 3 shows the effect of Withaferin-A on the activities of enzymatic antioxidants in the liver mitochondria of control and experimental animals. The activities of SOD, CAT, GPx and GR were found to be significantly ($p<0.001$) decreased in cancer induced group (Group-II) when compared with the control group (Group-I) and a significant increase ($p<0.01$) in enzyme activities can be observed in animals treated with Withaferin-A (Group III). Cancer bearing group treated with Withaferin-A (Group-IV) showed a much more significant ($p<0.05$) increase in the activities of these antioxidant enzymes. There was no significant difference in the enzymic activities of the control animals (Group-I) and the control treated with Withaferin-A (Group-V).

Similarly the levels of non-enzymic antioxidants such as GSH, vitamin E and vitamin C were found to be significantly ($p<0.001$) decreased in cancer bearing group (Group-II) when compared with the control group (Group-I). On administration of Withaferin-A (Group-III), there was found to be a significant ($p<0.05$) increase in the levels of these antioxidants when compared with cancer bearing animals. Animals subjected to post treatment with Withaferin-A (Group-IV) showed a significant ($p<0.01$) increase by bringing the levels of non-enzymic antioxidants to near normal levels when compared with the cancer induced group. There was no significant difference in the antioxidant levels between the control animals (Group-I) and drug control animals treated with Withaferin-A alone (Group-V).

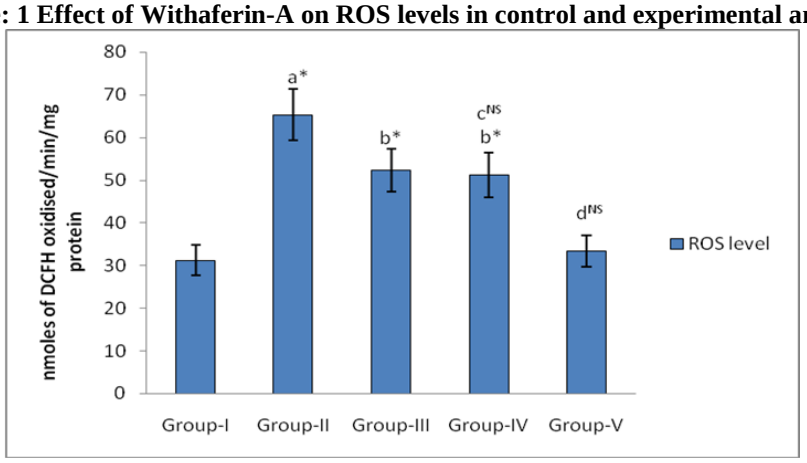
Membrane damage

Figure: 4 show the effect of withaferin-A on the activities of membrane bound ATPases in liver of control and experimental animals. A decrease ($p<0.001$) in the activities of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+}ATPase activity was seen in cancer bearing Group II animals when compared with control animals. There was a significant ($p<0.001$) increase in the activities of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+}ATPase in withaferin-A treated (Group III and Group IV) animals when compared with Group II animals. No significant difference in the activities of ATPase was seen between Group V animals and Group I controls.

Table: 4 represents the effect of withaferin-A on the activities of ATPases in erythrocyte membrane of control and experimental animals. HCC bearing animals (Group II) showed a significant decrease ($p<0.001$) in the activities of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+}ATPase when compared with control animals (Group I). This change in the ATPases activities were significantly ($p<0.001$) reverted in Group III and Group IV ($p<0.01$,

p<0.05) withaferin-A treated animals to near normal values. No significant difference was observed in the ATPase activities between withaferin-A alone treated (Group V) animals and Group I controls.

Figure: 1 Effect of Withaferin-A on ROS levels in control and experimental animals



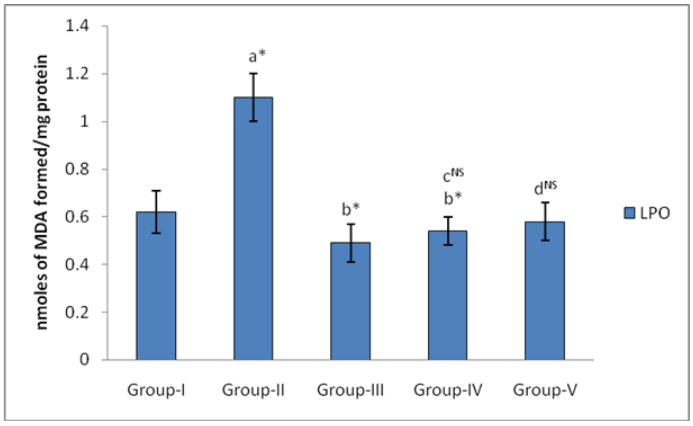
Each value is expressed as mean ± SD for six rats in each group.
a - as compared with group I; b - as compared with group II; c - as compared with Group III
Units: nmoles of DCFH oxidized/min/mg protein
Statistical significance - *p<0.001, #p<0.01, @p<0.05, NS-Not significant.

Table: 1 Effect of Withaferin-A on the level of free radicals in the liver of control and experimental animals

Particulars	Group I (Control)	Group II (DEN induced)	Group III (DEN + WFA-Pre)	Group IV (DEN + WFA- Post)	Group V (WFA alone)
H_2O_2	69.54±7.68	163.45±18.1 a^*	121.43±13.91 b^*	132.35±16.16 b^* c^{NS}	71.10±7.55 d^{NS}
OH^-	15.74±2.14	39.63±5.71 a^*	25.78±3.45 b^*	27.35±3.23 b^* c^{NS}	16.53±1.89 d^{NS}
O_2^-	55.41±5.45	132.52±15.67 a^*	99.51±11.88 b^*	103.75±13.08 b^* c^{NS}	54.31±6.34 d^{NS}

Each value is expressed as mean ± SD for six rats in each group.
H_2O_2:µmoles/1012 cells; OH- : MDA as nmol/1012 cells
O^{2-} : µmoles NBT reduced /10min/1012 cells.
a - as compared with group I; b - as compared with group II; c - as compared with Group III
Statistical significance - *p<0.001, #p<0.01, @p<0.05, NS-Not significant.

Figure: 2 Effect of Withaferin-A on the level of Lipid Peroxidation in liver of control and experimental animals



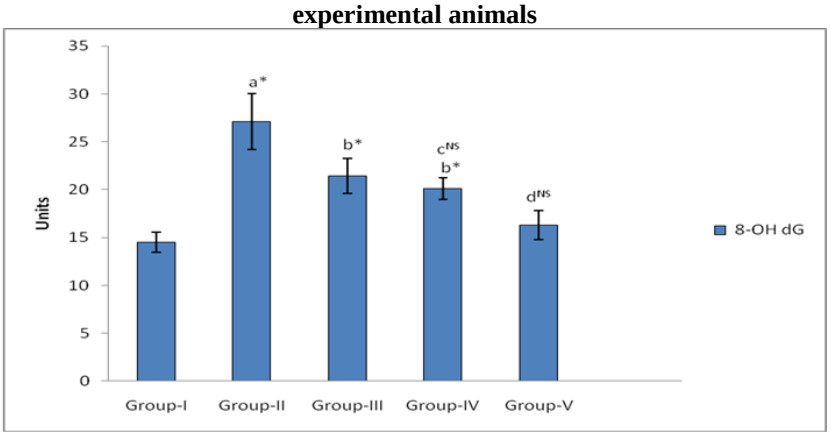
Each value is expressed as mean ± S.D. for six male wistar rats in each group.
a- as compared with Group I; b- as compared with Group II. c- as compared with Group III.
d- as compared with Group IV; NS- Not significant;
LPO: nmoles of MDA formed/min/mg protein.
Statistical significance: *p<0.001, $^{@}p$<0.01, $^{\#}p$<0.05, NS-Not significant

Table: 2 Effect of Withaferin-A on the level of protein carbonyls and DNA-Protein crosslink in liver of control and experimental animals

Particulars	Group I (Control)	Group II (DEN induced)	Group III (DEN + WFA-Pre)	Group IV (DEN + WFA-Post)	Group V (WFA alone)
Protein carbonyl	1.23±0.1	2.31±0.21 a^{*}	1.49±0.16 b^{*}	1.58±0.15 b^{*} c^{NS}	1.19±0.07 d^{NS}
DNA-Protein crosslinks	1.72±0.21	5.86±0.53 a^{*}	2.45±0.31 b^{*}	3.12±0.43 $b^{\#}$	1.84±0.18 d^{NS}

Each value is expressed as mean ± S.D. for six male wistar rats in each group.
a-as compared with Group I; b-as compared with Group II; c-as compared with Group III.
d-as compared with Group IV
Units: Protein carbonyl: nmoles/mg protein; DNA-Protein cross links: % of DNA protein cross links
Statistical significance: *p<0.001, @p<0.01, #p<0.05, NS-Not significant

Figure: 3 Effect of Withaferin-A on the levels of 8-hydroxyl deoxyguanosine in liver tissue of control and experimental animals



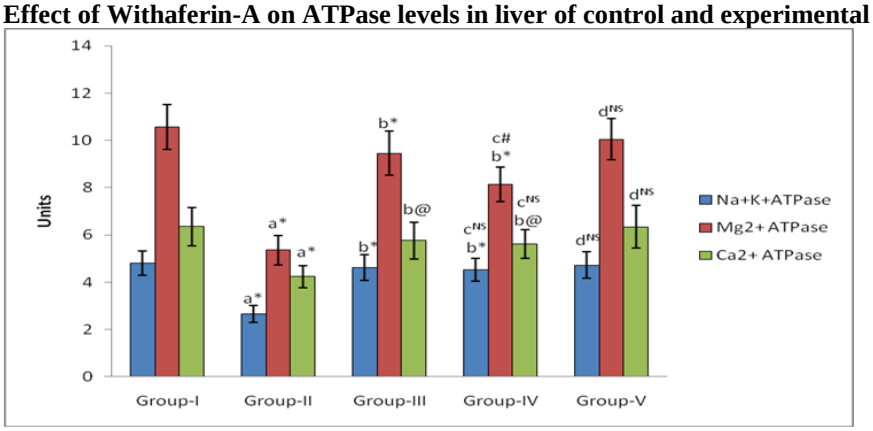
Each value is expressed as mean ± SD for six rats in each group.
Values are expressed as pmoles of 8-OH guanosine/mg of nuclear DNA
a - as compared with group I; b - as compared with group II; c - as compared with Group III
Statistical significance - *p<0.001, #p<0.01, @p<0.05, NS-Not significant.

Table: 3 Effect of Withaferin-A on antioxidant status in liver mitochondria of control and experimental animals

Particulars	Group I (Control)	Group II (DEN induced)	Group III (DEN + WFA- Pre)	Group IV (DEN + WFA- Post)	Group V (WFA alone)
SOD	7.56 ± 0.75	5.32 ± 0.71 a^{*}	6.71 ± 0.68 $b^{@}$	5.94 ± 0.67 $b^{@}c^{NS}$	7.24 ± 0.63d^{NS}
CAT	47.65 ± 3.77	28.53 ± 3.45 a^{*}	45.23 ± 4.34 b^{*}	36.29 ± 4.76 $b^{@}$ $c^{@}$	45.52 ± 3.54d^{NS}
GPx	5.53 ± 0.34	3.74 ± 0.35 a^{*}	6.17 ± 0.41 b^{*}	5.38 ± 0.38 $b^{\#}c^{NS}$	5.28 ± 0.37d^{NS}
GSH	46.51 ± 4.02	31.42 ± 2.47 a^{*}	43.02 ± 4.09 b^{*}	36.84 ± 4.57 $b^{\#}$ $c^{\#}$	44.53 ± 4.75d^{NS}
LPO	2.35 ± 0.14	3.51 ± 0.35 a^{*}	2.19 ± 0.23 b^{*}	2.01 ± 0.33 $b^{*}c^{NS}$	2.68 ± 0.15 $d^{\#}$

Each value is expressed as mean ± SD for six rats in each group.
Units- SOD: units/min/mg protein; CAT: µmoles of H_2O_2 liberated/min/mg protein;
GPx: µmoles of GSH oxidized/min/mg protein; GR: nmoles NADPH oxidized min/mg protein
GSH, Vitamin C, Vitamin E: µg/mg protein
a - as compared with group I; b - as compared with group II; c - as compared with Group III
Statistical significance - *p<0.001, $^{\#}$p<0.01, $^{@}$p<0.05, NS-Not significant.

Figure: 4 Effect of Withaferin-A on ATPase levels in liver of control and experimental animals



Each value is expressed as mean ± SD for six rats in each group.
Units: ATPase activities - µmoles of Pi liberated/min/mg protein
a - as compared with group I; b – as compared with group II; c – as compared with group III
Statistical significance - $^{*}p<0.001$, $^{\#}p<0.01$, $^{@}p<0.05$, NS-Not significant.

Table: 4 Effect of Withaferin-A on ATPase levels in erythrocyte membrane of control and experimental animals

Particulars	Group I (Control)	Group II (DEN induced)	Group III (DEN + WFA-Pre)	Group IV (DEN + WFA-Post)	Group V (WFA alone)
$Na^{+}K^{+}$ ATPase	2.78± 0.25	1.92±0.12 a^{*}	2.69±0.23 $b^{@}$	2.57±0.24 $b^{\#}$ c^{NS}	2.73±0.26 d^{NS}
Ca^{2+} ATPase	4.31±0.24	2.73±0.22 a^{*}	3.81±0.34 b^{*}	3.62±0.16 $b^{@}$ c^{NS}	4.12±0.23 d^{NS}
Mg^{2+} ATPase	2.5±0.56	1.63±0.26 a^{*}	2.51±0.43 b^{*}	2.11±0.36 $b^{@}$ $c^{\#}$	2.3±0.46 d^{NS}

Each value is expressed as mean ± SD for six rats in each group.
Units: ATPase activities - µmoles of Pi liberated/min/mg protein
a - as compared with group I; b - as compared with group II; c- as compared with group III
Statistical significance - *p<0.001, #p<0.01, @p<0.05, NS-Not significant.

IV. Discussion

Liver cancer is one of the most common neoplasms in the world. There are many pathological factors, such as ROS, involved in the process of cancer initiation and progression. One of the greatest challenges in cancer research today is the identification of specific cause of oxidative modification and accumulation of macromolecules specifically oxidative stress status. Proteins are one of the major categories of cancer related damage in macromolecules, which have been observed during various cells, tissues and organs (45). ROS can lead to oxidation of amino acid residue side chains containing reactive carbonyls, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation (46, 47). So the biomarkers of protein oxidation are often applied when a battery of markers of oxidative stress status is being studied. Therefore, the measurement of the protein oxidation is a clinically important factor for the prediction of the cancer process and liver related diseases. Accordingly, in our study, we found that increased protein carbonyl levels in liver cancer. Our result shows that the presence of carbonyl groups in proteins has therefore been used as a marker of ROS mediated protein oxidation. The fact that WFA has been found to reduce protein carbonyls in our study thereby reducing stress and free radical formation might be a reason in restoring the normal cell activity.

Lipid peroxidation products have also been shown to be mutagenic and carcinogenic (48) and has been implicated as the underlying mechanisms in numerous disorders and diseases such as cardiovascular diseases, cancer etc (49). Lipids are modified by ROS and visualized as a thiobarbituric acid reactive substance (TBARS). We measured the TBARS and protein carbonyls that serve as an indicator for intracellular oxidation in liver tissue. The increase in lipid peroxide may suggest a possible mechanism of tissue injury by reactive oxygen intermediates (50). This may lead to permanent alterations in the genetic material which may serve as an initial step during DEN induced hepatocarcinogenesis (51, 52). Similar results were seen in our group II animals due to action of DEN on liver, the hydroxyl radicals are generated which leads to oxidation of structural and functional proteins, membrane lipids and depletion of glutathione. It indicates, lipid peroxidation causes loss of membrane fluidity, impairs ions transport and finally leads to loss of cellular functions. In our study, all groups treated with DEN, have a significant increase in the levels of LPO as compared with normal group animals. This verifies the antilipid peroxidative role of WFA by its ability to scavenge free radical generation (53).

To further evaluate the roles of WFA against DEN induced devolvement of hepatocarcinogenesis, we examined the levels of 8-OHdG, a DNA base modified product generated by reactive oxygen species, which is a good marker for DNA oxidative damage due to aging, cancer and other degenerative diseases (54). ROS can cause strand breaks and base modifications in DNA. Particularly, oxidation of guanine residues to 8-OHdG is an oxidized nucleoside of DNA that is the most frequently detected and studied DNA lesion. Elevated levels of 8-OHdG were demonstrated in some carcinomas, including liver cancer (22). In our research we also found that there was increased serum 8-OHdG in liver cancer. Nevertheless, the greater oxidative DNA damage in the liver cancer, as a possible result of impaired antioxidant activity, implies an important role for oxygen free radicals in liver carcinogenesis. Therefore, the contents of 8-OHdG in serum could act as a sensitive biomarker for liver cancer patients, too. It has been regarded as a novel showed that WFA administration significantly decreased the levels of 8-OHdG in liver DNA compared with that in DEN group, which suggested that WFA could significantly prevent DEN induced oxidative DNA damage *in vivo* and appears to play a crucial role in mutagenesis.

ROS generated in the system is quenched and the body is protected against its deleterious effects by the antioxidants defense enzyme system (55, 56). The animal tumor cells lack these complex enzymes systems, which normally exert protection by scavenging toxic oxygen species such as superoxide radicals, hydrogen peroxide and lipid hydroperoxides (57). It was previously reported that the enzymatic antioxidants like SOD, CAT, GPx and GR activities are significantly decreased in the cancer induced animals (58). The decreased activity of SOD and CAT may be due to the inhibition of these enzymes by ROS and decrease in the GPx and GR may be due to the increased utilization of glutathione system (59). From our results the levels of SOD, CAT, GPx and GR were replenished on Withaferin-A supplementation. This reflects a favorable balance between potentially harmful oxidants and protective antioxidants. Furthermore, elevated SOD and CAT activities can play an inhibitory role in cell transformation. Similarly, Vitamin C, E and reduced glutathione comprise the non-enzymic antioxidant system that protects the cells against free radicals and ROS. Vitamins have a number of biological activities such as immune stimulation, scavenging the free radicals and alteration in metabolic activation of carcinogens (60). They can utilize reactive oxygen species metabolites, to protect the biomolecules and reduce oxidative DNA damage (61). In this regard, GSH is an important non-protein thiol, which in conjunction with GPx and GST plays a significant role in protecting cells against the cytotoxic and carcinogenic chemicals. It acts directly in free radical scavenging by donating hydrogen atom (62). GSH is also a substrate for the GSH peroxidase, playing a critical role in the elimination of hydrogen peroxide, as well as other organic hydroperoxides and toxic chemicals generated from the radiated membrane. From this, we observed that the levels of Vitamin C, E and GSH levels were decreased in cancer induced animals. On treatment with Withaferin-A the non-enzymic antioxidants levels were brought to near normal. This may be due to the antioxidant property of Withaferin-A.

The plasma membrane regulates numerous aspects of cell physiology and signaling and also protects cells against oxidative stress. The proteins and lipids involved in these functions of the plasma membrane are susceptible to oxidative modifications that may contribute to the dysfunction and degeneration of cells occur degenerative disorders (63). For example, lipid peroxidation and oxidative modifications of membrane ATPases are implicated in the pathogenesis of liver cancer (64). In this regard, erythrocytes are highly susceptible to oxidative damage due to the high cellular concentration of oxygen and hemoglobin, a potentially powerful promoter for the oxidative processes (65). Recently, one report revealed that an increased level of ATPase in human erythrocyte membrane during oxidative stress condition (66, 67). Injury to cell membrane by free radicals has been a recent focus in research since the vital activities of the cell are challenged. The three important ATPases of the plasma membrane are the Na^+K^+ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase. Na^+K^+ATPase uses energy derived from the hydrolysis of ATP to keep a high K^+ and a low Na^+ concentration in the cytoplasm which in turn provides the driving force for the net movement of other substance such as Ca^{++}, amino acids and H^+ (68). In the present study, reduction in the activities of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase were found in liver tumor bearing animals, which suggests the condition of malignancy and progression of cancer, could have altered membrane structure. In the current study, significant increase in the activities of these three ATPase enzymes in the group III & group IV animals suggest that decrease in levels of lipid peroxides could have contributed to an increase in the enzyme activities, indicating their protective role in maintaining membrane integrity. It can be deciphered that WFA conditions in hepatic cells, preventing any further damage to liver parenchyma, which in turn would have decreased the leakage of enzymes into circulation.

V. Conclusion

From the above study, it can be concluded that Withaferin-A has the capacity to modulate the activities of membrane bound enzymes and decrease the levels of lipid peroxides and protein carbonyls thus restoring the membrane integrity and suggesting its role as a reducing oxidative stress and antioxidant compound. This was

confirmed by the histopathological observations (published data). In conclusion, the results obtained in the present study suggest that Withaferin-A possesses a promising hepatoprotective and antioxidant effect in DEN intoxicated rats probably due to its antioxidant effects. However, further biochemical evidence at the molecular level is required to establish the actual mechanism of the drug action and research into this area is underway in our laboratory.

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