BENHA VETERINARY MEDICAL JOURNAL, VOL. 30, NO. 1:124-136,

Biochemical Studies on Evaluation of New Composite against Hepatocellular carcinoma-induced in Rats: Recent Therapeutic Approaches

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A B S T R A C T

The hepatoprotective effect of some natural and synthetic compounds against chemically induced hepatocellular carcinoma in rats was evaluated. One hundred male albino rats were divided equally into five groups. Normal control group, carcinogenic [Ferric nitrilotriacetate (Fe-NTA: 9 mg Fe/kg b.wt. i.p.) and chloroform (150 mg/ kg b.wt. orally)] - induced group, curcumin group (400 mg/kg. b.wt. orally), tetrachlorocuprate-lysine (25 mg/kg. b. wt. s.c.) and ascorbate (500 mg/kg. b. wt. orally) group and a mixture group (composed of curcumin, tetrachlorocuprate -lysine and ascorbate). Blood samples and liver tissue specimens were collected at the end of experiment (4 months) for determination of the following parameters: Tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), catalase and myeloperoxidase (MPO) in liver tissues, in addition to serum malondialdehyde (MDA) and reduced glutathione (GSH). Moreover, histopathological examination of liver tissues was done for results confirmation. The obtained results showed a significant elevation in MDA, MPO and immunological markers levels, with significant reduction in serum reduced glutathione and catalase activity in liver tissue in hepatocellular-carcinogen induced rats as compared to the control group. However, administration of rats with the compounds under investigation resulted in a significant reduction of MDA, MPO and immunological markers levels, and increased in reduced glutathione and catalase levels compared to the carcinogenic non treated group. Various pathological alterations were observed in liver of chemically induced-carcinogenic group interestingly, results supported the protective effect of the compounds under investigation and preserved the histological structures of liver tissues. These results concluded that basic curcumin, tetrachlorocuprate-lysine and ascorbate exert chemopreventative effect against hepatocellular carcinoma.

Keywords: Ferric nitrilotriacetate, curcumin, antioxidant, hepatocellular carcinogen.

(http://www.bvmj.bu.edu.eg) **(BVMJ-30(1): 124-136, 2016)**

1. INTRODUCTION

Hepatocellular carcinoma (HCC), also called malignant hepatoma, is the most common type of liver cancer. Most cases of HCC are secondary to either a viral hepatitis infection (hepatitis B or C) or cirrhosis (alcoholism being the most common cause of liver cirrhosis (Kumar et al., 2003). Ferric nitrilotriacetate (Fe-NTA) is one such environmental toxicant, a known substitute for pyrophosphate used in various kinds of detergents, Fe-NTA induces lipid peroxidation (LPO) which in turn generates oxidative stress and reactive oxygen species (ROS), also produces oxidative modifications in deoxyribonucleic acid (Iqbal et al., 1999). Fe-NTA plays an essential and fundamental role in nephrotoxicity and tumorigenesis (Kaur et al., 2007). Chloroform is an organic compound with formula CHCl3. It is one of the four chloromethanes (Rossberg et al., 2006). Chloroform may be released to the air as a result of its formation in the

chlorination of drinking water, wastewater and swimming pools. Other sources include pulp and paper mills, hazardous waste sites, and sanitary landfills. The major effect from acute (short-term) inhalation exposure to chloroform is central nervous system depression. Also, chloroform has been shown to be carcinogenic in animals after oral exposure, resulting in an increase in kidney and liver tumors (Agency for Toxic Substances,1997). Curcumin is the principal curcuminoid of the popular Indian curry spice turmeric, which is a member of the ginger family (Zingiberaceae). Curcumin can exist in at least two tautomeric forms, keto and enol. The enol form is more energetically stable in the solid phase and in solution. Akram et al., (2010) have suggested that in vitro and animal studies, the curcumin may have antitumor, antioxidant, antiarthritic. Curcumin inhibited the recruitment of RNA polymerase II to viral DNA, thus inhibiting the transcription of the viral DNA. Curcumin acts as a free radical scavenger and antioxidant, inhibiting lipid peroxidation and oxidative DNA damage. Curcuminoids induce glutathione S-transferase and are potent inhibitors of cytochrome P450. Ascorbic acid is a naturally occurring organic compound with antioxidant properties. It is a white solid, but impure samples can appear yellowish. It dissolves well in water to give mildly acidic solutions. Ascorbic acid is one form of vitamin C (Lachapelle et al., 2013). Tetrachlorocuprate, Cuprate loosely refers to a material that can be viewed as containing copper anions. Examples include tetrachlorocuprate ([CuCl4]2−) (Potassium Cuprate (III)" in Handbook of Preparative Inorganic Chemistry). Copper is a component of various intracellular and extracellular enzymes such as cytochrome oxidase, lysyl oxidase, ceruloplasmin and superoxide dismutase (Klasing et al., 1998). Copper complexes are often toxic to cells, therefore tumor cells were killed, while normal cells in the whole body remained alive for the lower level of copper (Daniel et al., 2005).

2. MATERIAL AND METHODS

2.1. Chemical and Reagents

Nitrilotriacetate was obtained from Thermo Fisher Scientific Inc- England and Ferric Nitrate from El-Nasr chemical company- Egypt.

2.1.1. Preparation of Fe-NTA solution:

Fe-NTA was prepared freshly and immediately before its use. To prepare Fe-NTA, ferric nitrate solution was mixed with four-fold molar excess of disodium salt of NTA and the pH is adjusted to 7.4 with sodium bicarbonate solution (Awai et al., 1979). Chloroform was obtained from Thermo Fisher Scientific Inc- England. Ascorbic Acid was obtained from TS laboratory company- Egypt. Curcumin was obtained from Loba Chemie Company – India and mixed with Sodium bicarbonate at a concentration of (1:4) to form basic curcumin. Tetrachloro cupprate and Lysine were obtained from Sigma Aldrich-USA.

2.1.2. Preparation of Bis (L-Lysine-Tetrachlorocuprate):

L-Lysine HCl and CuCl2 in a 2:1 molar ratio were mixed together and grinded in an agate mortar for about 30 minutes at room temperature to get the best homogeneity. The mixture was gradually turned to light green indicating the formation of Tetrachlorocuprate complex (Adams et al., 2010).

2.2. Animals and Grouping

This work was carried out on 100 male albino rats, weighting 40-60gm., purchased from the animal house colony of the National Cancer Institute (NCI), Cairo University, Egypt. Animals were housed under normal environmental conditions of standard temperature, humidity and diurnal environment of light and dark and fed a standard diet, which composed of (24% proteins, 5.55% fibers, 5.5% ash) and drink tap water ad libitum. Rats were

divided into five main equal groups, 20 rats each and classified as follows: Group (1): Rats were fed standard food and drinking tap water ad-libitum for 4 months and served as control group. Group (2): Rats were given intraperitoneal injection of Fe-NTA (9 mg Fe/kg body weight) (Summya et al., 2013) and chloroform (150 mg/ kg b.wt.) dissolved in corn oil, orally (Afrah et al., 2014) for 4 months. Group (3): Rats were administered orally with basic curcumin (400 mg/kg) (Yumei et al., 2008) half an hour prior to Fe-NTA and chloroform administration and continued till the end of experiment (4 months). Group (4): Rats were received Bis (tetraclorocuprate- Lysine) dissolved in ethyloacetate (25 mg/kg. b. wt. s.c.) (Frechilla et al., 1999) and ascorbate (500 mg/kg. b. wt.) (Adejuwon et al., 2008) orally and daily half an hour prior to the administration of Fe-NTA and Chloroform till the end of 4 months. Group (5): Rats treated daily with Bis (L- Lysine) tetrachlorocuprate combined with ascorbate and basic curcumin half an hour prior to the administration of Fe-NTA and chloroform till the end of 4 months.

2.3. Sampling

At the end of the experiment (4 months), all animals were sacrificed, blood samples were collected and serum was separated by centrifugation at 2500 r.p.m for 15 minutes, the clean clear serum was separated by Pasteur pipette and kept in a deep freeze at -20 ºc until used for biochemical analysis. Also, livers tissue specimens of the experimental animal's groups will be quickly removed**,** perfuse immediately with ice-cold saline (0.9%w/u) and homogenized (Glas-Col, Terr Hauter, USA) in chilled phosphate buffer (0.1 M, pH 7.4) containing Kcl (1.17%w/u). The homogenate was centrifuged at 3000 rpm for 10 min at 4ºc and clear supernatants were used for biochemical analysis. Additionally, a portion of freshly excised rat liver, of all groups, was dissected and immediately fixed in 8% phosphate buffered formalin and used for histopathological examination.

2.4. Biochemical analysis:

Determination of MPO, MDA, GSH and CAT were analyzed according to the methods described by (Joris et al., 2006), (Ohkawa et al., 1979), (Beutler et al., 1963) and (Aebi, 1984 & Fossati et al., 1980) respectively. Determination of TNF-α and IL-1 using ELISA kit according to the methods described by (Chen et al., 1998) and (Liu et al., 1995) respectively.

2.5. Statistical Analysis

All data were expressed as the mean ± SD. Statistical comparison between different groups were done by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparison (Graphpad Prism version 5.03, San Diego, CA, USA). $P< 0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Effect of basic curcumin, tetrachloro-cuprate-lysine and ascorbic acid on the MDA, GSH, MPO and catalase activity:

The obtained data in table (1) revealed that, the mean levels of the MDA of the carcinogen-treated group were (6.22±0.58), which showed a significant increase compared to the mean levels of the control group (2.35 ± 0.19) at the same period of treatment ($p \leq 0.05$). Whereas the mean levels of the MDA were (3.79±0.14) in curcumin-treated group, (3.46±0.12) in tetrachlorocuprate-lysine+ascorbic acid-treated group and **(**3.12±0.02) in the mixture-treated group at the same period of treatment ($p \leq 0.05$) which showed a significant decrease compared to the mean levels of the carcinogen-treated group.

The mean levels of the reduced glutathione of the carcinogen-treated group were (14.67±1.11), which showed a significant decrease compared to the mean levels of the control group (29.37± 0.99) at the same period of treatment ($p \leq 0.05$). Whereas the mean levels of the GSH were (24.13±0.7) in

curcumin-treated group, (27.88±0.61) in tetrachlorocuprate-lysine+ascorbic acid-treated group and (29.50±0.60) in the mixture-treated group at the same period of treatment ($p \leq 0.05$) which showed a significant increase compared to the mean levels of the carcinogen-treated group, table (1). The obtained data in table (1) showed that, the mean levels of the MPO of the carcinogen-treated group were (1.79±0.07), which indicated a significant elevation compared to the mean levels of the control group (0.22± 0.01) at the same period of treatment ($p \leq 0.05$). Whereas the mean levels of the MPO were (0.84±0.06) in curcumin-treated group, (0.82±0.05) in tetrachlorocuprate-lysine+ascorbic acid-treated group and (0.82±0.05) in the mixture-treated group at the same period of treatment ($p \leq 0.05$) which resulted in a significant reduction compared to the mean levels of the carcinogen-treated group. The obtained data in table (1) showed that, the mean levels of the catalase of the carcinogen-treated group were (70.53 ± 3.119), which indicated a significant reduction compared to the mean levels of the control group (122.5±1.76) at the same period of treatment ($p \leq 0.05$). Whereas the mean levels of the Catalase were (96.35± 1.85) in curcumin-treated group, (101.0 ± 4.14) in tetrachlorocuprate-lysine+ascorbic acid-treated group and (111.2± 1.88) in the mixture-treated group at the same period of treatment ($p \leq 0.05$) which resulted in a significant reduction compared to the mean levels of the carcinogen-treated group.

3.2. Effect of the different compounds on tissue Interleukin-1 and TNF- α activity:

The obtained data in table (2) revealed that, the mean levels of the Interleukin-1 of the carcinogen-treated group were (113.5±1.21), which showed a significant increase compared to the mean levels of the control group (30.89± 1.011) at the same period of treatment ($p \leq 0.05$). Whereas the mean levels of the Interleukin-1 were (86.50±1.97) in curcumin-treated group, (77.10±1.41) in tetrachlorocuprate- lysine + ascorbic acid-treated group and (61.52±3.93) in the mixture-treated group at the same period of treatment ($p \leq 0.05$) which showed a significant decrease compared to the mean levels of the carcinogen-treated group. The mean levels of the TNF- α of the carcinogen-treated group were (117.6 ± 2.42), which showed a significant increase compared to the mean levels of the control group (33.04±0.97) at the same period of treatment ($p \leq 0.05$). Whereas the mean levels of the TNF- α were (69.95 ± 2.29) in curcumin-treated group, (69.07 ± 1.18) in tetrachlorocuprate-lysine+ascorbic acid-treated group and (47.40 ±1.71) in the mixture-treated group at the same period of treatment ($p \leq 0.05$) which showed a significant decrease compared to the mean levels of the carcinogen-treated group, table (2).

4. DISCUSSION

Lipid peroxidation play an important role in carcinogenesis (Banakar et al., 2004), is the most studied biologically free radical chain reaction. Lipid peroxidation may lead to the formation of several toxic byproducts such as malondialdehyde (MDE) and 4-hydroxynonenal which can attack cellular targets including DNA and lead to mutagenicity. An excellent model of in vivo free radical induced damage, associated with extensive lipid peroxidation, is the ferricnitrilotriacetate (Fe-NTA) model. Administration of Fe-NTA leads to increasing oxidative stress that starts from the plasma compartment, where Fe-NTA finds the ideal environment to react with oxidizable lipids (Deiana et al., 2007). This may explain the elevated levels of MDA in rats administrated with Fe-NTA. The obtained results of this work demonstrated that, feeding rats with basic curcumin decreased the lipid peroxidation level compared to the carcinogen group, in agreement with the study of Sankar et al., (2012) who showed that, the presence of curcumin significantly decreased MDA as a marker of lipid peroxidation. Lipid

Table.1) Effect of basic curcumin, tetrachlorocuprate-lysine and ascorbic acid on the serum GSH, MAD and tissue CAT and MPO activities in carcinogen-treated rats:

Parameter / Groups	GSH (µmol/ L)	MDA (µmol/ L)	CAT (U/g)	MPO (U/g)
Control	29.37± 0.99[a]	2.35 ± 0.19 [a]	122.5±1.76 [a]	0.22± 0.01 [a]
Carcinogen	14.67±1.11	6.22±0.58	70.53 ± 3.119	1.79±0.07
Curcumin+ Carcinogen	24.13±0.7[b]	3.79±0.14 [b]	96.35± 1.8 [b]	0.84±0.06 [bc]
Tetraclorocuprate -lysine+Ascorbic+ Carcinogen	27.88±0.61 [b]	3.46±0.12 [b]	101.0 ± 4.14 [b]	0.82±0.05 [bc]
Mixture+ Carcinogen	29.50±0.60 [b]	3.12±0.02 [b]	111.2± 1.88 [b]	0.37±0.04 [b]

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P<0.05).

Table.2) Effect of basic curcumin, tetrachlorocuprate-lysine and ascorbic acid on the tissue TNF- α and IL-1 activities in carcinogen-treated rats:

Parameter / Groups	IL-1 (pg/ml)	TNF- α (pg/ml)	
Control	30.89± 1.011[a]	33.04±0.97[a]	
Carcinogen	113.5±1.21	117.6 ± 2.42	
Curcumin+ Carcinogen	86.50±1.97[bc]	69.95 ± 2.29[bc]	
Tetraclorocuprate -lysine+Ascorbic+ Carcinogen	77.10±1.41[bc]	69.07 ± 1.18[bc]	
Mixture+	61.52±3.93[b]	47.40 ±1.71[b]	Carcinogen

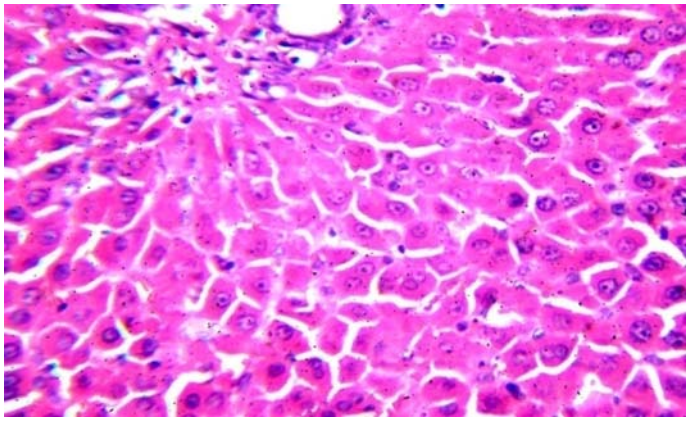

Fig (1) A photomicrograph of section of normal-looking liver of control group showing blond hepatocytes and normal architecture (400 X).

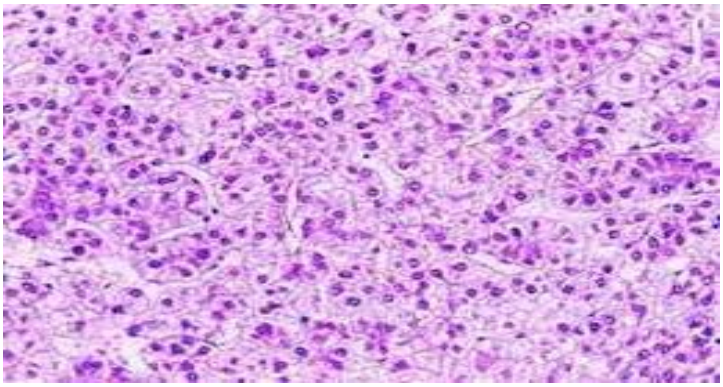

Fig (2) A photomicrograph of section of liver tissue in carcinogenic group showing malignant nuclei (X400).

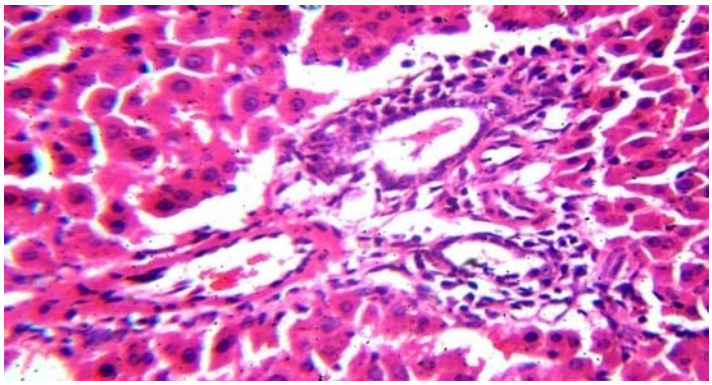

Fig (3) A photomicrograph of section of liver of curcumin treated carcinogenic group showing normal-looking portal tract (X400).

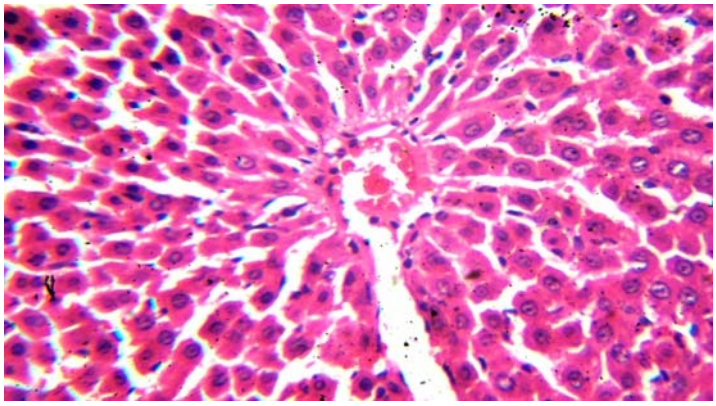

Fig (4) A photomicrograph of section of liver of tetrachlorocuprate-lysine and ascorbic acid treated carcinogenic group showing normal trabecula of hepatocytes and central vein (X400).

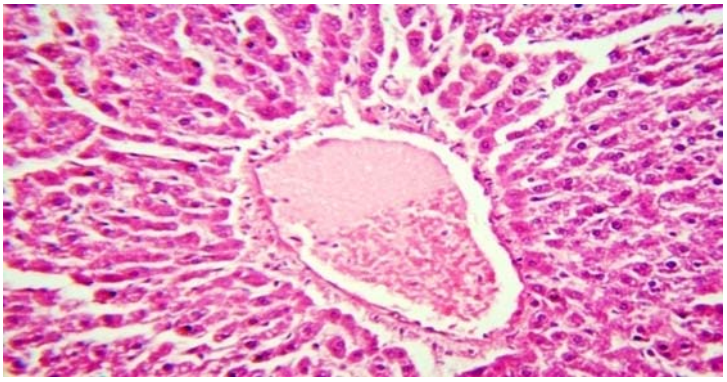

Fig (5) A photomicrograph of section of liver of Curcumin, tetrachlorocuprate-lysine and ascorbic acid treated carcinogenic group showing trabecula of hepatocytes (1-2 cell thick) and central vein (X400).

peroxidation was decreased in the presence of ascorbic acid. This result was in agreement with Elias et al., (2012) who showed that, Vitamin C (ascorbic acid) which is a major water-soluble antioxidant is believed to decrease lipid peroxidation either directly or indirectly by regenerating vitamin E. Vitamin C is an important free radical scavenger in extracellular fluids, trapping radicals and protecting biomembranes from peroxide damage. Malondialdehyde (MDA) concentration was decreased in plasma and liver tissues, by Cu administration (Jianbo et al., 2011). This explains the decreased levels of MDA in rats administrated with tetrachlorocuprate. Glutathione (GSH) plays an important role in a multitude of cellular processes, including cell differentiation, proliferation, and apoptosis, and disturbances in GSH homeostasis are involved in the etiology and progression of many human diseases including cancer (Nicola et al., 2013). Compared with the control, GSH concentrations in treated rats significantly decreased after exposure to Fe-NTA in agreement with the reports of (Athar & Iqbal., 1998) who showed the decreased levels of GSH in rats administrated with Fe-NTA. The present study showed that administration of basic curcumin increased the GSH levels compared to the carcinogen non treated group. Similarly, Chiagoziem et al., (2014) demonstrated that administration of curcumin improved the GSH. It was clear from the present study that a significant increase in serum GSH levels was induced after administration of tetrachlorocuprate compared with that received Fe-NTA and Chloroform. This improvement in the glutathione levels may

be referred to supplementation with copper increase GSH levels (Derouiche et al., 2013) and presence of ascorbic acid which is important in the production of glutathione (Duke and Atchley, 1984).

Catalase is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (Biology in focus, 2008). In the present study, a decrease in liver catalase activity was observed in rats injected with Fe-NTA compared to control group. These results were in agreement with Athar and Iqbal (1998) who showed that the catalase activity was significantly decreased. In our study, the mean values of catalase activity in rats administrated with basic curcumin showed a significant elevation as compared to carcinogen non treated group in agreement with the reports of Prabhakar et al., (2007).

It was clear from the present study that a significant increase in serum catalase activity was induced after administration of ascorbic acid which combined with tetrachlorocuprate and in agreement with the reports of Garg and mahajan., (1993).

Myeloperoxidase (MPO) is a peroxidase enzyme, most abundantly expressed in neutrophil granulocytes and produces hypohalous acids to carry out their antimicrobial activity (*Klebanoff*, 2005).

It was clear from the present study that a significant increase in liver myeloperoxidase activity was induced after administration of Fe-NTA was agreement with the reports of Muneeb et al., (2012).

The present study also showed that, administration of basic curcumin significantly decreased the MPO levels compared to the carcinogen group. Also, *Zhe* et al., (2014) demonstrated that, treatment with curcumin significantly reduced the expression of MPO.

It was shown that administration of ascorbic acid caused a decrease in MPO activity (*Murat* et al., 2008).

Interleukins are a group of cytokines (secreted proteins and signal molecules) that were first seen to be expressed by leukocytes (*Brocker* et al*., 2010)*. Interleukin-1 alpha and interleukin-1 beta (IL-1α and IL-1ß) are cytokines that participate in the regulation of immune responses, inflammatory reactions, and hematopoiesis (Barthelmes et al., 2011). It is obvious from the present study that Interleukin-1 activity in rats administrated with Fe-NTA and Chloroform was significantly elevated compared with its corresponding values in normal group and in agreement with the reports of Ahmad et al., (2005). The results of this work demonstrated that, feeding rats with basic curcumin decreased the Interleukin-1 activity compared to the carcinogen group. Moreover, Gaddipati et al., (2003) reported that, oral administration of curcumin resulted in significant restoration of the cytokines included interleukin-1 to depleted levels.

It was clear from the present study that, a significant decrease in liver interleukin-1 levels was observed after administration of ascorbic acid when compared to carcinogen group. The obtained results were nearly similar to Tomofuji et al., (2009) who demonstrated that, gene expression encoding inflammation, including interleukin-1 alpha and interleukin-1 beta, was more than two fold down-regulated by vitamin C intake.

Tumor necrosis factor (TNF) is a multifunctional cytokine that plays important roles in diverse cellular events such as cell survival, proliferation, differentiation, and death. As a pro-inflammatory cytokine, TNF is secreted by inflammatory cells, which may be involved in inflammation-associated carcinogenesis. Recent studies have focused on sensitizing cancer cells to TNF-induced apoptosis through inhibiting survival signals such as NF-κB, by combined therapy (Xia *&* Yong., 2008). In addition to apoptosis, TNF can also induce necrotic cell death. Reactive oxygen species (ROS) play a critical role in mediating necrotic cell death because ROS scavenger butylated hydroxyanisole (BHA)

can effectively block this pathway (*Lin* et al., 2004).

The present study showed that administration of Fe-NTA increased the TNF-α levels compared to the control group. Similarly, Summya et al., (2013) demonstrated that. chronic exposure of Fe-NTA for 16 weeks in tumor group induced over expression of TNF-α as compared to control group. Also, Muneeb et al., (2012) reported that, elevated levels of TNF-α could be detected in serum of rats exposed to tumor promotion with Fe-NTA. Compared with the carcinogen group, TNF-α concentration in treated rats significantly decreased after administration of curcumin which had been shown to markedly reduce serum TNF- α (Gulcubuk et al., 2006). This explains the decreased levels of TNF-α in rats administrated with basic curcumin.

In the present work, a significant decrease in liver TNF-α level was observed in rats administrated with ascorbic acid compared with carcinogen group. Also, *Masoumeh* et al., (2015) revealed that, ascorbic acid administration could reduce the levels of TNF-α. Moreover, Senturk et al., (2004) demonstrated that, ascorbic acid administration significantly decreased the concentrations of serum tumor necrosis factor- α. It was clear from the present study that a significant decrease in liver TNF-α levels was induced by administration of cupper-lysine. Also, Jianbo et al., (2008) demonstrated that, the reduction in backfat depth may be due to copper from Cu-lysine altering TNF-α metabolism in lambs.

Histopathological examination of the liver tissues of the different experimental groups illustrated that, Fe-NTA and chloroform administrated group showed macrovesicular steatosis with prominent sinusoid nucleoli. Hepatic arteriole showed Hyalinosis. dilated central venule as well as wide area of coagulative necrosis and malignant nuclei in compared to that of control group. These results agree with Kannappan (2014) who reported that, an iron chelate, ferric nitrilotriacetate (Fe-NTA), induces necrosis as a consequence of lipid peroxidation and oxidative tissue damage that eventually leads to high incidence of cancer in liver and kidney. Fe-NTA acts through the generation of free radicals and by enhancing the rate of DNA synthesis with simultaneous decrease in antioxidant defenses. .Also, Chloroform may be caused enhancement of cancer inductionin was in agreement with Byron et al., (1995) who showed that, Chloroform produces cancer by a non genotoxic-cytotoxic mode of action, with no increased cancer risk expected at noncytotoxic doses. Rat's liver administrated with curcumin revealed that, significant improvement in the histological structure of liver tissue compared to the carcinogen group. Similarly, Ajaikumar et al., (2008) reported that, curcumin has been found to inhibit the proliferation of various tumor cells in culture, prevents carcinogen-induced cancers in rodents, and inhibits the growth of human tumors in xenotransplant or orthotransplant animal models either alone or in combination with chemotherapeutic agents. Also, the results have indicated that, tetrachlorocuprate-lysine in combination with ascorbic acid administration showed significant improvement in the histological picture compared to the carcinogen group. Also, Netke et al., (2003) reported that the synergistic anticancer effect of ascorbic acid, proline, lysine on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients. Moreover, Casini et al., (2007) reported that, the interactions with protein targets of the ruthenium(III) complex imidazolium trans- [tetrachloro(dimethyl sulfoxide) (imidazole) ruthenate(III)], NAMI-A, an effective anticancer and anti-metastatic agent now in clinical trials.

*Conclusion***:** Basic curcumin has potent chemopreventative activity against a wide variety of tumors and has great potential in the prevention and treatment of hepato-carcinogenesis, Moreover, Tetra-chlorocuprate- lysine in combination with ascorbic acid exert chemopreventative effect against hepatocellular-carcinoma,

through have antioxidant and free radicals scavenging activity and trapping of activated metabolites of chemical carcinogen.

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