-Original Basic Research-

Vitamin C and diallyl sulfide as chemo-sensitizers to cisplatin in treating hepatocellular carcinoma

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Abstract

Background

The resistance to chemotherapy is a major obstacle in the treatment of hepatocellular carcinoma (HCC), necessitating the discovery of additional agents. The use of natural products in this respect is extensively under investigation.

Methods

Two hundred and ten male albino rats were used, divided into 14 groups. Selected groups were pre-treated with vitamin C (ascorbic acid, AA) and/or diallyl sulphide (DAS). Hepatocellular dysplasia was initiated by a single intra-peritoneal (IP) injection of diethyl nitrosamine (DENA), diabetes was induced by a single IP injection of Streptozotocin (STZ). Other groups were treated with cisplatin (CP) alone or combined with AA and/or DAS for 14 weeks. This work aims to check if naturally occurring materials can help in improving response to CP chemotherapy using ploylol profile as a new index in management of HCC.

Results

The results revealed that DENA significantly increased relative liver weight, serum ALT, AST and GGT activities, AFP, TNF-α and IL-6 levels, expression of aldose reductase (AR), sorbitol dehydrogenase (SDH) and Bcl2 proteins in the liver with decrease in body weight, expression of Bax protein and Bax/Bcl2 ratio in the liver. These effects were more pronounced in DENA+STZ group. These parameters showed relative correlation to AFP levels in HCC, in response to AA and/or DAS treatment except for SDH. Treatment with CP and/or AA and/or DAS significantly modulated most of these parameters except for SDH which showed no significant change in response to the suggested treatment.

Conclusions

In conclusion, AA and/or DAS showed apparent chemo-sensitizing, anti-inflammatory, AR inhibitory, apoptotic inducing and anti-diabetic activities, indicating new aspects for use as adjuvant to chemotherapy. Induction of diabetes in hepatocellular dysplasia -bearing rats showed higher resistance to chemotherapy. Ploylol profile is a useful prognostic tool in HCC patients with diabetic interference.

Key words:

Ascorbic acid; Chemo-sensitization; Diabetes; Diallyl sulphide; Hepatocellular carcinoma; Diethyl nitrosamine

INTRODUCTION

One of the key challenges with hepatocellular carcinoma (HCC) treatment is that patients can develop resistance to chemotherapy. [1] Tumor chemo-resistance is inversely proportional to patient survival and often considered the cause of ineffective cancer therapy regimes. [2]

Patients with HCC can be classified into two groups depending on response to chemotherapy: the first group has intrinsic drug resistance and the second acquired drug resistance after the first treatment with a chemotherapeutic agent.[3] The main possible mechanisms for chemo-resistance of tumor cells are (i) decrease of drug concentration in the cell due to activation of transporter proteins; (ii) reduced drug activation or increased detoxification of the drug within the cell; (iii) alterations of drug target and increased repair of the damaged target; (iv)abrogation of apoptosis (i.e. mutation of the p53 gene). The ratio between apoptotic promoters and repressors also determines the chemo-sensitivity of cells to apoptotic stimuli**.** [4]

Conventional cancer therapies, including surgery, chemotherapy and radiotherapy, as a single modality have a limited but important role in the overall treatment of most solid tumors. [5] Thus, the strategies of cancer treatment using combined therapies or combined agents with distinct molecular mechanisms are considered more promising. [6] Therefore, common cancer therapies combined with certain dietary compounds may exert enhanced antitumor activity through synergic action or compensation of inverse properties. Combined treatment may also decrease the systemic toxicity caused by chemotherapies or radiotherapies decreasing the used doses. [2]

Cis-diamminedichloroplatinum (cisplatin, CP) is an important anticancer drug used to treat solid tumors. [7] It induces cytotoxicity by interference with transcription and/or DNA replication mechanisms. Additionally, CP kills tumors via induction of apoptosis by the activation of various signal transduction pathways, including calcium signaling, death receptor signaling, and activation of mitochondrial pathways. Unfortunately, neither cytotoxicity nor apoptosis are exclusively induced in cancer cells, thus, CP might also lead to diverse side-effects such as neuro- and/or renal-toxicity or bone marrow-suppression. [8]

Vitamin C (ascorbic acid, AA) an essential nutrient and active reducing agent, is involved in numerous biological effects.[9, 10] Its chemo-preventive potential in cancers have been widely reported. However, the definite role of AA in cancer treatment remains controversial. [11-13] In general, data from *in vitro* and *in vivo* experiments and population-based studies do not indicate that high doses of AA show any form of toxicity with an evidence for a strong protective role against cancer. [14, 15]

Other studies shows that AA in combination with certain pharmacological agents produces a synergistic or additive effect on the growth inhibition of tumor cells *in vitro* and *in vivo*.[12, 16] Recently, it was reported that AA elevated CP of tumor cells to CP through enhancing apoptosis by regulating p53.[17]

Consumption of garlic (Allium sativum L.) is known to beneficial for the prevention of life style-related diseases. The therapeutic use of garlic in cancer have been widely studied.[18, 19] Diallyl sulfide (DAS) is a flavor derived from garlic and is sequentially converted to diallyl sulfoxide (DASO) and diallyl sulfone (DASO2) by cytochrome P450 2E1 (CYP2E1). This component decreased the incidence of cancers,[20] inhibiting tumor cell growth *in vitro*[21] and suppressing the formation of DNA-adducts caused by N-nitroso compounds.[22]

The present work was conducted to check whether naturally occurring materials can help in improving response to CP chemotherapy and if ploylol profile can assist in more accurate HCC follow up.

MATERIALS AND METHODS

Animals

Two hundred and ten male albino rats (3 months old) weighing 145-180 g, were used in this study. They were divided into equal 14 groups (15 per group). They were kept under constant environmental and nutritional conditions for two weeks then placed on a standard 26% casein diet with water *ad libitum* during the whole period of the experiment. Individual body weights were recorded weekly.

Chemicals

DENA, STZ, AA, DAS and CP were purchased from Sigma chemical company, St Louis, MO, USA. All other chemicals and solvents were of analytical grade and obtained from Sigma chemical company unless otherwise specified elsewhere.

Induction of Hepatocellular Dysplasia and Diabetes

A single sub-necrogenic IP dose of DENA (125 mg/kg body weight in 0.9% normal saline) was administered in the second week to initiate hepatic carcinogenesis.[23]

Selected groups were given a single IP dose of freshly prepared STZ solution in the fifth week (as 65 mg/kg body weight in 0.05%M citrate buffer, pH 4.5), injected within 10 min of dissolution. [23] The diabetic state was ascertained by monitoring blood glucose levels using a standard Randox glucose kit. Blood glucose levels above 200mg/dl were considered diabetic.[23]

Doses for Chemo-sensitizers and CP

AA: A single daily IP dose of 4 g/kg body weight (pH 7).[24]
DAS: A daily oral dose of DAS in corn oil (200 mg/kg body weight).[25]
CP: A single IP, daily dose of 2 mg/kg body weight, for two weeks, then twice daily for another two weeks, the total treatment duration is four weeks after complete induction of HCC.[26]

Experimental Design

A- Non-diabetic groups: 6 groups, each comprised 15 rats:
1- Normal control: Received same volume of IP saline vehicle.
2- HCC (DENA only): At 2nd week, rats received a single sub-necrogenic dose.
3- DENA + CP: At 2nd week, rats received a single sub-necrogenic IP injection of DENA. At 10th week, rats received CP.
4- DENA + CP + DAS: At 2nd week, rats received a single dose of DENA. Then, at 10th week rats received CP accompanied with oral DAS, for 4 weeks.
5- DENA + CP + AA: At 2nd week, rats received a single dose of DENA. Then, at 10th week, received CP accompanied with a daily single IP dose of AA.
6- DENA + CP + DAS + AA: At 2nd week, rats received a single IP dose of DENA. Then, at 10th week, rats received CP accompanied with DAS plus AA.

B- Diabetic groups: 8 groups, each comprised of 15 rats:
7- Diabetic control (STZ): At 5th week, rats received a single IP dose of STZ.
8- STZ+DAS: At 5th week, rats received a single dose of STZ. Then at 10th week, rats were given DAS for 4 weeks.
9- STZ+AA: At 5th week, rats received a single dose of STZ. Then at 10th week, rats were given daily doses of AA for 4 weeks.
10- Diabetic HCC group (DENA+STZ): At 2nd week, rats received a single dose of DENA. At 5th week, rats received a single dose of STZ.
11- DENA + STZ + CP: At 2nd week, rats received DENA. At 5th week, rats received STZ. At 10th week, rats received CP.
12- DENA + STZ + CP + DAS: At 2nd week, rats received DENA. At 5th week, rats received STZ. At 10th week, rats received CP accompanied by DAS.
13- DENA + STZ + CP + AA: At 2nd week, rats received DENA. At 5th week, rats received STZ. At 10th week, rats received CP accompanied by AA.
14- DENA + STZ + CP + DAS + AA: At 2nd week, rats received DENA. At 5th week, rats received STZ. At 10th week, rats received CP accompanied with DAS plus AA for four weeks.

Sample Preparation

By the end of the experiment at 14th week, all rats were fasted overnight then killed by exposure to increasing concentration of CO_2.

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Blood samples 5 ml blood was obtained by cardiac puncture and divided into two portions. Two ml of blood samples were heparinized for determination of erythrocyte sorbitol content. The remaining blood was left to clot for 1 hour at 37º C, kept in 4º C overnight to allow the clot to shrink, then centrifuged at 10 000 rpm at 4ºC to separate serum samples and kept at -80º C for further use.

Liver tissue After killing of rats, livers were quickly removed, washed thoroughly in ice-cold phosphate buffered solution (PBS) and well blotted. Individual liver weights were accurately recorded, divided into two portions, one for histological examination; the second was immediately frozen in liquid nitrogen, kept at -80º C for determination of polyol pathway enzymes and apoptotic markers in the liver by western blotting.

Liver Histopathology

The posterior lobe of the liver was immediately fixed in 10% neutral formalin for 24 h then changed to absolute ethanol for dehydration and embedded in paraffin. Sections were stained with hematoxylin and eosin for microscopical examination.

Erythrocytes Sorbitol Content

Erythrocytes from heparinized blood were separated by centrifugation at 3 000 g for 30 min. The cells were washed three times with cold isotonic saline. In the final washing, the cells were centrifuged at 2 000 g for 5 min to obtain a consistently packed cell preparation. The packed cells (1 ml) were then incubated with 4 ml Krebs−Ringer bicarbonate buffer (pH 7.4), containing 50 mM glucose in the presence or absence of samples at 37°C at 5% CO_2 for 60 min. The erythrocytes were washed with cold saline, centrifuged at 2000g for 5 min, precipitated by adding 6% of cold perchloric acid (3 ml), and centrifuged again at 2 000 g for 10 min. The supernatant was neutralized with 2.5 M potassium carbonate at 4 °C and used for sorbitol determination.[27, 28]

Preparation of Hepatic Cytosol

Upon removal from the freezer, portions (approx. 100 mg) of livers from each rat were pulverized separately in a mortar under liquid Nitrogen. The finely ground material from each sample was re-suspended in 1 ml of ice-cold 50 mM Hepes buffer, pH 7.5, containing 150 mM NaCl and 1 mM dithiothreitol. This buffer was fortified with complete EDTA-free protease inhibitor cocktail tablets at a dose of one tablet/10 ml buffer (Roche diagnostics Ltd, Lewes, East Sussex, UK). The pulverized hepatic material was placed on ice and homogenized mechanically by three separate 30 second pulses using an Omni homogenizer. The resulting extracts were subjected to two centrifugation steps at 4°C (15 000g for 45min, followed by 100 000 g for 90 min). The final supernatants (cytosols) were kept for biochemical analyses.[29]

Western Blotting

Up on determination of individual protein concentrations of the liver tissue samples using Bradford assay,[30] samples were prepared for loading, amounts of loading buffer and distilled water needed to be added were calculated depending on the protein concentrations of samples to produce 2 mg/ml sample stock, then the mixture was left in a boiling water bath for 5 min. AR, SDH, Bcl2, Bax and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary and secondary antibodies were obtained from Santa Cruz Technology, Inc, UK. GAPDH was used as a loading control to ensure equal loading and even transfer from the gel to the membrane across the whole gel. Electrophoresis was carried out in a Bio-Rad Mini-Protean II Cell apparatus (Bio-Rad Laboratories, Hemel Hempstead, UK) using a discontinuous buffer system. Electroblotting was performed in a Bio-Rad Mini Trans-Blot Cell. Aliquots of hepatic samples were examined on SDS-PAGE and stained with Coomassie R250 immediately before immunoblotting to ensure equal loading of samples. Cross-reacting bands were visualized by enhanced chemiluminescence and resulting images were analyzed densitometrically.

Biochemical Analysis

Blood glucose levels and serum ALT, AST, GGT activities were measured using a using Randox kits on a Daytona autoanalyzer. Rat TNF- α was determined using ELISA kit from ABCAM, UK and rat IL-6 was determined using Endogen® IL-6 ELISA kit from Pierce Biotechnology following the instructions of the manufactures.

Statistical Analysis

Data were expressed as mean ± SD for 10 remaining rats in each group, as mortality rates were about 40% among all groups. Significant difference between groups was analyzed by one-way ANOVA test and correlation coefficients were calculated using Graph pad Prism 5 software.

RESULTS

Figure 1-3 show representative photomicrographs of liver sections from all groups. Normal control showed normal hepatic lobular architecture, with granulated cytoplasm and small uniform nuclei. Diabetic control showed massive fat accumulation and infiltration of mononuclear inflammatory cells**.** Groups treated with DENA and/or STZ showed cloudy swelling, focal areas of necrosis, portal inflammation, bile stasis and proliferation of bile ducts. Hepatocytes showed partial loss of architecture and significant tumor nests. Hepatocellular dysplasia is growing in nests separated from one another by thin walled sinusoids, bearing some resemblance to normal hepatocytes but they are slightly larger, have more irregular and prominent hyperchromatic nuclei. These manifestations were more pronounced in groups treated with DENA+STZ, compared to groups treated with DENA alone (Figure 2). Treatment with CP alone or combined with AA and/or DAS showed apparent improvement. All sections showed more or less normal architecture. Collectively, most of the histological manifestations observed in cancer control were greatly reduced especially when AA and/or DAS were used in plus CP (Figure 3).

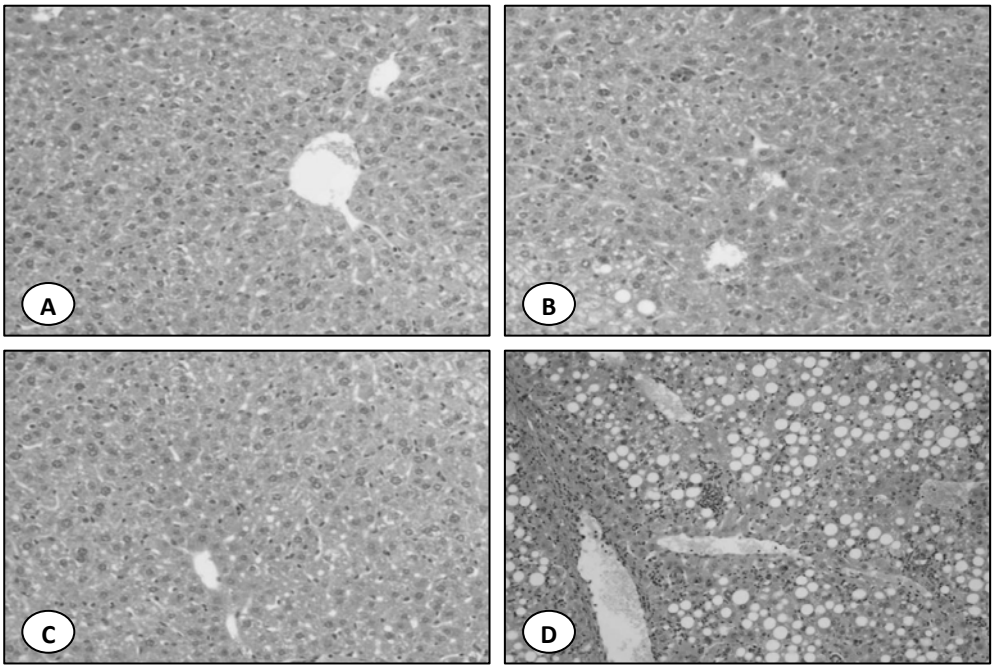

Figure 1 Representative photomicrographs of liver sections from control groups.
A: Normal control**, B:** AA control**, C:** DAS control. All sections showed normal hepatic lobular architecture, normal hepatocytes with granulated cytoplasm and small uniform nuclei**, D:** Diabetic (STZ) control showed massive fat accumulation and inflammatory infiltration in the form of mononuclear cells. (H & E, × 200).

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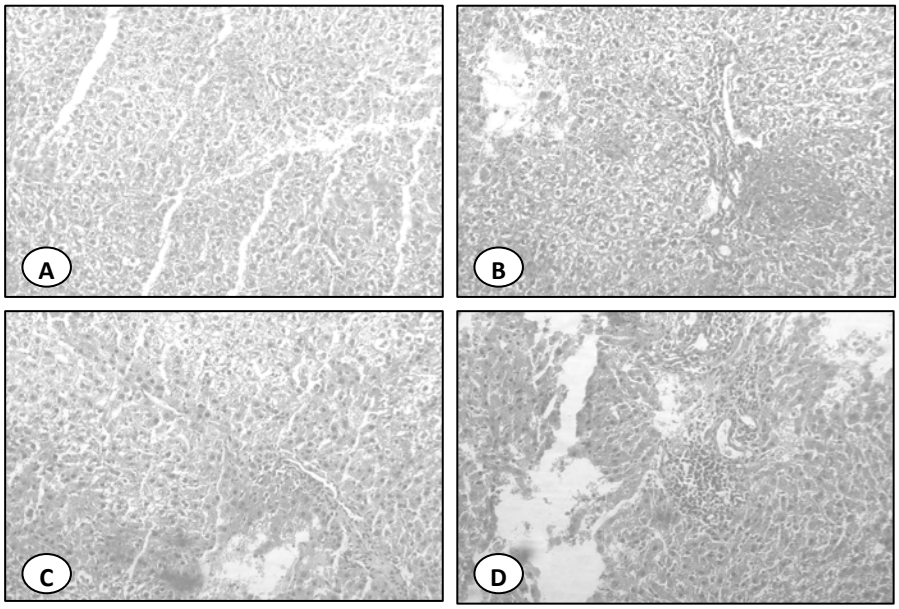

Figure 2 Representative photomicrographs of liver sections from DENA and STZ groups
A and B: Animals treated with DENA only, **C and D:** Animals treated with DENA+STZ. All sections showed hydropic degeneration or cloudy swelling, focal areas of necrosis, bile stasis and portal inflammation. Affected cells showed loss of architecture, growing in nests and thick cords that are separated from one another by thin walled sinusoids. Cytologically, affected cells bear some resemblance to normal hepatocytes but they are slightly larger, have more irregular and prominent hyperchromatic nuclei. These effects were more pronounced in groups treated with both DENA+STZ (H & E, × 200).

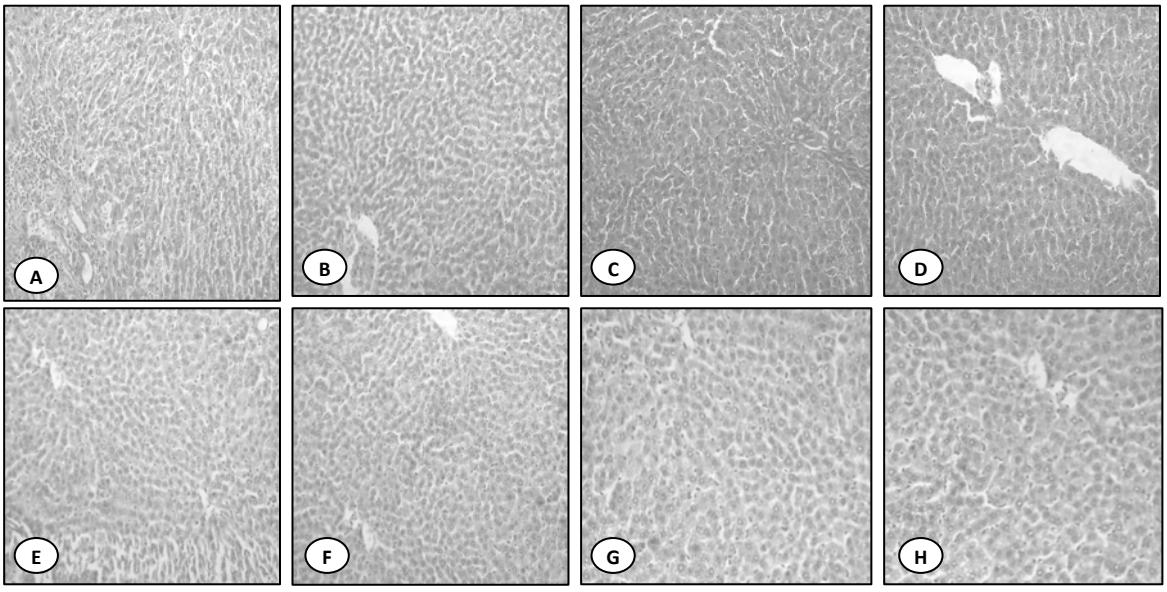

Figure 3 Representative photomicrographs of liver sections from AA and/or DAS against CP treated groups
A: Animals treated with DENA+CP , **B:** Animals treated with DENA +CP +AA, **C:** Animals treated with DENA +CP+DAS**, D:** Animals treated with DENA +CP+AA +DAS, **E:** Animals treated with DENA+STZ +CP only, **F:** Animals treated with DENA+STZ +CP+AA, **G:** Animals treated with DENA+STZ+CP+DAS,**H:** Animals treated with DENA+STZ +CP+AA+DAS (H & E, × 200).

Figure 4(A) shows representative western blotting for polyol enzymes (AR and SDH). AR protein expression was nearly undetectable in normal control, AA and DAS control. AR expression showed significant increase in cancer and/or diabetic controls ($P < 0.001$), compared to normal control. Groups treated with CP alone had no or mild effect, but when combined with AA and/or DAS showed significant decrease in AR expression ($P < 0.001$), compared to their respective controls. SDH expression showed significant increase in cancer and cancer diabetic controls ($P < 0.001$), compared to their corresponding controls. Diabetic control showed significant decrease in expression of SDH ($P < 0.001$), compared to normal control. Treatment with CP alone or combined with AA and/or DAS caused no significant change to SDH protein expression in all treated groups, compared to their respective controls.

Figure 4(B) shows representative western blotting for anti-apoptotic Bcl2 and pro-apoptotic Bax expression in the liver of groups treated with DENA +/- STZ demonstrated significant increase in expression of Bcl2 protein ($P < 0.001$) compared to normal control. The same effect was also observed in DENA +STZ treated group when compared to DENA control, while diabetic control showed significant decrease in Bcl2 protein expression in the liver ($P < 0.001$) compared to control. On the contrary, Bax showed significant increase in groups treated with DENA +/- STZ ($P < 0.001$) compared to their corresponding controls, diabetic control showed significant decrease ($P<0.001$), compared to normal control. CP alone or combined with AA and/or DAS significantly alleviated these effects.

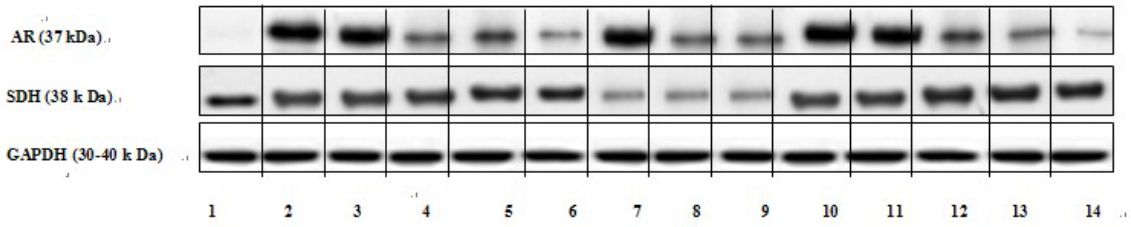

Figure 4 A: Western Blotting for AR, SDH protein expression in liver tissue

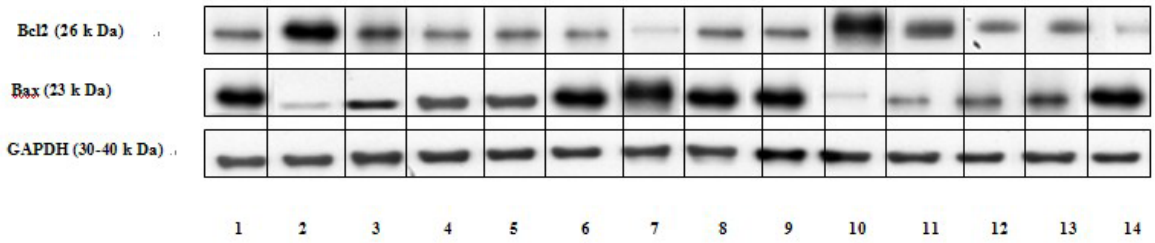

Figure 4 B: Western Blotting for Bcl2 , Bax protein expression in liver tissue

Figure 5 shows that Bax/Bcl2 ratio was significantly decreased in groups treated with DENA +/- STZ ($P<0.001$) compared to their corresponding controls. On the other hand, diabetic control showed significant increase in Bax/Bcl2 ratio ($P<0.001$) compared to normal control. Treatment with CP + AA and/or DAS significantly corrected this defected ratio.

Table 1 shows initial body weights in all groups ranged between 145-180 g, while final body weights differed significantly among groups. Cancer (DENA) and/or diabetic (STZ) groups showed significantly decreased final body weights, compared to control, with significant increase in relative liver weight (absolute liver weight/total body weight).
Table 2 shows that groups treated with DENA +/- STZ showed significant increase in ALT, AST and GGT activities, compared to their corresponding controls, while diabetic groups showed non significant change in ALT activities but significant increase in AST and GGT activities, compared to normal control. All STZ treated groups showed significant increase in blood glucose levels, while DENA control showed significant hypoglycemia, compared to normal control. Treatment with CP alone or combined with AA +/- DAS significantly normalized liver enzyme activities.
Table 3 demonstrates that serum AFP levels were significantly increased in DENA treated groups, compared to normal control. This effect was more pronounced in DENA+STZ treated groups, while diabetic control showed non significant change. CP +/- AA and/or DAS significantly decreased serum AFP levels. Inflammatory markers (TNF-α and IL-6) showed similar pattern but diabetic control showed significant increase. On the other hand, erythrocyte sorbitol levels were not significantly influenced by CP alone, but significantly lowered with AA and/or DAS especially in combination.

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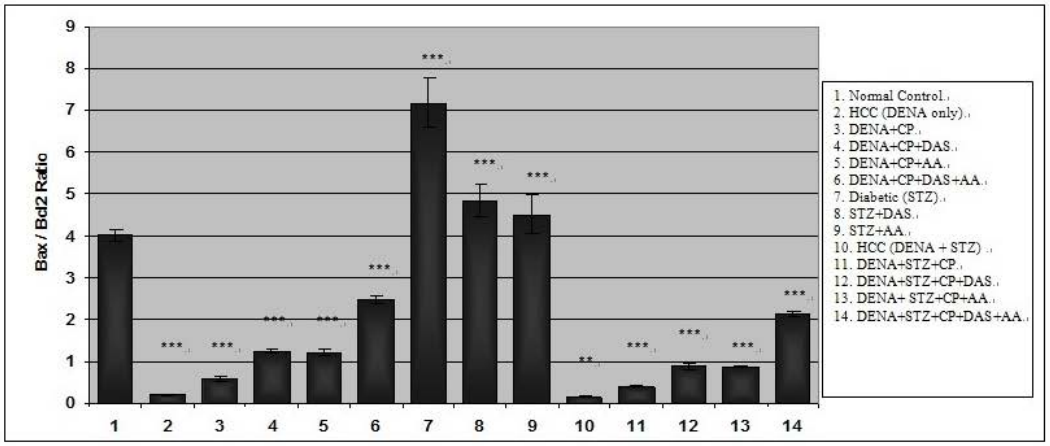

Figure 5 Bax / Bcl2 Ratio

Data were expressed as $X \pm$ SD of three separate experiments. Significant difference between groups is analyzed by one-way ANOVA test, where: (* $P < 0.001$: highly significant , ** $P < 0.01$: significant , * $P < 0.05$: mildly significant , ns $P > 0.05$: non significant). Groups 2,7 were compared to group 1 (normal control). Groups 3,4,5,6,10 were compared to group 2 (cancer control).Groups 8,9 were compared to group 7 (diabetic control). Groups 11,12,13,14 were compared to group 10 (cancer diabetic control).**

Table 1 Body and liver weights

Parameter \ Group	1 Normal Control	2 DAS Control	3 AA Control	4 Cancer (DENA) Control	5 DENA+CP	6 DENA+CP+ DAS	7 DENA+CP+ AA	8 DENA+CP+ DAS+AA	9 Diabetic(STZ) Control	10 STZ+DAS	11 STZ+AA	12 DENA+STZ	13 DENA+STZ+ CP	14 DENA+STZ+ CP+DAS	15 DENA+STZ+ CP+AA	16 DENA+STZ+ CP+DAS+AA
Initial Body Weight	164.9±8.95	169.4±8.23	160.7±11.65	160.8±11.35	167.5±7.73	157.8±9.98	158±5.84	162±10.02	168.8±8.56	159.4±9.53	157.3±8.99	165.8±8.18	165.8±8.78	160.6±11.82	159.9±10.81	158.3±10.50
Final Body Weight	254.6±8.20	259.6±9.58	260.7±10.78	213.7±11.41***	223±12.82$$$	214.3±12.07$$	216.4±6.57$$$	224.9±9.52$$$	234.7±7.24***	242.5±9.23&&.	239.6±7.45&.	213.3±15.21	218.6±10.10£££	218.6±12.20£££	216.8±11.95£££	223.7±12.34£££
Absolute Liver weight	5.7±0.40	5.8±0.41	5.9±0.41	10.4±0.90***	9.2±0.29$	9.0±0.2$$$	8.80±0.19$$	8.1±0.22$$$	6.1±0.45	6.1±0.24	5.9±0.16	11.0±1.02$	9.8±0.19£££	9.2±0.25£££	9.1±0.23£££	8.6±0.25£££
Relative Liver Weight	0.02±0.002	0.02±0.001	0.03±0.002	0.05±0.004***	0.04±0.002$$$	0.04±0.003$$$	0.04±0.001$$$	0.04±0.002$$$	0.03±0.003*	0.03±0.002&.	0.02±0.001&.	0.05±0.004$$$	0.04±0.003£££	0.04±0.002£££	0.04±0.002£££	0.04±0.003£££

Data are expressed as $X \pm$ SD of ten rats in each group.Significant difference between groups is analyzed by one-way ANOVA test, where: (*** $P < 0.001$: highly significant , ** $P < 0.01$: significant , * $P < 0.05$: mildly significant , ns $P > 0.05$: non significant).* : Groups 2,5,4,9 were compared to group 1 (normal control) .$: Groups 5,6,7,8,12 were compared to group 4 (cancer control)& : Groups 10,11 were compared to group 9 (diabetic control).£ : Groups 15,14,15,16 were compared to group 12 (cancer diabetic control)

Table 2 Blood glucose levels and serum liver enzyme activities

Parameter \ Group	1 Normal Control	2 DAS Control	3 AA Control	4 Cancer (DENA) Control	5 DENA+ CP	6 DENA+CP+ DAS	7 DENA+CP+ AA	8 DENA+CP+ DAS+AA	9 Diabetic (STZ) Control	10 STZ+ DAS	11 STZ+AA	12 DENA+ STZ	13 DENA+ STZ+ CP	14 DENA+ STZ+ CP+DAS	15 DENA+ STZ+ CP+ AA	16 DENA+ STZ+ CP+ DAS+AA
Blood Glucose (mg/dl)	83.3±4.25	79.3±4.11	78.2±3.00	69.2±5.74*	75.3±3.06	76.6±0.96	74.3±3.87	78.6±1.82	331.9±7.61***	293.9±9.30&&&	290.3±7.17&&&	311.4±6.99$$$	306.7±5.17	259.7±9.08£££	250.5±5.48£££	195.1±7.48£££
ALT (IU/L)	41.5±3.49	39.0±1.50	40.7±2.91	137.1±4.07***	102.3±5.34***	91.4±2.30***	97.0±4.85***	78.7±4.47***	48.3±4.13	45.8±4.31	44.9±4.98	154.2±6.31$$$	120.1±6.31£££	99.0±6.98£££	101.0±3.49£££	90.6±5.53£££
AST (IU/L)	88.4±4.63	89.1±3.54	87.1±2.81	238.4±5.58***	184.3±5.53$$$	167.8±8.22$$$	169.3±6.79$$$	135.1±5.99$$$	100.3±5.99	84.8±4.62&	83.7±4.73&&	243.9±5.31$$$	197.5±4.90£££	174.6±5.11£££	173.4±5.35£££	157.5±6.89£££
GGT (IU/L)	19.5±1.90	19.6±2.34	19.8±1.98	80.7±3.20***	58.2±2.43$$$	48.6±4.88$$$	54.5±2.10$$$	43.3±3.79$$$	40.8±1.90***	36.9±3.05	39.5±3.76	113.9±7.02$$$	90.6±2.66£££	76.2±5.98£££	79.0±5.86£££	67.2±5.10£££

Data are expressed as X± SD of ten rats in each group.Significant difference between groups is analyzed by one-way ANOVA test, where: (*** $P < 0.001$: highly significant , ** $P < 0.01$: significant , * $P < 0.05$: mildly significant , ns $P > 0.05$: non significant).* : Groups 2,3,4,9 were compared to group 1 (normal control) .$: Groups 5,6,7,8,12 were compared to group 4 (cancer control).& : Groups 10,11 were compared to group 9 (diabetic control).£ : Groups 13,14,15,16 were compared to group 12 (cancer diabetic control)

Table 3 Serum alpha-fetoprotein, sorbitol and inflammatory markers

Parameter \ Group	1 Normal Control	2 DAS Control	3 AA Control	4 Cancer (DENA) Control	5 DENA+ CP	6 DENA+ CP+ DAS	7 DENA+ CP+ AA	8 DENA+ CP+ DAS+AA	9 Diabetic (STZ) Control	10 STZ+ DAS	11 STZ+AA	12 DENA+ STZ	13 DENA+ STZ+ CP	14 DENA+ STZ+ CP+DAS	15 DENA+ STZ+ CP+ AA	16 DENA+ STZ+ CP+ DAS+AA
AFP(ng/ml)	2.4±0.15	2.5±0.17	2.5±0.16	6.9±0.39***	5.1±0.21$$$	4.6±0.11$$$	4.4±0.16$$$	4.0±0.10$$$	2.3±0.15	2.4±0.13	2.6±0.17	8.5±0.26$$$	6.3±0.31£££	5.7±0.20£££	5.5±0.20£££	**5.0±0.15£££**
TNF-α(pg/ml)	16.0±0.68	14.3±1.02	16.1±1.06	83.8±2.77***	69.5±2.96$$$	61.7±3.92$$$	63.4±3.33$$$	48.7±2.29$$$	60.4±2.11***	48.4±4.51&&	54.0±4.71&	101.3±3.55$$$	78.5±2.16£££	69.5±2.67£££	71.6±4.40£££	**61.9±3.44£££**
IL-6(pg/ml)	41.5±2.38	37.5±3.06	38.5±3.10	134.0±6.71***	103.8±3.73$$$	92.7±3.47$$$	89.0±3.20$$$	70.7±3.90$$$	63.7±5.05***	52.0±5.14&	52.2±2.78&	151.6±4.15$$$	119.0±8.70£££	103.3±5.85£££	105.7±3.86£££	**85.0±4.98£££**
Sorbitol(nmole/ g hemo-globin)	16.1±0.42	16.2±1.04	14.4±1.05	67.1±3.43***	68.5±3.04	61.7±3.46$	48.8±3.88$$$	44.4±5.53$$$	100.5±4.71***	94.8±3.36&&&	79.1±2.41&&&	136.0±6.26$$$	138.5±8.63	117.9±6.99£££	102.1±5.33£££	**95.7±3.90£££**

Data are expressed as X± SD of ten rats in each group.Significant difference between groups is analyzed by one-way ANOVA test, where: (*** $P < 0.001$: highly significant , ** $P < 0.01$: significant , * $P < 0.05$: mildly significant , ns $P > 0.05$: non significant).* : Groups 2,3,4,9 were compared to group 1 (normal control).$: Groups 5,6,7,8,12 were compared to group 4 (cancer control).& : Groups 10,11 were compared to group 9 (diabetic control).£ : Groups 13,14,15,16 were compared to group 12 (cancer diabetic control). Significant difference between groups is analyzed by one-way ANOVA test, where: (*** $P < 0.001$: highly significant , ** $P < 0.01$: significant , * $P < 0.05$: mildly significant , ns $P > 0.05$: non significant).

DISCUSSION

Combining the strengths of several treatment modalities, is a proven tactic in fighting cancer. Certain dietary components may serve as potent agents for enhancing the therapeutic effects of chemotherapy, radiotherapy, or other standard therapeutics for the treatment of resistant cancers.[26, 31] In the present work, AA, DAS didn't show any histological changes in liver of normal rats, while induction of diabetes, more or less induced some inflammatory changes. The relative liver weight was not changed in these groups, but was increased in diabetes due to decreased body weight. Both ALT and AST were not changed, while GGT was significantly elevated, this might be attributed to diabetic induced inflammation that possibly affected biliary pathways. This could be confirmed by the increased values of TNF-α, IL-6 and ES contents among diabetic animals. However, induction of liver cancer by DENA manifested tumor tissue changes that were exaggerated by co-morbid diabetes induction. Relative liver weights were significantly elevated in both groups. Both ALT, AST and GGT activities, TNF-α, IL-6 and ES contents were significantly elevated in both groups, while blood glucose was only elevated in diabetized group. Bax/ Bcl2 ratio, which is an index for apoptotic potential of the hepatocyte was greatly depressed in DENA and more depressed when combined to STZ, indicating lower control of hepatocarcinogenesis. Recent observation showed that AA had little synergistic action to CP through modulation of Bax pathway.[32]

Disturbed serum transaminases in cancer group is in accordance with several studies using DENA-induced hepato-carcinognesis.[33] The increased serum GGT activities could reflect a persistent increase of oxidative and other types of cellular stresses, manifested by depressed glutathione maintenance within the cells.[34, 35]

Elevated AFP levels observed in DENA control group is still believed to be a hallmark for HCC development.[36,37]However, false-negative or -positive rate with AFP level may be as high as 40%, especially for early diagnosed small size HCC (<3 cm). It is sometimes very difficult to make the distinction between tumors and falsely elevated AFP levels because of benign liver diseases.[38, 39] This initiated our interest to look for another markers known to change clearly during cancer/diabetes or at least exaggerate carcinogenesis as diabetic co morbidity to HCC. Polyol profile (AR, SDH and ES) were recently observed to be key markers during hepatocarcinogenesis activated by diabetes.[40]

CP is one of the most potent antitumor agents, displaying clinical activity against a wide variety of solid tumors. [41] Its cytotoxic mode of action is mediated by an interaction with DNA to form DNA adducts, primarily intra-strand crosslink adducts, which activate several signal transduction pathways. [42-44] Apoptotic signals, however, can be attenuated, and the resistance that ensues is a major limitation of CP-based chemotherapy. [45, 46]

In the present study, CP alone or combined with AA and/or DAS showed apparent improvement in most of the histological manifestations observed after hepatocellular dysplasia induction, especially when AA and/or DAS were used in combination with CP. This results certainly means that these additions to CP could efficiently work as chemo-sensitizers to CP as powerful as therapeutics if given as combination. [40] AA was reported to elevate CP of tumor cells to CP through enhancing apoptosis by regulating p53. [17]

Biochemical results could clearly augment this observation, specially the decreased inflammatory infiltration and increased apoptotic index in AA+DAS+CP groups. These combinations also alleviated greatly the diabetic impact on HCC as mirrored in normalized poylol profile. Previous reports showed that patients with cancer have lower serum AA levels even when intakes are greater than controls. The oxidized form of AA (dehydroascorbic acid, DHA) may be an added stress, especially during chemotherapy. [47]

Polyol profile effectively contributed in both hepatocellular dysplasia and diabetes initiation. AR expression showed significant increase in cancer +/- diabetic rats. Groups treated with CP alone had no or mild effect while if preceded by AA +/- DAS, showed significant decrease in AR expression. SDH expression significantly increased in DENA and DENA diabetic groups. Diabetic control showed significant decrease in expression of SDH compared to normal control. Treatment with CP alone or in combination with AA +/- DAS caused no significant change to SDH protein expression in all treated groups, compared to their respective controls. It means that CP when given alone, or preceded by DAS plus AA may decrease ES content through suppressing AR activity, meaning, depressing sorbitol formation although its null effect on SDH means alternatively that this treatment fail in salvage of the de novo formed sorbitol.

Recent reports also demonstrate that DAS is a novel, selective and highly potent modulator of P-glycoprotein-mediated multidrug resistance (P-gp-MDR) in rodent liver. The MDR-reversing properties of DAS might also be attributed to its lipophilic nature and electrophilic sulfur atom. Cytochrome P450 enzyme-mediated oxidation at the sulfur atom of DAS

produces diallyl sulfoxide and diallyl sulfone. These metabolites are further converted to epoxide intermediates, responsible for this MDR modulatory effect. [48]

It was also noticed that both AA and DAS ameliorated serum levels of inflammatory markers (TNF-α, IL-6), thus offered an anti-inflammatory environment improving CP action. These agents may exert their chemo-sensitizing effect via regulation of NF-κB pathway. [49] It is believed that AA +/- DAS treatment may inactivate NF-κB, thus contributes to growth inhibition and apoptosis, since NF-κB-induced apoptosis resistance has been implicated in chemotherapeutic failures in cancer treatment. [50] AA +/- DAS significantly inhibited Bcl2 gene joined with activated Bax gene. They achieved higher Bax/Bcl2 ratio which reflected higher rate of apoptosis in cancer cells, suggesting that combination treatment may regulate important molecules in the apoptotic pathway. [51]

These naturally derived substances also caused an obvious AR inhibition with subsequent lowering in sorbitol levels, an effect which was not observed with CP alone. Several reports have shown that over-expression of AR in many tumor cells renders these cells resistant to chemotherapy and also demonstrate that inhibition of AR enhances sensitivity to chemotherapeutic drugs. [40, 52, 53]

CONCLUSION

Present data clearly demonstrate the chemo-sensitizing benefit of AA and DAS combination to CP therapy. These natural substances potentiated the effect of CP on almost all the parameters that could be affected in the course of hepatocarcinogenesis. They restored liver integrity and activated apoptosis in hepatocellular dysplasia, offering an anti-inflammatory micro environment which improved CP action. They can be used as adjuvant to enhance antitumor activity of conventional chemotherapeutic drugs with little or virtually no side-effects nor further increase in the medication burden on the patient. Their early use in the disease process may retard or prevent the appearance of resistant neoplastic clones. They helped in restoring better liver integrity by modulation of serum activities of ALT, AST, GGT and they also normalized serum AFP levels. These drug combinations can be recommended for future chemotherapeutic disciplines, although human studies still in queue.

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