

# Isolation of Withaferin-A from *Withania Somnifera* plant root and its effects on cancer rats

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## Abstract

**Background:** Hepatocellular carcinoma (HCC) is the most universal primary malignant tumor of the liver. It is the third most common cause of cancer related deaths in men and the ninth in women caused by chemical carcinogens. Chemotherapy is a systemic treatment, which works throughout the body to kill cancer cells. **Aim and Objectives:** The purpose of this study is to investigate the oxidative biomarkers and hepatoprotective efficacy of Withaferin-A (WFA) against hepatocellular carcinoma in experimental rats. **Materials and Method:** Group I - Control animals treated with 0.5% of Carboxymethyl cellulose (CMC) orally throughout the experimental period. Group II- Hepatocellular carcinoma was induced by providing 0.01% DEN through drinking water for 15 weeks. Group III - Animals were treated with Withaferin-A at the dosage of 50mg/kg body.wt, orally for 21 days before administration of DEN as in Group II. Group IV - Hepatocellular carcinoma induced animals treated with Withaferin-A at a dosage similar to group III for 21 days, i.e. after the administration of DEN for 12 weeks. Group V -Animals treated with Withaferin-A (as in Group III) alone for 21 days. **Results:** In this study, we observed that WFA protects the rats from DEN induced HCC when administered at a lethal dose of 50 mg/kg for 21 days. The levels of lipid peroxidation, protein carbonyls and liver marker enzymes were markedly increased in cancer bearing animals. In contrast, the activities of the enzymic and Non-enzymic antioxidants were found to be decreased in liver of cancer bearing animals. The cancer bearing animals when treated with Withaferin-A showed significantly decreased levels of liver marker enzymes with simultaneous reversion in the activities of antioxidants levels when compared to the cancer induced animals. **Conclusion:** These observations suggest that the treatment with WFA effectively reduced oxidative stress, as assessed by decreased levels of various oxidants and improved the level of diverse antioxidants. The obtained results it is concluded that withaferin-A is capable of restoring the liver architecture by increasing the antioxidant status in hepatocarcinogenic rats.

**Keywords:** Withaferin-A, Oxidative Stress, Antioxidant Enzymes, DEN, Hepatocellular Carcinoma.

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worldwide because of its poor prognosis. It is the third most common cause of death from cancer traced out in developing countries<sup>1</sup>. It is estimated that 748,300 new liver cancer cases and 695,900 cancer deaths occurred worldwide in 2008<sup>2</sup>. It accounts for approximately 85% of primary malignant tumors of the liver. The enzymes in liver are thought to reflect oxidative damage in the cell by getting increased under different conditions of oxidative stress. In this research our aim is to establish whether the liver enzymes and antioxidant levels can be used as a new parameter for the assessment of carcinogenic risk of hepatocellular carcinoma. Diethyl nitrosamine (DEN) is one of the important environmental carcinogens used to induce liver cancer in experimental animal model systems especially rats<sup>3</sup>. In this connection cellular and hepatocytes membranes are especially susceptible

## INTRODUCTION

Cancer is a most dreaded complication throughout the world. Particularly hepatocellular carcinoma is a major health problem and it is the sixth most common cancer

representing a prominent target for oxidative stress. Enhanced oxidative stress leads to increased lipid peroxidation and altered membrane potential can cause profound changes in the mechanism of signal transduction like receptor densities, activation of second messenger and intracellular calcium signaling. A recent report has revealed that, the administration of DEN leads to the generation of lipid peroxidation products in general and develops chemiluminescence, reflecting the formation of the reactive oxygen species in the preneoplastic nodules in the rat liver<sup>4</sup>. The Reactive Oxygen Species (ROS) may participate in the multistage carcinogenesis by causing oxidative DNA damage and mutations in proto-oncogenes and in tumor suppressor genes by activating signal transduction pathways<sup>5</sup>. Hence the LPO are good markers for free radical induced tissue damage in the cancer cells. Similarly previous one report illustrate that various antioxidants and vitamins were shown to offer protection against DEN-induced hepatocarcinogenesis in animal models<sup>6</sup>. Various molecules can inhibit the formation of free radicals associated with carcinogenesis. The help of antioxidant enzymes can counteract the deleterious action of ROS as radical scavengers, inhibiting LPO and protect from cellular and molecular damage from various diseases<sup>7</sup>. Bioactive compounds from plant origin have the potential to subside the biochemical imbalances induced by various toxins associated with free radicals formation. In accordance the investigation of anticancer agents from plant origin used in traditional medicine seems to be important. Recently, it is reported that withania Somnifera (WS) plant extracts and plant products have been identified as good protectors against the free radicals by triggering antioxidant gene expression as a promising therapeutic drugs<sup>8</sup>. Withaferin-A (WFA) is one of the main withanolid active principles isolated from the root extracts of *Withania somnifera* which showed antitumor and antioxidant effects in animals<sup>9</sup>. This root extract prevented the rise of experimentally induced lipid peroxidation in rabbits and mice<sup>10</sup>, and it induced an increase in the levels of antioxidant and anti-inflammatory activity, but not yet fully investigated<sup>11</sup>. Therefore, we isolated and identified a compound withaferin-A from the root powder of *Withania somnifera* and its effect was tested against DEN induced hepatocellular carcinoma rats.

## MATERIALS AND METHODS

### Animal care and Experimental Design

Male Wistar Albino rats, 6-8 weeks of age and 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). The animals were divided into five groups with six rats in each group. Group I - Control animals treated with 0.5% of Carboxymethyl cellulose (CMC) orally throughout the experimental period. Group II- Hepatocellular carcinoma was induced by providing 0.01% DEN through drinking water for 15 weeks. Group III - Animals were treated with Withaferin-A at the dosage of 50mg/kg body.wt, orally for 21 days before administration of DEN as in GroupII. Group IV - Hepatocellular carcinoma induced animals treated with Withaferin-A at a dosage similar to group III for 21 days, i.e. after the administration of DEN for 12 weeks. Group V -Animals treated with Withaferin-A (as in Group III) alone for 21 days.

### Statistical analysis

Mean and SEM were calculated using standard procedures. For statistical analysis, one-way analysis of variance (ANOVA) was used, followed by the Newman-Keuls multiple comparison test using SPSS version 10 (SPSS, Chicago, IL). Mean differences with  $P < 0.001$ ,  $P < 0.01$  and  $P < 0.05$  were considered statistically significant.

## RESULTS

### Identification of Isolation of Withaferin-A

The compound withaferin-A was eluted with 95:5 of 5%  $\text{CHCl}_3$ : MeOH. General molecular formula of this compound is  $\text{C}_{28} \text{H}_{38} \text{O}_6$ . The structure of the compound was confirmed on the basis of IR,  $^1\text{H}$  NMR, and MS spectrum. The following data were the results of IR and NMR. MS  $m/z$  470.113, M.p. 245°C, (Fig:1).  $^1\text{H}$ NMR ( $\text{CDCl}_3$  500MHz): d 0.91(s, 3H, H-18), 1.03 (d,  $J=7$  Hz, 3H, H-21), 1.20 (s, 3H, H-19), 1.27 to 1.34 (m, 3H, H-11, 15), 1.64 to 1.65 (m, 2H, H-9, H-12), 1.68 to 1.91 (m, 2H, H-8, H-12), 1.91 (s, 3H, H-27), 2.0 (s, 3H, H-28), 2.52 to 2.57(m, 4H, 17-OH, H-4,23), 2.68 to 2.73(m, 1H, H-4), 3.06(d,  $J=3.7$  Hz, 1H, H-6), 4.3 to 4.41 (m, 1H, H-22), 5.86 (dd,  $J=10.1$ , 2.6Hz, 1H, H-2), 6.59 to 6.62 (m, 1H, H-3)(Fig:2). IR (KBr)  $\lambda_{\text{max}}$  1715  $\text{cm}^{-1}$  (C=O), 3434  $\text{cm}^{-1}$  (-OH), 1464  $\text{cm}^{-1}$ (C=C) (Fig:3). The yield of this compound was 300mg from 400gms of crude extract. The drug was prepared freshly before use. It was dissolved in a few drops of ethanol and a homogeneous suspension was made with normal saline containing 0.5% carboxymethyl cellulose (CMC) and used for the experimental study.

Figure 3 Mass Spectroscopy Spectrum of Withaferin-A.

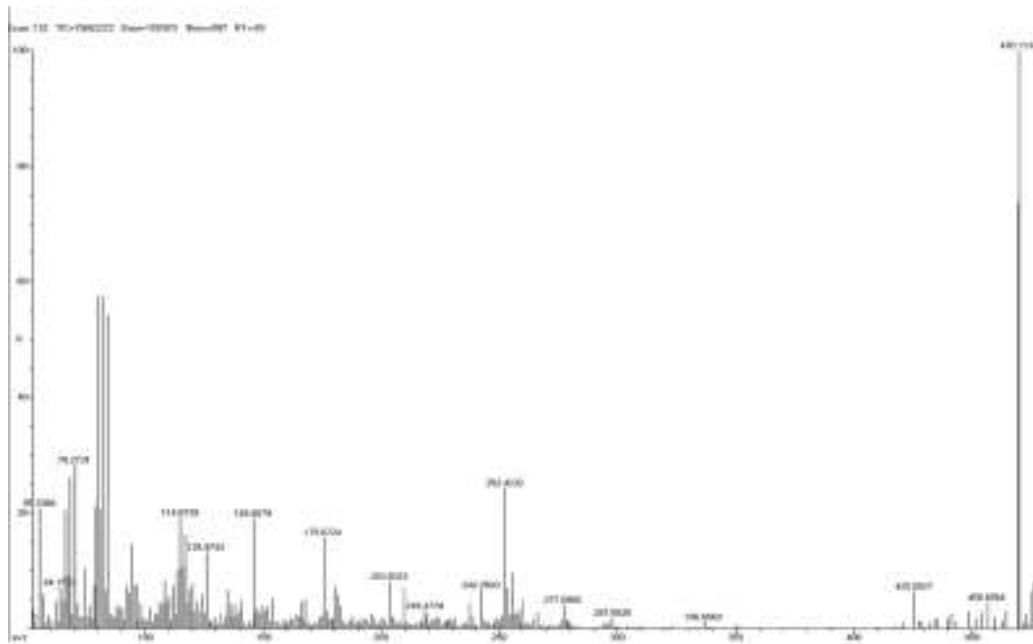
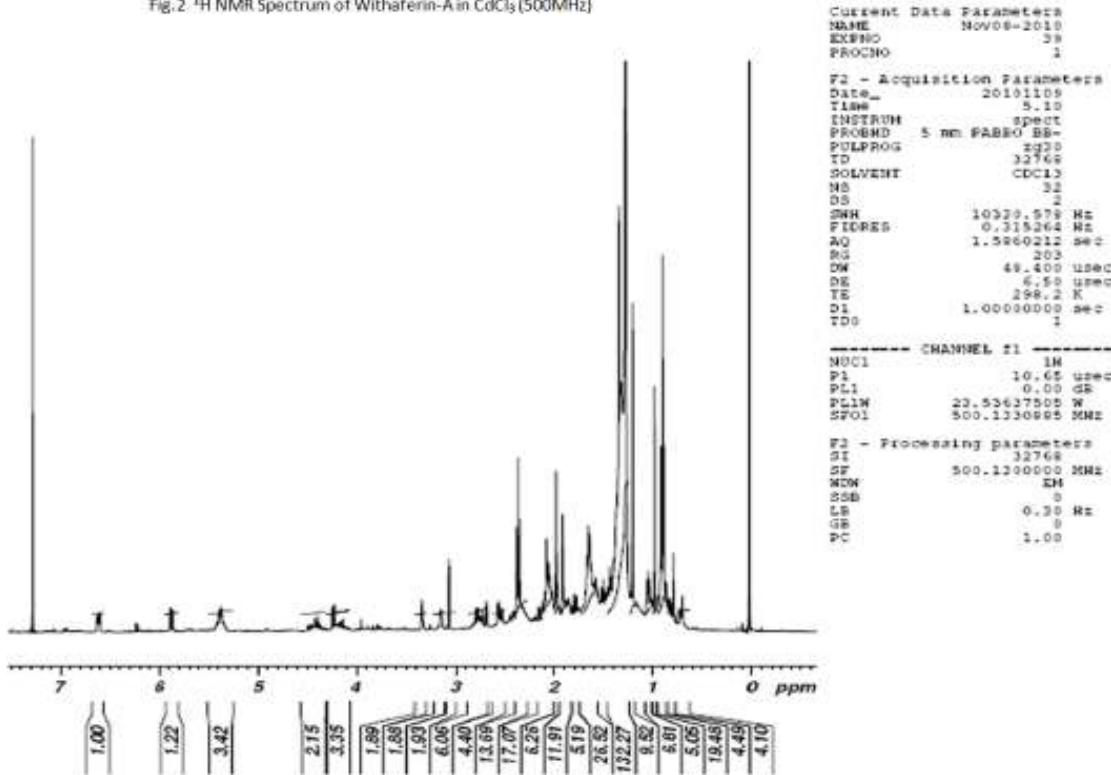
Fig. 2  $^1\text{H}$  NMR Spectrum of Withaferin-A in  $\text{CDCl}_3$  (500MHz)

Fig. 3 IR Spectrum of Withaferin-A using KBr Solvent

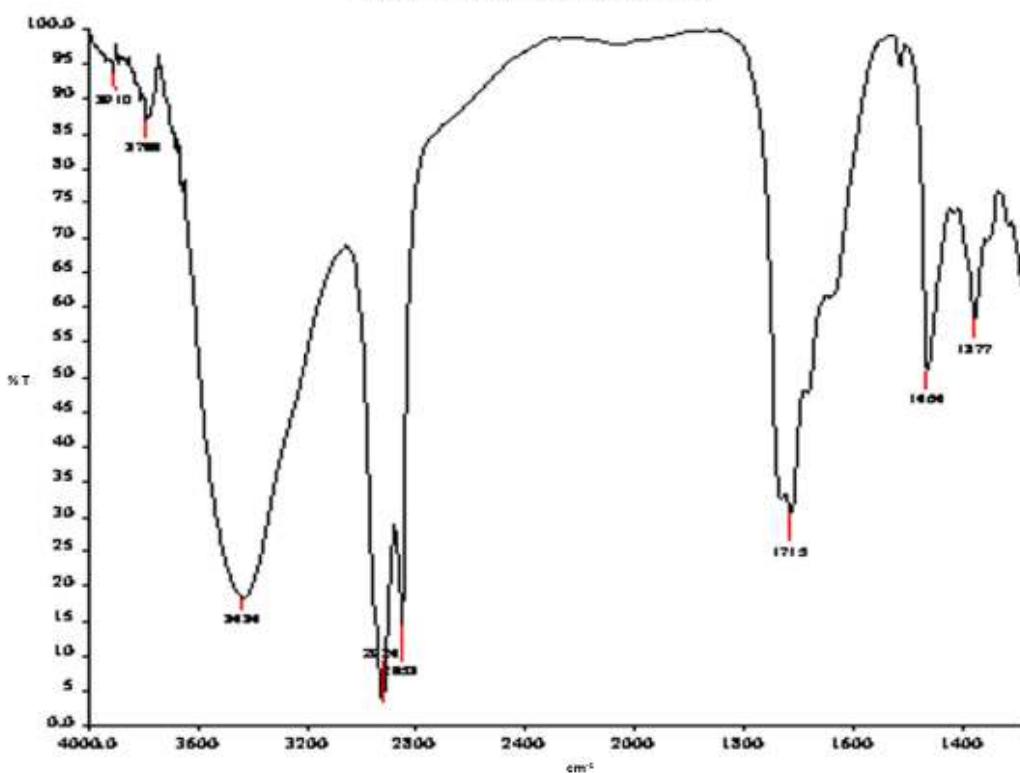


Table 1: Effect of Withaferin A on body weight and liver weight in control and experimental animals

Particulars	Group I (Control)	Group II (DEN induced)	Group III (Pretreated)	Group IV (Post treated)	Group V (WA alone)
Body weight (gm)	191±11.03	147.02±10.01a*	165.04±12.02b <sup>#</sup>	169.12±10.1b <sup>*c</sup>	187.12±11.10
liver weight (gm)	5.64±0.54	7.80±0.64a*	6.13±0.57b <sup>#</sup>	5.68±0.56b <sup>*c</sup>	5.53±0.52 <sup>NS</sup>

Each value is expressed as mean  $\pm$  S.D. for six male wistar rats in each group. a: compared with Group I; b: compared with Group II; c: compared with Group III; d:

compared with Group IV; NS- compared with Group I. Statistical significance: \* $p$ <0.001, <sup>@</sup> $p$ <0.01, <sup>#</sup> $p$ <0.05, NS- Not significant.

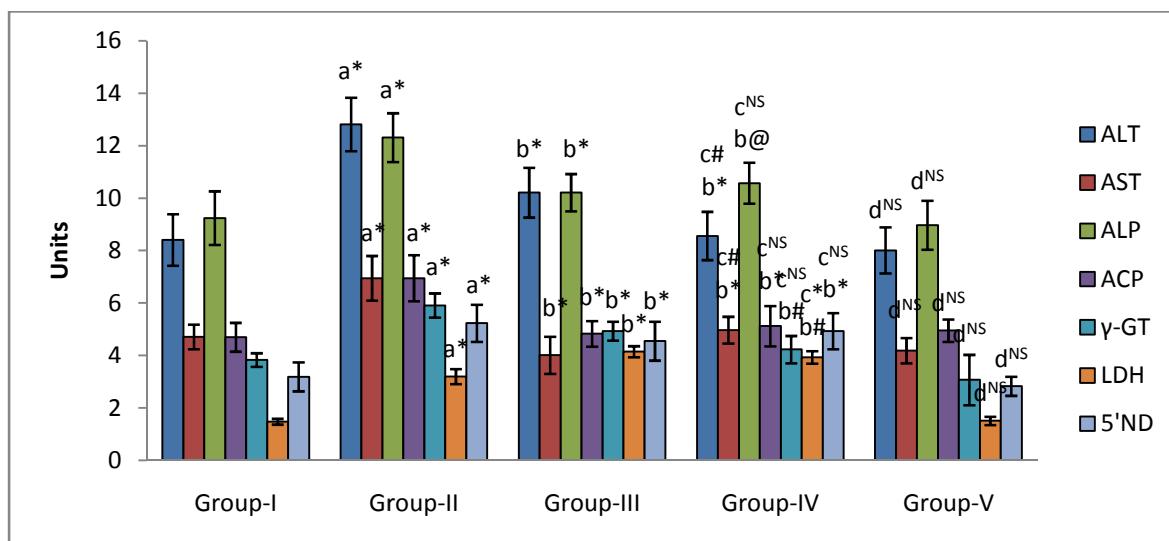


Figure 4: Effect of Withaferin-A on the activity of some marker enzymes in liver of control and experimental animals

Each value is expressed as mean  $\pm$  SD for six rats in each group. Units – ALT, AST and LDH:  $\mu$ moles of pyruvate liberated/min/mg protein. ALP and ACP:  $\mu$ moles of phenol liberated/min/mg protein; 5'ND – nmoles of Pi liberated/min/mg protein.  $\gamma$ - GT – nmoles of p-

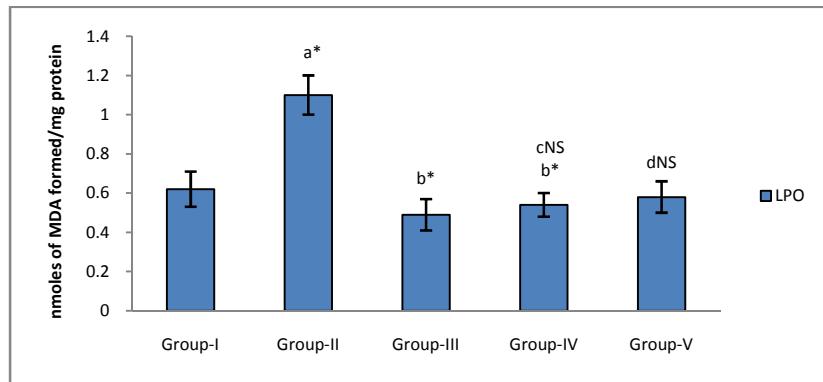
nitroaniline formed/min/mg protein; a - as compared with group I; b - as compared with group II; c - as compared with group III; Statistical significance - \*  $p<0.001$ , #  $p<0.01$ , @  $p<0.05$ , NS-Not significant.

**Table 2:** Effect of Withaferin-A on antioxidant status 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)
SOD	7.98 $\pm$ 0.92	4.37 $\pm$ 0.49 a*	7.96 $\pm$ 1.26 b*	7.41 $\pm$ 0.83 b* c <sup>NS</sup>	7.43 $\pm$ 0.94 d <sup>NS</sup>
CAT	61.23 $\pm$ 7.98	41.56 $\pm$ 5.39 a*	57.35 $\pm$ 6.39 b*	55.62 $\pm$ 6.46 b@ c <sup>NS</sup>	62.32 $\pm$ 6.83 d <sup>NS</sup>
GPx	6.47 $\pm$ 1.07	3.99 $\pm$ 0.49 a*	5.82 $\pm$ 0.78 b@	5.24 $\pm$ 0.64 b# c <sup>NS</sup>	6.67 $\pm$ 1.02 d <sup>NS</sup>
GR	4.12 $\pm$ 0.41	2.64 $\pm$ 0.39 a*	3.54 $\pm$ 0.89 b#	3.45 $\pm$ 0.45 b# c <sup>NS</sup>	3.95 $\pm$ 0.51 d <sup>NS</sup>
GSH	5.17 $\pm$ 0.56	3.64 $\pm$ 0.49 a*	4.72 $\pm$ 0.51 b@	4.65 $\pm$ 0.43 b@ c <sup>NS</sup>	5.39 $\pm$ 0.52 d <sup>NS</sup>
Vit C	3.28 $\pm$ 0.29	2.44 $\pm$ 0.25 a*	2.98 $\pm$ 0.28 b@	3.02 $\pm$ 0.35 b@ c <sup>NS</sup>	3.16 $\pm$ 0.34 d <sup>NS</sup>
Vit E	1.82 $\pm$ 0.23	0.83 $\pm$ 0.08 a*	1.23 $\pm$ 0.11 b*	1.48 $\pm$ 0.16 b* c#	1.76 $\pm$ 0.19 d <sup>NS</sup>

Each value is expressed as mean  $\pm$  SD for six rats in each group. Units- SOD: units/min/mg protein; CAT:  $\mu$ moles of  $H_2O_2$  liberated/min/mg protein; GPx:  $\mu$ moles of GSH oxidized/min/mg protein; GR: nmoles NADPH oxidized min/mg protein. GSH, Vitamin C, Vitamin E:  $\mu$ g/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 5:** Effect of Withaferin-A on the level of Lipid Peroxidation in liver of control and experimental animals

Each value is expressed as mean  $\pm$  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 3:** 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 $\pm$ 0.1	2.31 $\pm$ 0.21 a*	1.49 $\pm$ 0.16 b*	1.58 $\pm$ 0.15 b* c <sup>NS</sup>	1.19 $\pm$ 0.07 d <sup>NS</sup>
DNA-Protein crosslinks	1.72 $\pm$ 0.21	5.86 $\pm$ 0.53 a*	2.45 $\pm$ 0.31 b*	3.12 $\pm$ 0.43 b#	1.84 $\pm$ 0.18 d <sup>NS</sup>

Each value is expressed as mean  $\pm$  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

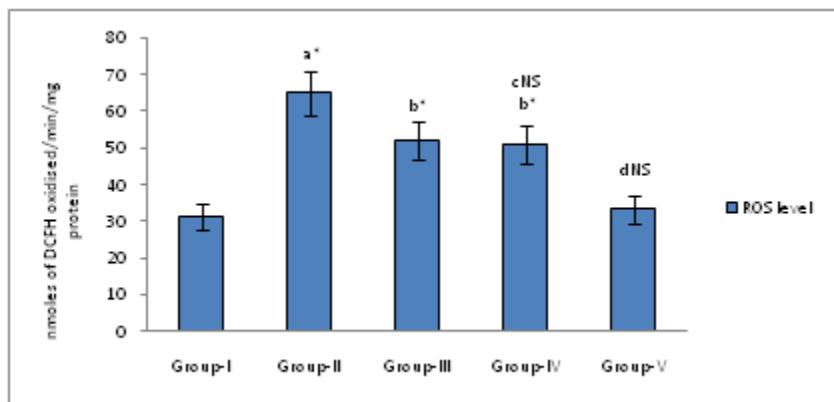


Figure 6: Effect of Withaferin-A on ROS levels in control and experimental animals

Each value is expressed as mean  $\pm$  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.

## DISCUSSION

Hepatocellular carcinoma (HCC) is considered to be closely related with oxidative stress mediated metabolisms. Therefore, the liver backgrounds such as hepatocytes nodules, focal proliferation, viral hepatitis, abnormal expression of related genes and proteins play crucial roles in the process of carcinogenesis. All liver cancer is developed on the basis of hyperplastic nodules and persistence to cell proliferation and evolution<sup>4</sup>. Reactive oxygen species (ROS) are involved in a diversity of important phenomena in medicine, such as radiation effects, chemotherapeutic effects, mutagenesis, carcinogenesis and aging<sup>29</sup>. In this paper, we hypothesize protective effect of withaferin-A in DEN induced hepatocellular carcinoma. Among the targets of ROS, DNA appears most important in tumor biology since it is firmly established that cancer is a genetic disease<sup>30</sup>. ROS induce several kinds of DNA damage, including strand breakage, base modification and DNA-protein cross-linkage<sup>31</sup>. So the herbal treatment for cancer has almost no side effects and is relatively cheap and locally available. They are effective in reducing the tumour growth in the system<sup>32</sup>. There are few reports available in connection with an active principle withaferin-A from WS tested against cancer<sup>33</sup>. Based on these reports, we have decided to isolate the active principle from ethanolic extract of WS roots and to test its efficacy on hepatocellular carcinoma bearing rats. The structural elucidation of withaferin-A is required extensive chemical work and spectral interpretation. Proton NMR spectroscopy and mass spectrometry have played important roles in determining the structures of

Withanolides in the last fifteen years. In spite of extensive proton NMR and mass spectral data available for this class of compounds<sup>34,35</sup>, it is not always possible to establish the various substitution patterns on the withanolide skeleton unambiguously, e.g., the complete structures of several withanolides still remain unknown<sup>36</sup>. The structure of withaferin-A has been elucidated by mass spectral studies and NMR<sup>38,39,40</sup>. Although the <sup>13</sup>C NMR data of several hundred steroids have been published<sup>37</sup>, the application of <sup>13</sup>C NMR spectroscopy to the structure elucidation of this class of compounds has been largely ignored. The IR spectrum displayed intense absorption band at 3434(OH), 1715(C=O) and 1464 cm<sup>-1</sup>(C=C) assignable respectively, to hydroxyl, enone and  $\alpha$ ,  $\beta$ -unsaturated  $\delta$ -lactone functionalities. The EI MS spectrum of compound showed the molecular ion at *m/z* 470.113 corresponding to the molecular formula C<sub>28</sub>H<sub>38</sub>O<sub>6</sub>. The corresponding <sup>1</sup>H NMR spectrum of the root (Fig:2) was clear enough to indicate the presence of withaferin-A skeleton. The <sup>1</sup>H signals at d 0.91(s, 3H, H-18), 1.03 (d, J=7 Hz, 3H, H-21), 1.20 (s, 3H, H-19), 1.91 (s, 3H, H-27), 2.0 (s, 3H, H-28), 2.68 to 2.73(m, 1H, H-4), 4.3 to 4.41 (m, 1H, H-22), 5.86 (dd, J=10.1, 2.6Hz, 1H, H-2) and corresponding <sup>13</sup>C signals at d  $\delta$  203.21, 177.84, 139.62, 129.03, 122.04, 57.33 were identified by NMR spectrum provided sufficient evidence for the presence of withaferin-A. Assignments of resultant chromatograms were performed by matching with the chromatogram of purified withanolides<sup>41</sup>. The analysis suggested that withaferin-A is the major metabolites present in the root as shown by NMR. Diet plays a major role in cancer aetiology and prevention. Although inconsistency exists across studies that have investigated the relationship between diet and cancer, the basic assertion that dietary factors influence cancer risk is not really a matter of debate. On the other hand, inadequate intake levels of some micronutrients can be critical to genomic stability, increasing the risk of genetic-related diseases like cancer<sup>42</sup>. In the present study, the

administration of Diethylnitrosoamine to rats, as expected, resulted in loss of appetite, weakness, hair fall, fluid retention and loss of body weight. This might be due to the mechanism action of the DEN. Decrease in body weight of cancer bearing rats is due to catabolism of fats and proteins. This feature has paramount importance because nutritional depreciation causes body weight loss, which may in parallel results in increased tumor volume. In our study initial and final body weight of control and experimental rats were measured whereas the food and fluid intake were evaluated on daily basis. We observed a significant reduction in the intake of food and fluid, whereas an increase in body weight accompanied by reduction in tumor incidence was also observed when the cancer bearing rats were treated with withaferin- A. In addition to this, an increase in the liver weight and regression in the body weight of HCC bearing animals shows the severity of hepatocarcinogenesis in DEN administered Wistar Albino rats<sup>43</sup>. The abnormalities were modulated by Withaferin-A and these modulations showed the possible biological function of Withaferin-A highlighting its anticancer effect. These aspects strongly suggest that cancer bearing rats are influenced by environmental factors, including diets, and therefore cancer is largely preventable. Biochemical tumor markers are used to screen particular tumor condition for diagnosis, prognosis and for assessing the response to therapy. These enzymes are unique and the rise in their activities indicates the number of transformed cells in cancer conditions. The role of transaminase in biological system is well known, which indicates the severity of an advanced cancer condition. Increased levels of transaminase activity are also seen in hepatocellular carcinoma<sup>44</sup>. Liver damage caused by DEN generally reflects on the instability of liver cell metabolism which leads to distinctive changes in the serum enzyme activities<sup>45</sup>. So in the present study, the major marker enzymes of hepatic injury namely AST, ALT and ALP are determined to assay the activity of the WFA on hepatocyte membrane. The activities of ALT and AST in liver were normalized after treatment with Withaferin-A. The  $\gamma$ GT is an enzyme embedded in the hepatocyte plasma membrane, mainly in the canalicular domain; again the liberation of this enzyme into serum indicates damage to the cell and injury to the liver. It is important to point out that serum  $\gamma$ GT activity is considered to be one of the best indicators of liver damage<sup>46</sup>. The enzymes ALP and ACP are membrane bound enzymes and its alteration is likely to affect the membrane permeability. The ALP and ACP levels were elevated in cancer bearing animals<sup>47</sup> which reflects the pathological alteration in biliary flow. The activities of these enzymes were brought back to near normal on treatment with Withaferin-A.

Similarly LDH is a cytoplasmic enzyme which catalyses the oxidation of lactate to pyruvate and vice versa. LDH is a marker for membrane integrity and is a regulator of many biochemical reactions in the body tissues and fluids<sup>48</sup>. The discharge of LDH reflects a non-specific alteration in the plasma membrane integrity. There is a marked increase in the enzyme activity of LDH in hepatocellular carcinoma animals, indicating the enhanced glycolysis during tumor growth<sup>49</sup>. The activity of LDH was brought back to normal on treatment with Withaferin-A. 5'- Nucleotidase was found to be increased significantly in hepatocellular carcinoma bearing animals similar to reports in the sera of patients with solid tumor<sup>50</sup>, treatment with WFA attenuated the decreased activities of these enzymes. From this it was suggested that WFA aids in parenchymal cell regeneration in liver, thus protecting membrane integrity, thereby decreasing enzyme leakage. Reactive oxygen species (ROS) are constantly generated and eliminated in the biological system. They play an important role in normal biochemical functions and abnormal pathological functions. The oxidative stress hypothesis of carcinogenesis reports suggest that many carcinogens can generate free radicals that damage cells, thereby predisposing them to malignant conversions. ROS generated in the system is quenched and the body is protected against its deleterious effects by the antioxidants defence system<sup>51</sup>. 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<sup>53</sup>. It was previously reported that the enzymic antioxidants like SOD, CAT, GPx and GR activities are significantly decreased in the cancer induced animals<sup>54</sup>. 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<sup>55</sup>. 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<sup>56</sup>. They can utilize reactive oxygen species metabolites, to protect the biomolecules and reduce oxidative DNA damage<sup>57</sup>. 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<sup>58</sup>. 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. Increased incidence of oxidative stress and lipid peroxidation are implicated in carcinogenic processes<sup>59</sup>. MDA is a low-molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. Hence, it is of interest to assess MDA as a marker of oxidative stress. Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidant defense system that quench or scavenge them and thereby protect the body against pathogenesis<sup>51</sup>. Free radicals are involved both in the initiation as well as promotion stage of tumourigenesis and their biochemical reactions in each stage of the metabolic process are associated with cancer development<sup>60</sup>. It is evident from the results that increased level of LPO was found in cancer-bearing animals when compared to control group. On the contrary, reduced level of LPO was observed in WFA treated animals indicating that it is a good free radical scavenger activity. The binding of chemical carcinogens or their metabolically activated products to DNA is believed to be an essential part of the carcinogenic process<sup>61</sup>. The DEN induced genetic lesions consist of DNA–protein cross links, chromosomal aberrations, sister chromatin exchanges and base modifications<sup>62</sup>. The DEN induced DNA–protein complexes may be formed by the generation of active oxygen species during the intracellular proteins<sup>63</sup>. The reactive oxygen species induced damages and levels were reduced by the supplementation of dietary antioxidants<sup>64</sup>. The details of DNA protein cross-links repair in rat cells remain largely unknown. In the present study, the levels of DNA–protein cross links are found to be high in DEN induced liver cancer bearing animals which may be due to carcinogenic affect of DEN on macromolecules. It has been established that ROS could modify the chemical structure of proteins with formation of protein carbonyls due to oxidative cleavage of the main peptide backbone of amino acids<sup>65</sup>. Protein carbonyl content has been the most commonly used marker of protein oxidation. Recent study shows that oxidative stress elevates the protein carbonyl content in plasma of HCC patients<sup>66</sup>. As a hallmark of protein oxidation, total protein carbonyl content was measured in

our present study in the livers of DEN exposed animals. In line with previous observations<sup>67,68</sup>, we have observed an elevated hepatic level of protein carbonyl formation in DEN-treated animals, indicating oxidative protein damage. We have also noticed that dietary WFA completely abrogated DEN-induced enhanced protein carbonyl formation, which implicates the ability of this dietary agent in attenuating oxidative stress in the liver. The results of our present study strongly suggest that the antiproliferative effect of WFA showed the reduced lipid peroxidation, protein carbonyl, DNA-protein crosslinks activity and marked improvement in hepatic architecture with reduced morphological cell surface changes in DEN induced rat hepatocarcinogenesis.

## CONCLUSION

From the experimental studies, we suggest that Withaferin-A is having a hepatoprotective effect in DEN induced animals. Withaferin-A is a potent free radical scavenger and is known to modulate the activities of antioxidant enzymes due to their interaction with various biomolecules. Moreover, our study suggests that WFA can act with promising antioxidant properties and as a antimetastatic agent, it can act as a targeted therapy for liver cancer and may be important for cancer prevention, should be further investigated.

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