Original Research Article

Study on the Anticarcinogenic Efficacy of Withaferin-A in DEN Induced Hepatocellular Carcinoma: Morphology and Histopathology

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide, and the burden of this devastating cancer is expected to increase further in coming years, due to late diagnosis, lack of definitive treatment and poor prognosis. In their context, we need to find out a new compound that will improve the overall prognosis of HCC. In this regard, naturally occurring polyphenols are receiving increased attention because of their promising efficacy in several cancer models. A large amount of data have indicated the therapeutic benefits of Withaferin-A against cancer. However, it remains unclear whether these benefits are similar and equally effective both in prevention and in treatment of HCC.
Aim: To evaluate the anticancer property of Withaferin-A against hepatocellular carcinoma in experimental rats.

Materials and Methods: For the experiment, rats were divided into different groups and treated with Withaferin-A either from day 1 of DEN administration for 21 days (pre-HCC), or after the development of HCC, i.e., 12-15 weeks after DEN administration (post-HCC), and compared with untreated HCC-bearing rats.

Results: Decreased level of serum tumor marker α-fetoprotein along with the marked difference in morphology and histology of the treated HCC compared with untreated HCC-bearing rats demonstrates the suppressive effect of Withaferin-A against cancer.

Conclusion: Our results indicate that the administration of Withaferin-A is effective in experimental rats for the prevention and treatment of HCC.

Key words: Hepatocellular Carcinoma, Histopathology, DEN, AFP, Withaferin-A.

INTRODUCTION

Liver disease is a life threatening public health problem. At present, approximately 300 million people are having liver problems. The prevalence of cancer in India is estimated to be around 2.5 million, with about 800,000 new cases and 550,000 deaths per annum. [1] Most hepatocellular carcinoma (HCC) develops in the background of chronic liver disease. The clinical management choice for HCC is very complex. For the optimum clinical outcomes, we need the most reliable classification of tumors, based on the histopathologic evaluation and the treatment based on this evaluation. [2] Since the early
detection of HCC is difficult due to the lack of specific diagnostic markers and asymptomatic nature of the tumor, patients usually present with the advanced stage of HCC. \[3\] For making a more accurate final diagnostic report, pathologist should be familiar with histopathologic lesions of hepatocellular carcinoma. \[4-6\] Moreover, tumor angioinvasion leads to poor prognosis. \[7\] Evaluation of the macroscopic (tumor size, masses, hepatic nodularity, growth pattern) and microscopic features (grade of differentiation, vascular invasion) of the tumor helps in arriving at an accurate diagnosis and better prognosis. \[8\] Liver tumor classification was revised recently according to the 2010 WHO classification. \[9\] A recent paper by Ramakrishna et al \[10\] showed that DEN is a causative agent of liver cancer if administered for longer periods in rats.

Not all the markers profiles are particularly useful in diagnosis of HCC. However, in practice, AFP is being routinely used for the diagnosis of HCC. Elevated alpha-fetoprotein level gives an overall status and stage of HCC. \[11\] Furthermore, altered morphology and tissue structure plays a major role in pathogenesis of cancer. Therefore, the study of liver tissue architecture, morphology, size, masses and histology is important for comprehensive management of HCC patients. \[12\] Precise diagnosis is of vital importance for therapy and prognosis. Currently large number of new treatments is under trials for HCC. Therefore, the understanding of above-mentioned parameters is very important for prevention and treatment of liver diseases. Recent developments in chemotherapeutic agents, such as sorafenib, have shown promising results in transiently reducing the tumor burden. \[13\] However, chemotherapeutic agents, in general, are not cancer-specific in targeting and often exhibit a broad spectrum of toxicities. Improving quality of life in future is a legitimate goal of regional therapy. In view of these facts, attempts have been made to study the necessity to find a better treatment compound that selectively targets and blocks the tumor-specific pathways in HCC. The present study was conducted to evaluate the anti-carcinogenic activity of Withaferin-A against DEN induced hepatocellular carcinoma in rats.

Based on the literature review Withaferin-A (WFA), which is the active component of *Withania somnifera*, has potent anti-tumor and antioxidant properties and is remarkably non-toxic. \[14,15\] However, protective activity of Withania somnifera was not been scientifically investigated against DEN induced hepatic cancer. Hence, an attempt was made to investigate the efficacy of Withaferin-A against liver cancer.

**Objectives:** The objectives of this study are:

1) To study the effect of Withaferin-A on histopathology and morphology of hepatocellular carcinoma.

2) To assess tumor marker status.

**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, 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
with 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:** The animals were divided into five groups with six rats in each group. Group I served as control animals treated with 0.5% CMC (vehicle) orally. Group II animals were treated with N-Nitrosodiethylamine (DEN) (0.01% dissolved in water) daily and kept for 12 weeks to induce liver cancer. The method of cancer induction was adopted from Ramakrishnan et al. [16] with slight modifications and treatment conditions were accordingly decided in the current study. Group III comprised of control animals treated orally with Withaferin-A (50mg/kg b.wt/day) for three weeks prior 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. Control animals treated with Withaferin-A alone as in group III served as group V.

After the experimental period, the animals were killed by cervical dislocation; blood and liver tissues were used for the further analysis. The liver tissues were excised immediately and were washed in ice-cold saline to remove any extraneous matter and to observe tumor morphology. Liver specimens were fixed in 10% neutral buffered formalin for histopathological and further studies.

For histology, tissues were routinely fixed in phosphate-buffered 10% formalin (Polysciences Co., Warrington, PA, USA), dehydrated by graded ethanol, embedded in Paraplast Plus wax (McCormick Scientific, IL, USA), sectioned at 5 microns, mounted on slides, oven-dried and deparaffinized. The tissue sections were subjected to H&E staining and viewed under a light microscope. Analysis of serum tumor markers was done. The α-fetoprotein (AFP) level was quantified based on the ELISA principle using the Fully Automated Bayer ADVIA Centaur chemiluminescent system (Bayer Corp., Pittsburgh, PA, USA). In brief, a 96-well plate pre-coated with AFP antibody was incubated with an anti-AFP antibody conjugated with horse-radish peroxidase to measure signal intensities generated from the enzyme-substrate reactions. Protein content was estimated following the method of Lowry et al. [18]

**Statistical Analysis:** Data is presented as the mean ± 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. A commercial software SPSS version 10.0 was employed to find out the statistical significance between the groups and p < 0.05 was considered statistically significant.

**RESULTS**

**Effect of Withaferin-A on the Changes in liver and body weights**

Table: 1 represents the body weight and liver weight changes of the control and experimental rats. Body weight and liver weight were noted from the day of tumor induction, until the completion of the experimental period. The weights were noted periodically once in a week. The control rats showed a gradual increment in their body weight throughout the experimental period. There found to be a significant (p<0.001) decline in the body weight from 10<sup>th</sup> week of experiment and continued constantly till the end of the experiment and an increase in liver weight...
of the cancer induced rats (Group-II) when compared with the normal control rats (Group-I). In Withaferin-A treated group III and group IV animals, there found to be a significant (p<0.05) reduction in the body weight and a significant (p<0.001) decrease in liver weight when compared with cancer bearing group II animals. However, there found to be no significant difference in the body and liver weights of control animals and the control group treated with Withaferin-A (Group-V).

**Effect of Withaferin-A on Tumor Marker Enzymes**

**Fig: 1** represents the tumor marker alpha fetoprotein (AFP) levels in serum of control and experimental rats. The level of AFP significantly (p<0.001) increased in cancer bearing rats (group II) when compared to the control (group I) rats. Withaferin-A treated cancer bearing rats showed a significant (p<0.001) decrease in the level of AFP, in both pre (group III) and post-treated (group IV) rats compared to cancer bearing (group II) rats.

**Effect of Withaferin-A on Morphological changes in liver**

**Fig: 1** shows the effect of Withaferin-A on morphological changes in liver of control and experimental animals. Gross liver pathology of the control animals (Group I) showed yellowish liver with pale red tinge, with these changes increasing with time. The liver of control animals showed normal architecture with normal size and shape when compared with group II animals. The liver on the 10th and 13th weeks after DEN treatment showed grayish-white visible multi-nodules on the outer surface, about 1 mm in diameter in group II animals. The liver showed a significant widening of intercellular spaces between hepatocytes, elongated microvilli over large regions of the cell surface, multiple nodules, size and shape, many invaginations of the cell membrane and irregularly shaped biliary canaliculi when compared with control animals (Group I). Foci appeared in random fashion in all the three liver zones. This is considered to be due to the effect of DEN as both initiator and promoter i.e. as a complete carcinogen.

Liver of WFA pretreated animals (Group III) showed a noticeable recovery in the liver architecture with normal size and shape when compared with group II animals. Liver of WFA post treated animals (Group IV) showed perceptible recovery in the liver architecture with normalizing of cell surface, cell membrane, reduced number of nodules, size and shape when compared to Group II animals. The portal areas showed no or very limited increase in the number of cells. However, there found to be no significant changes appeared between the control animals (Group-I) and the control animals treated with Withaferin-A (Group-V).

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

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (gm)</td>
<td>137±14.28</td>
<td>171±19.06</td>
<td>154±17.08</td>
<td>152±17.04</td>
<td>163±17.83</td>
</tr>
<tr>
<td>Final body weight (gm)</td>
<td>189±12.2</td>
<td>145±11.19</td>
<td>163±13.3</td>
<td>167±11.21</td>
<td>185±12.28</td>
</tr>
<tr>
<td>Liver weight (gm)</td>
<td>5.63±0.59</td>
<td>7.78±0.69</td>
<td>6.11±0.61</td>
<td>5.65±0.62</td>
<td>5.51±0.58</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ±SD for six rats in each group.

Body weight and Liver weight: 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.
Fig. 1 Effect of Withaferin-A on AFP in serum of control and experimental animals
Each value is expressed as mean ±SD for six rats in each group.
Comparisons were made between: 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.

Fig.1 On gross examination: Plate showing the morphological changes in liver of control and experimental animals. The Group I indicates the normal morphology of liver but Group II showed marked alterations like foci, nodules and increased the size. In Group III and IV (Withaferin-A treated) showing marked recovery (no foci and nodules) when compared to control animals. No significant alterations were observed in Group V when compared to control animals.
Fig.2 On microscopy: Plate showing histological observation (20X) of liver tissue in control and experimental animals. Group I control animals showed normal architecture with central vein, hepatocytes and nuclear size. The Group II cancer bearing animals showed huge infiltration of central vein multinucleated hepatocytes, loss of radiating hepatocytes when compared to control (Group I). Group III and IV pre and post treated with Withaferin-A shows noticeable recovery of radiating hepatocytes, central vein and nuclear size. Group V Withaferin-A alone administrated showed normal architecture and slight sinusoidal infiltration.

**Effect of Withaferin-A on liver histopathology**

Fig: 2 show the histopathological studies on the liver of control and experimental animals. Group I: Histopathology of the normal Liver stained with Haematoxylin and eosin, showed normal hexagonal architecture with normal central vein, radiating hepatocytes with normal cell and nuclear size. Group II: Liver of HCC bearing animals showed narrowing and huge infiltration of central veins with multinucleated hepatocytes and loss of the radiating hepatocytes architecture. The nuclear size and hepatocytes also showed wide variation when compared to the normal liver. Group III: Liver of Withaferin-A (WFA) pretreated animals showed noticeable recovery in the liver architecture with restoring of radiating hepatocytes, central vein and normalized hepatocytes and nuclear size. Group IV: Liver of WFA post treated animals showed perceptible recovery in the liver architecture with normalizing of radiating hepatocytes, central vein, hepatocytes and nuclear size. Group V: Liver of WFA alone treated animals showed normal architecture (hexagonal radiating hepatocytes and normal nuclear size) with slight sinusoidal infiltration.
DISCUSSION

Cancer or malignant neoplasm is a class of diseases in which a group of cells displays uncontrolled growth, invasion and even sometimes metastasis. [19,20] It continues to be a serious public health problem throughout the world as the most feared diagnosis. It is the third leading cause of human death after cardiovascular diseases in developing as well as in developed countries. [21] Currently, the treatment for cancer primarily includes surgery and chemotherapy, but the curative effects of the existing therapeutic drugs are not good enough and they have plentiful side effects. The development of more effective drugs for treating patients with cancer has been a main attempt over the past 50 years. However, little is known about the relationship between systemic chemotherapy and histologic changes in the cancer that may be responsible for the post chemotherapy liver. However, the question remains whether these hepatic injuries have any clinical significance. In the current study, we attempted to find the anticancer effect of WFA against hepatocellular carcinoma. Evaluation of pathological features (macroscopic and microscopic) provides accurate diagnosis and prognosis. [8,22]

First, we observed in standardization experiment (unpublished data), of the 10 animals receiving DEN, 4 died from effects of an acute hemorrhage into the peritoneal cavity. Of the other 6 rats, 3 only were killed in good condition as the sole survivor after 15 weeks. Its liver had several nodules. The others were killed because they were ill. An outstanding feature of the necrosis was its very hemorrhagic character and the liver lesion was frequently accompanied by massive hemorrhagic ascites and bleeding into the gastro intestinal tract. [23] Uniformly shrunken fibrotic livers were not seen. Architectural consideration was more challenging to recognize as HCC, even though observation of morphological changes and architecture are the as best tools for diagnostics of malignancy. [4-6] The anatomical structural studies were performed to confirm the occurrence of morphological changes at the cellular level. The control and the drug control animals showed normal architecture view. The DEN induced animals showed the presence of visualized multiple nodules, foci, gross irregular shape and enlargement of liver size (Fig.1B). A normal liver is shown for comparison (Fig.1A). Numerous small translucent cysts were frequently seen in addition to the solid nodules. All had nodular and small hemorrhage from the liver surface. From the results, we observed Withaferin-A treated groups showing marked recovery (no foci and nodules) when compared to control animals. It indicates that Withaferin-A has potential protective effect against cancer.

Histopathological analysis was helpful in confirming the necrosis of cells, damage that had occurred in bile ducts and central vein dilation. In the current histological study, cancer-bearing animals showed damage to hepatocytes with granular cytoplasm, narrowing and huge infiltration of central veins with multinucleated hepatocytes and loss of radiating hepatocytes architecture, which is due to toxicity of carcinogen. These pathological anomalies were altered on administration of Withaferin-A for 21 days (Fig.2C,D) indicating it’s protective effects on hepatocytes when compared with cancer bearing rats. The proliferating hepatocytes had a vacuolated cytoplasm and showed nuclear changes similar to those noted in the foci of the previous study. [10, 24, 25] Similar results were noticed during the six sets of experimental animals that foci appears in 10th week after DEN treatment through drinking water, indicate clearly that oval cell appearance is correlated with hepatic
carcinogenesis (Fig. 2B) especially in Group II cancer bearing animals. It is important to mention that the dose of DEN used for selection is carcinogenic. In agreement, Magda Ismail Youssef et al. [26] demonstrated gross structural alterations in rats liver treated with DEN, with predominantly basophilic, eosinophilic and some large nuclei in the cells.

Relative liver weight is an important parameter in judging the pathological condition of the liver. [23] During the experimental period, water consumption, food intake and behavioral changes are some of the physical alterations that were observed. Control animals showed a gradual increment in their body weight throughout the experimental period. A strong suppression of body weight in DEN treated animals was observed. The body weight starts declining from 10th week of experiment and continued constantly until the end of the experiment in DEN treated group, indicates severity. However, WFA treatment reduced the body weight loss in the HCC induced animals (Table: 1). Decrease in the relative liver weight by WFA treatment is an indication of improvement of the pathological condition of liver. These improvements correlate with the histopathological results. Similar findings were observed when weight of liver was assessed. WFA alone treated group did not show any change (Table: 1). From this study, we came to the inference that WFA reduces the liver weight in HCC, but has no role in normal liver. This change is associated with improvement of disease.

The biomarkers analyzed in the present study have their metabolic origins in various biochemical pathways, which are important for the assessment of the risk, involved in the process of carcinogenesis. Alpha-fetoprotein (AFP) is the major immunomodulatory glycoprotein expressed by the immature liver cells in the fetus, widely used in the diagnosis and management of HCC. [27, 28] The high levels of AFP are found in patients with advanced HCC. [29] AFP is also present in some hepatic injury and liver diseases including HCC. [11,30,31] Thus, the AFP is an excellent candidate for monitoring and applying new therapy strategies to HCC. [32] Our study also showed an increased level of AFP in the carcinogen-administered animals confirming the presence of HCC, and Withaferin-A treatment significantly reduced the elevation of AFP. This supports the inference that Withaferin-A treatment affects the rate of HCC growth perhaps due to its antiproliferative effects.

CONCLUSION

Hence our present study suggests that Withaferin-A inhibits proliferation of cancer cells and protects the cells from cytotoxic damage induced by carcinogen. Our results suggest that Withaferin-A treatment offers a promising effect by acting as a potential chemotherapeutic and chemo preventive drug with less toxic effect against liver carcinogenesis. Further phytochemical studies are required to establish this compound, which can bring promising results in cancer chemotherapy.

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