

Curcumin / BSA: New approach for hepatocellular carcinoma treatment

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ABSTRACT

The systemic availability of curcumin is very low after oral administration; this limits their therapeutic potential. This study aims to increase the bioavailability of curcumin; the highest reproducible solubility modality will be applied on an experimental carcinogenesis models in order to evaluate its chemo-preventive, chemotherapeutic effects and antitumor potential. We found that administrating of curcumin (200 mg/kg I.P. bound to 5% BSA in PBS, pH 7.4) results in a significant inhibitory effect on tumor in vivo. An anti-oxidant effect and anti-tumor effect of curcumin in vivo was observed. A significant reduction in anti-oxidants and tumor markers levels in tumor treated animals when compared with untreated ones. As well as Bcl2 expression was reduced. Conclusion: curcumin bound BSA has a strong inhibitory activity against tumors. The anti-tumor mechanism may be mediated by preventing oxidative damage and induction of apoptosis improved animals' chances of survival and they become healthier.

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KEYWORDS

Curcumin;
Bovine serum albumin;
Apoptosis;
Hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC), the most common type of liver cancer, is the third leading cause of cancer deaths worldwide^[1]. It is one of the five most common cancers worldwide. HCC is a high-grade malignancy showing a rapid infiltrating growth, early stage metastasis, poor therapeutic response and disappointing prognosis even after successful curative resection surgery^[2]. Surgery, including transplantation, remains the only potentially curative modality for HCC, yet the recurrence rate for this particular cancer is high and long-term survival rate is rather poor^[3]. Cancer

chemoprevention is a rapidly growing area of oncology which can make a significant progress in the prevention and treatment of carcinogenesis by administration of various drugs with chemical or natural entities depending on their anti-mutagenic properties^[4]. Cancer chemoprevention is a rapidly growing area of oncology which can make a significant progress in the prevention and treatment of carcinogenesis by administration of various drugs with chemical or natural entities depending on their anti-mutagenic properties. It is, therefore, essential that new therapeutic options are needed for cancer therapy with attention to toxicity and side effects, besides the major treatment modalities including

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surgery, immunotherapy and radiotherapy^[5,6].

Curcumin (diferuloylmethane) is a major constituent of the yellow spice turmeric derived from the rhizomes of *Curcuma longa*. It is safe and nontoxic and has demonstrable antitumor, anti-inflammatory, apoptotic, and antioxidant properties. It was shown previously that curcumin inhibits tumor metastasis, invasion, and angiogenesis^[7-9]. Therefore, it is regarded as a high potential to develop into modern drug. Curcumin's chemo-preventive activity in animal model systems has led investigators to study its potential impact upon tumor cell growth and apoptosis. Several reports document an anti-proliferative effect on cultured cells such as on colon cancer and breast cancer cells. This may, in part, be because of programmed cell death because at high concentrations curcumin can induce apoptosis such as in human leukemia cells^[10]. Curcumin acts as a scavenger of oxygen species, such as hydroxyl radical, superoxide anion, and singlet oxygen, and it interferes with lipid per-oxidation. Curcumin possibly used in the prevention and treatment of cancer^[11-12]. Curcumin suppresses a number of key elements in cellular signal transduction pathways pertinent to growth, differentiation, and malignant transformation^[13]. Unfortunately, the solubility of curcuminoids in aqueous solutions is exceedingly low. This restricts their systemic availability in orally administered formulations and limits their therapeutic potential^[14] because most of the curcumin is metabolized in the intestine.

Several studies recently confirm the high affinity nature of curcumin binding to Bovine Serum Albumin (BSA)^[15]. Curcuminoids were highly soluble in solutions of purified albumin, a major component of serum. Solid curcuminoids would thus allow only limited surface access of the solvent to disrupt the defined structures. At saturating BSA concentrations (14–20%), the amount of DMSO-dissolved curcuminoids converted into BSA solubilized curcuminoids was about 92–96%. At pH 7.0, the protein molecule acquired more negative charges, which in turn help expand the protein a bit further allowing the binding of more dye molecule (curcumin) without altering the affinity^[16]. Hence, the concentrations at saturation obtained by adding either solid or DMSO dissolved curcuminoids to 5% BSA were about two-fold higher. So, these promising novel formulations, which appear to provide longer circula-

tion, better permeability, and resistance to metabolic processes^[17]. Curcumin bio-conjugates containing albumin improve medicinal properties over curcumin, suggesting increased cellular uptake.

MATERIALS AND METHODS

Tumors

Hepatocellular carcinoma (HCC) was induced by an intraperitoneal injection of N-diethylnitrosamine "DENA" (200 mg/kg; Sigma Chemical Company)^[18] dissolved in saline.

Curcumin

Crude curcumin was obtained from Fluka, Buchs, Switzerland, was dissolved in dimethylsulphoxide (DMSO), and then dissolved in 5% (Bovine Serum Albumin (BSA) in Phosphate Buffer Saline (PBS); pH 7.4 solution used during the treatment to increase the bioavailability of curcumin.

Experimental design

Albino male Wister rats' 190-200 g body weights were raised at the experimental animal house of the faculty of Science, Zagazig University. The animals were maintained in controlled environment of temperature, humidity and light. They were fed on a commercial standard diet and tap water. Rats were divided according to their weight into four groups, each one includes 8 rats. Group I, were received a saline solution by I.P. injection represented as a negative control group; Group II, were received DENA (200 mg/kg I.P.) served as a positive control group. Group III, were injected I.P. with curcumin one week before DENA (tumor induction), represented as preventive group, Group IV, were injected I.P. DENA, represented a therapeutic group, then curcumin was injected every week for five weeks to the last groups. The rats of four groups were maintained under the same conditions and were observed carefully everyday to the end of experiment. At the end of the experiment, the blood samples and liver tissues were collected from animals for biochemical and histological assays.

Biochemical analyses

a) Estimation of α -L- fucosidase in serum

Serum α -L- Fucosidase (AFU) activity was deter-

mined by using Biodiagnostic kit method (Biodiagnostic Company, Dokki, Giza, Egypt). The AFU assay is based on the enzymatic cleavage of the synthetic substrate p-nitrophenol and L-Fucose. The yellow color of p-nitrophenol in an alkaline medium can be measured at 405 nm^[19].

b) Anti-oxidants assays

(i) Estimation of nitric oxide in serum

In acid medium and in the presence of nitrite the formed nitrous acid diazotise sulphanilamide and the product are coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish-purple color which can be measured at 540 nm in a spectrophotometer^[20].

(ii) Estimation of malondialdehyde in serum

The lipid peroxidation products were estimated by the formation of thiobarbituric acid (TBA) and quantified in term of MDA where, thiobarbituric acid (TBA) reacts with MDA in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product, the absorbance of the resultant pink product can be measured at 534 nm in a spectrophotometer^[21].

(iii) Estimation of glutathione reduced in erythrocyte lysate

Glutathione reduced (GSH) level was determined by using Biodiagnostic kit method. This method based on the reduction of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound can be measured at 405 nm. The reduced chromogen is directly proportional to GSH concentration^[22].

(iv) Estimation of glutathione-S-transferase in plasma

Glutathione-S-Transferase (GST) level was determined by using Biodiagnostic kit method. This bio-diagnostic GST assay measure total GST activity (cytosolic and microsomal) by measuring the conjugation of 1-chloro-2,4- dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase the absorbance at 340 nm (UV method). The rate of increase the conjugation is directly proportional to GST activity in the sample^[23].

(v) Estimation of total antioxidant capacity in plasma

Total Antioxidant Capacity (TAC) level was determined by using Biodiagnostic kit method. The determination of the antioxidative capacity is performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide (H₂O₂). The antioxidants in the sample eliminate a certain amount of provided H₂O₂. The residual H₂O₂ is determined colorimetrically by an enzymatic reaction which involves the conversion of 3, 5-dichloro-2-hydroxybenzensulphate to a colored product can be measured at 505 nm^[24].

Histological study

Liver specimen was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. After de-paraffinization and dehydration, the paraffin blocks were stained with hematoxylin and eosin for microscopic examination. Light microscopy was used to evaluate the pathological changes in the liver tissues.

Chromosomal aberration

After treatment, the animals of all groups were sacrificed at the sampling time of 24 h by cervical dislocation (colchicine was given at a dose 4 mg/kg of the body weight, 2 hrs prior to killing to arrest the metaphase stage). Cytogenetic analysis was performed as per protocol of Preston *et al.*,^[25]. The bone marrow was flushed out from both femurs using Hank's balanced salt solution (pH 7.2). The cells were centrifuged at 1000 rpm for 5 min. and the pellet was re-dispersed in hypotonic solution of 0.56% (w/v) KCl for 30 min. at 37°C to permit osmotic swelling of cells. Swollen cells were fixed in ice- cold Carnoy's fluid, dropped to slides and stained with phosphate-buffered 5% Giemsa solution. A total of 75 well- spread metaphase plates per animal in each group were analyzed for chromosomal aberrations at a magnification of 100×.

Statistical analysis

All results were analyzed by SPSS software (version 14). Data were expressed as mean ± SD. The student's t test was used for statistical analysis of differences between each two groups. Comparison of mean values of studied variables among different groups was

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done using ANOVA test. Pearson's correlation coefficient was used to quantify the relationship between the studied parameters. $P < 0.01$ was considered to be significant^[26].

RESULTS

Data obtained from TABLE (I) summarize the anti-tumor and anti-oxidant effects of Curcumin as a chemopreventive natural product.

Effect of curcumin on AFU activity

A marked significant increase in AFU activity and oxidative stress in DENA induced HCC "group III" compared to negative control group "I". Anti-tumor effect of Curcumin showed a significant decrease in the activity of AFU enzyme by 35.3% and 38.7% in therapeutic and preventive groups, respectively; compared to HCC group ($p < 0.05$) as represent in Figure (1).

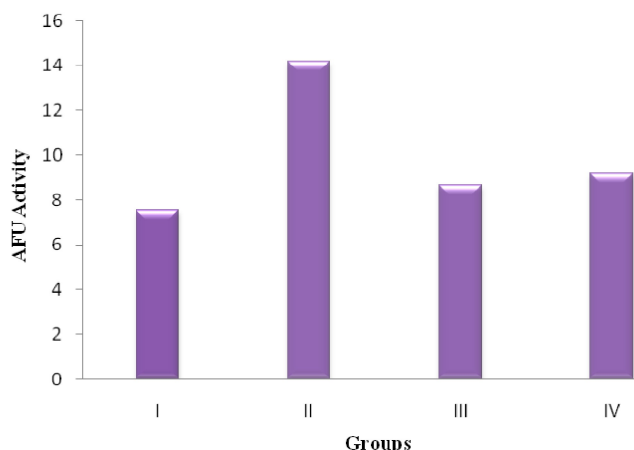


Figure 1 : Mean Difference of AFU activity in the studied groups

Effect of curcumin on oxidative stress

MDA was significantly decreased by 41.6% and 44.8% in therapeutic and preventive groups, respectively. NO was significantly reduced by 36.1% and 37.7% in therapeutic and preventive groups, respectively; ($p < 0.05$), show Figure (2).

Also, there were a significant decrease in the GST, GSH, and TAC levels in both therapeutic and preventive groups (43.4%, 33.3%; 18.6%, 14.9%; 27.03%, and 27.9%, respectively) compared to HCC group ($p < 0.05$), Figure (2).

Effect of curcumin on liver sections

Histopathological examination of liver sections from control animals revealed normal architecture. While, DENA group showed appearance of Hepatocellular carcinoma with enlarged hyper-chromatic nuclei and scattered mitosis, distorted architecture with moderate to dense inflammation represented by the presence of inflammatory cells and increase number of leukocyte cells and ballooning degeneration of hepatocytes. This results in irregular arrangement of hepatocytes with dilated central blood sinusoids. Nevertheless, Curcumin administration as a treatment, induce mild liver affection either before or after treatment; i.e. Preventive IV and Therapeutic III Groups"; the hepatocytes and sinusoid showed normal morphology, reflecting a well preserved liver parenchyma (H & E, $\times 200$). Curcumin alleviated the DENA induced alterations seen in the above group, reduction of inflammation and re-arrangement of hepatocytes as normal case (Figure 3 A, B, C and D).

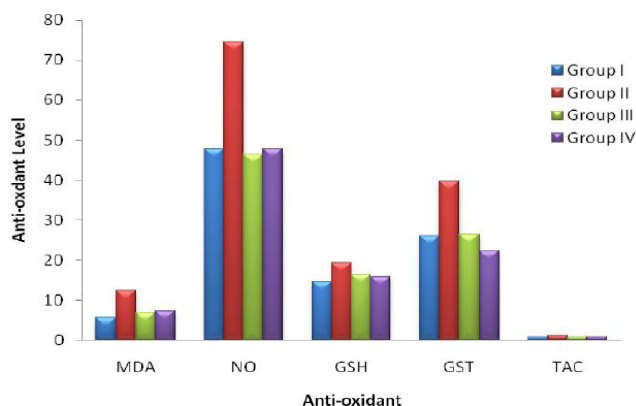


Figure 2 : Mean Difference of Anti-Oxidants levels in the studied groups

Effect of curcumin on chromosomal aberrations

TABLE (II) shows the effect of Curcumin on the chromosomal aberrations. The bone marrow cells of the control groups showed normal metaphase spread having 42 chromosomes (Figure 4). While structural and numerical aberrations were found in HCC groups (rats treated with DENA alone). The data revealed that, the endoreduplication break, gap, deletion, ring chromosomes, and dicentric chromosomes were the most frequent types of structural chromosomal aberrations (97%, 95%, 92%, and 93%, respectively) (Figure 5). But the numerical aberrations showed up in the form of

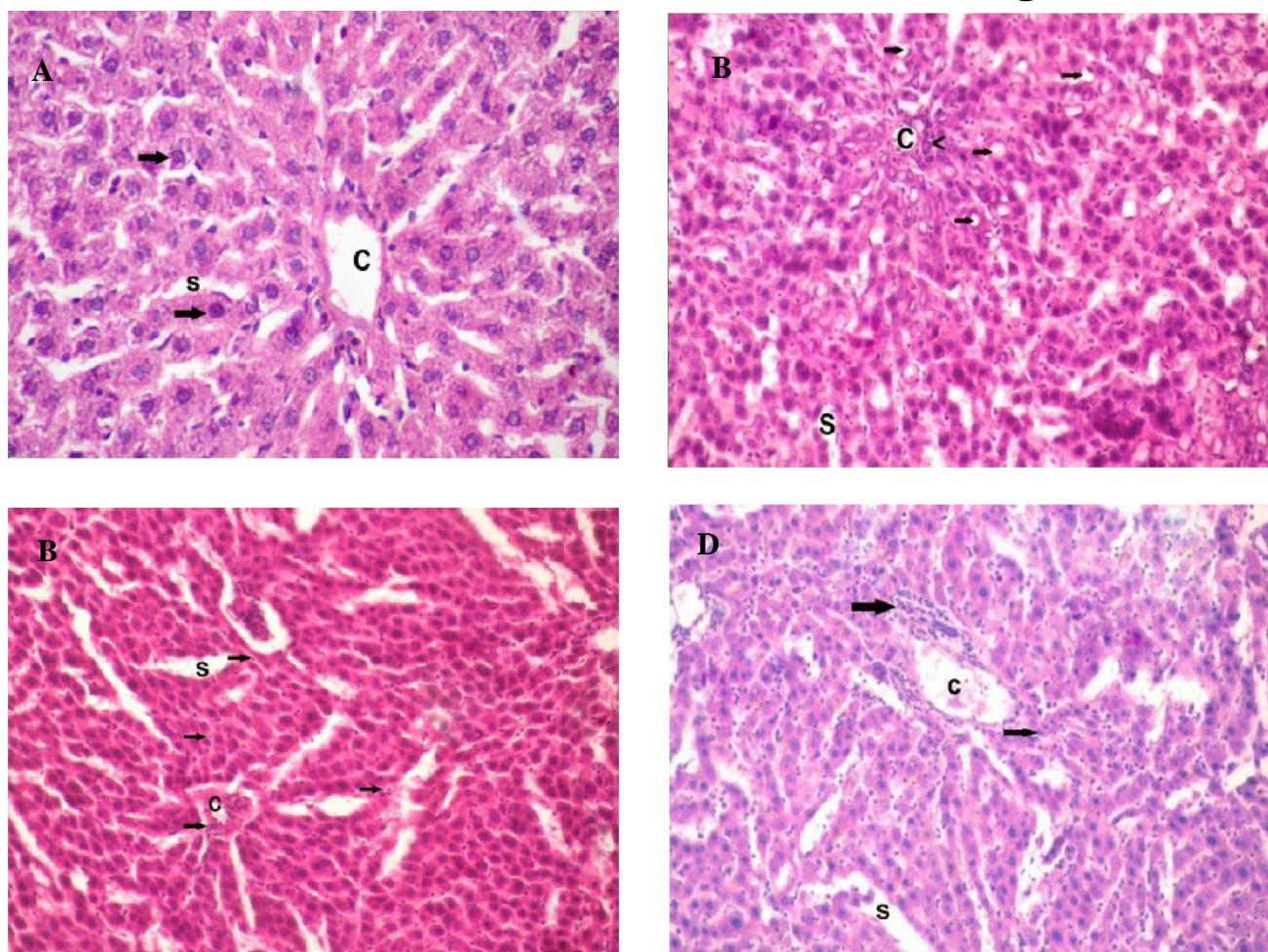


Figure 3 : Light photomicrograph of rat liver in the studied groups Hx & E X200. (A): photomicrograph of Negative Control “Group I” showing classical hepatic lobule consisting of hepatocytes with light stained nucleus and prominent nucleolus (arrow) and liver sinusoids (s) radiated from the central vein (C). (B): photomicrograph of Positive Control “Group II” showing classical hepatic lobule with many leukocyte cells (arrow head). Many hepatocytes show vacuolated cytoplasm with peripheral condensed nuclei (arrow) as signs of apoptosis. Note irregular arrangement of hepatocytes and dilated blood sinusoids (s). (C)& (D): photomicrograph of Treated Groups (Group III and Group IV) showing classical hepatic lobule small central vein (c) surrounded with many inflammatory cells (arrow). Note Hepatocytes were re-arranged again as normal.

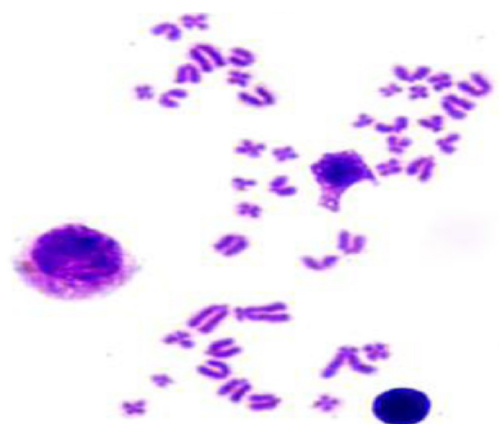


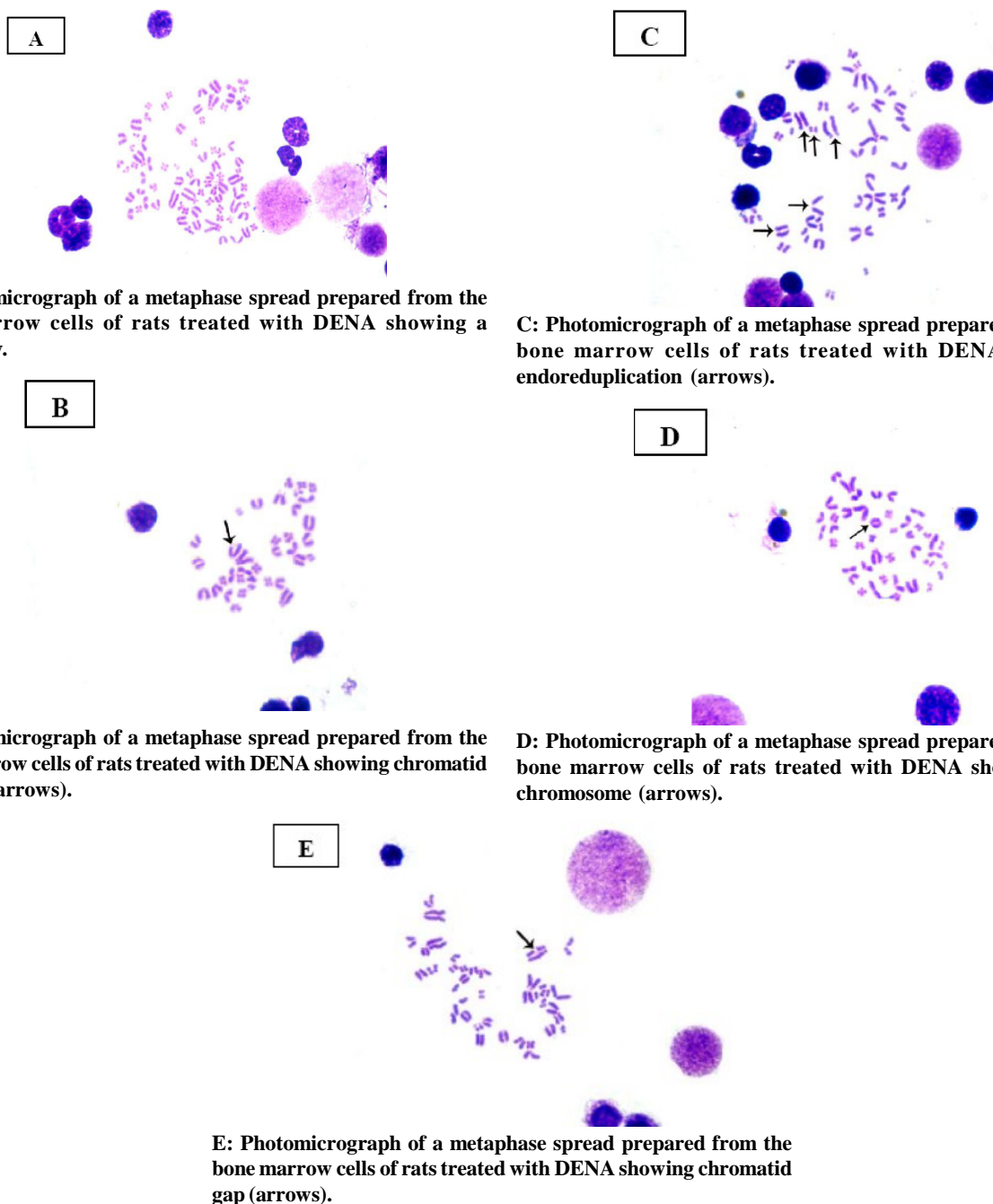
Figure 4 : Photograph showing metaphases plate from bone marrow cells of control rats (Negative Control Group I) showing 42 chromosome of normal shape.

aneuploidy and polyploidy, 87% and 95%, respectively. Moreover, the number of aberrations per cell reduced by Curcumin administration, significantly by 85.1% and 90.8% in therapeutic (III) and preventive (IV) groups, respectively (Figure 6).

Correlations between different studied parameters among different groups

TABLE (II) summarizes the correlations between different parameters. In studied groups, there were positive significant correlations between serum AFU and MDA, NO, GST, GSH, and TAC; respectively ($r=0.915$; $r=0.914$; $r=0.838$; $r=0.921$; $r=0.930$, and $r=0.648$, $p<0.01$; respectively). Also, there were posi-

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A: Photomicrograph of a metaphase spread prepared from the bone marrow cells of rats treated with DENA showing a polyploidy.

C: Photomicrograph of a metaphase spread prepared from the bone marrow cells of rats treated with DENA showing endoreduplication (arrows).

B: Photomicrograph of a metaphase spread prepared from the bone marrow cells of rats treated with DENA showing chromatid deletion (arrows).

D: Photomicrograph of a metaphase spread prepared from the bone marrow cells of rats treated with DENA showing ring chromosome (arrows).

E: Photomicrograph of a metaphase spread prepared from the bone marrow cells of rats treated with DENA showing chromatid gap (arrows).

Figure 5 : Photomicrographs of a metaphase spread prepared from the bone marrow cells of rats treated with DENA “Positive control group II” showing numerical (A) and structural (B, C, D, E) chromosomal aberrations.

tive significant correlations between serum MDA and NO, GST, GSH, and TAC ($r=0.938$; $r=0.862$, $r=0.939$, $r=0.960$, $p<0.01$; respectively) in the studied groups. There were positive significant correlations between serum NO and GST, GSH, and TAC ($r=0.908$; $r=0.940$, $r=0.976$, $p<0.01$; respectively). Our data illustrated positive correlations between GST and GSH, TAC ($r=0.874$, $r=0.938$, $p<0.01$; respectively).

DISCUSSION

Curcumin (diferuloylmethane) a low molecular weight polyphenol derived from the rhizomes of *Curcuma spp.*, has been shown to prevent cancer in the skin, fore-stomach, duodenum, and colon of mice and in the tongue, colon, mammary glands, and sebaceous

TABLE 1 : Anti-tumor and Anti-oxidant activities of curcumin in different studied groups

Parameter		Group I	Group II	Group III	Group IV
AFU (U/L)	$M \pm S.D$	7.55 \pm 0.28	14.17 \pm 1.64	8.68 \pm 0.64**	9.17 \pm 0.72**
	% Change	-----	-----	35.29	38.74
MDA (nmol/ml)	$M \pm S.D.$	5.62 \pm 0.05	12.35 \pm 0.48	6.81 \pm 1.04**	7.21 \pm 0.58**
	% Change	-----	-----	41.62	44.86
NO (μ mol/l)	$M \pm S.D.$	47.78 \pm 0.63	74.63 \pm 1.29	46.48 \pm 1.25**	47.67 \pm 1.31**
	% Change	-----	-----	36.12	47.67 \pm 1.31**
GSH (mg/dl)	$M \pm S.D.$	14.69 \pm 0.24	19.42 \pm 0.14	16.51 \pm 0.12**	15.79 \pm 0.09**
	% Change	-----	-----	18.69	14.98
GST (U/L)	$M \pm S.D.$	26.07 \pm 0.72	39.67 \pm 2.45	26.44 \pm 1.56**	22.44 \pm 1.01**
	% Change	-----	-----	43.43	33.35
TAC (mM/L)	$M \pm S.D.$	0.78 \pm 0.01	1.11 \pm 0.04	0.80 \pm 0.02**	0.81 \pm 0.02**
	% Change	-----	-----	27.03	27.93

The Significant difference: $P^{**} < 0.01 \rightarrow$ high significant $P^* < 0.05 \rightarrow$ significant

TABLE 2 : Anti-clastogenic effects of curcumin on the total chromosomal aberrations induced by diethylnitrosamine in rat bone marrow cells in different studied groups:

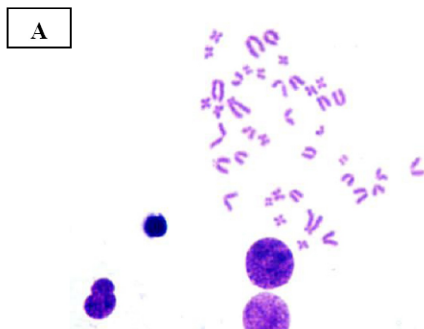
Examined groups	Total ex. cells	Structural aberrations					Numerical aberrations			Total aberrations
		Break	Gap	Deletion	Centro atten.	Ring chro.	Dicentric	Polyploidy	Aneuploidy	
Negative control group	400	2	2	3	1	2	2	1	2	15
% Change		--	--	--	--	--	--	--	--	--
Positive control group	400	70	40	40	55	25	30	20	15	295
% Change		97	95	92.5	98	92	93.3	95	86.7	94.9
Preventive group	400	10	18	7	2	00	2	4	1	44
% Change		85.71	55	82.5	96.36	100	93.33	80	93.33	85.1
Therapeutic group	400	8	7	3	4	2	0	1	2	27
% Change		88.57	82.5	92.5	92.73	92	100	95	86.66	90.85

The Significant difference: $P^{**} < 0.01 \rightarrow$ high significant $P^* < 0.05 \rightarrow$ significant

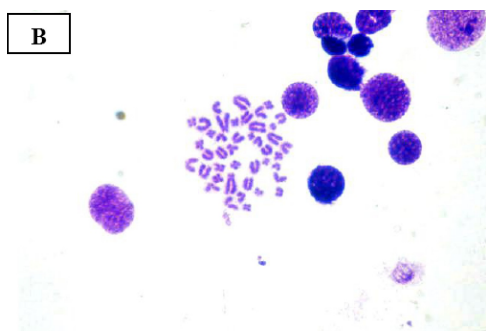
glands of rats^[27]. It is well known that the systemic availability of Curcumin is very low after oral administration, because most of Curcumin is metabolized in the intestine^[28]. The anti-tumor effect of Curcumin has been attributed in part to the suppression of cell proliferation, reduction of tumor load and induction of apoptosis in various cancer models both *in vitro* and *in vivo*^[29]. Curcumin inhibits multiple levels within transcriptional network to restrict cell proliferation. We found a marked significant increase in AFU activity in DENA induced HCC "group III" compared to negative control group "I". Anti-tumor effect of Curcumin/BSA showed a significant decrease in the activity of AFU enzyme by 35.3% and 38.7% in therapeutic and preventive groups, respectively; compared to HCC group ($p < 0.05$) as

shown in Figure (1). In addition, our results revealed that, Curcumin administration (200 mg/kg b.w; 5% BSA, I.P.) decrease the oxidative stress induced by DENA in HCC group; as illustrated in TABLE (1). Levels of MDA, NO, GST, GSH, and TAC were significantly decrease in therapeutic and preventive groups compared to HCC group (41.6%, 44.8%; 36.1%, 37.7%; 43.4%, 33.3%; 18.6%, 14.9%, and 27.0%, 27.9%, $p < 0.01$; respectively) as shown in Figure (2). Diethylnitrosamine (DENA) has been suggested to cause oxidative stress and cellular injury due to enhanced formation of free radicals^[30]. It is metabolized to its reactive ethyl radical metabolites that interact with DNA causing mutation, which lead to carcinogenesis^[31]. In hepato-carcinogenesis, fucosylation of some sugar

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A: Photomicrograph of a metaphase spread prepared from the bone marrow cells of rats take DENA that induced HCC firstly, and then take treatment showing normal count and shape of all chromosomes.



B: Photomicrograph of a metaphase spread prepared from the bone marrow cells of rats take curcumin followed by DENA that induced HCC, and then take treatment showing a normal count and shape.

Figure 6: Photomicrographs of a metaphase spread prepared from the bone marrow cells of rats treated with Curcumin "Treated groups". (A): Therapeutic group III; B: Preventive group IV.

proteins increase and this increase may lead to the elevation of AFU activity^[32]. As Curcumin has potential in the prevention and treatment of cancer^[33], protects from liver injury^[34]. Curcumin is an effective scavenger of free radicals such as superoxide anion radicals, hydroxyl radicals and nitrogen dioxide radicals^[35]. So, Curcumin may reduce the toxicity of heterocyclic aromatic amines; and inhibit the ability of nitrosamines to cause liver cancer. Our results are in agreement with Monica *et al.*^[36], who concluded that Curcumin exerted cell growth and apoptotic effects to free radicals generation in hepatic cancer cell line. Also, we are in accordance to Priyadarsini *et al.*,^[37] who found the anti-oxidant activity of Curcumin that inhibit lipid peroxidation in rat liver. Chuang *et al.*,^[38] results indicate that Curcumin effectively inhibits DENA induced hepatocarcinogenesis; as a series of intermediate biological markers showed a remarkable increase in HCC

but, eating Curcumin containing diet reversed the levels to normal values. Our biochemical findings were further supported by the histopathological examination of liver sections, which illustrated that liver tissue of DENA treated rats showed damage, resulting in malignant cell formation. On the contrary, liver tissues of Curcumin treated rats showed more or less normal hepatic lobular architecture in both therapeutic and preventive groups, as illustrated in Figure (3 A, B, C, and D). Administration of DENA produce high significantly chromosomal aberrations 94.9% compared to control group "I" as shown in Figure (4). The data revealed that, the numerical aberrations showed up in the form of aneuploidy and polyploidy (86.7%, 95%, respectively). The centromeric attenuation, endoreduplication break, gap, and deletion were the most frequent types of structural chromosomal aberrations (98%, 97%, 95%, and 92%, respectively), while other types of damage came next in descending values, ring and dicentric chromosomes (92% and 93.3%, respectively) as shown in Figure (5). But Curcumin administration weekly before and after HCC induction was significantly reduced the chromosomal aberrations (numerical and structural) by 85.1% and 90.8% in both therapeutic and preventive groups; respectively, as shown in Figure (6). This reduction effect of Curcumin may be due to the anti-mutagenic potential that is related to anti-oxidant and anti-carcinogenic activity of Curcumin^[39]. We agreed with Irulappan and Natarajain^[40], who found the total number of aberrations was significantly reduced in root tip cells pretreated with Curcumin.

CONCLUSION

Finally, it could be concluded that our in vivo studies provide a support for the hypothesis of the anti-apoptosis and strong anti-oxidative property of curcumin. The ability of curcumin to induce apoptosis in cancer cells without cytotoxic effects on healthy cells.

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