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Protective and therapeutic role of nano-curcumin against hepatocarcinogenesis in rats

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ABSTRACT

Curcumin medicinal applications are hindered by its well-known unstable metabolic state and also insufficient absorption and bioavailability. In recent years, nano-based drug delivery system has effectively improved the aqueous solubility and bioavailability of curcumin. The current study was established to evaluate the protective and therapeutic effect of nano-curcumin (Nano-Cur) in hepatocellular carcinoma induced in rats. Fifty white albino rats were divided into 5 groups 10 rat of each. Group I served as negative control while group II received DEN with a daily dosage of (20 mg/kg body weight) orally by gavage during twelve weeks. Group III rats received Nano-Cur (20 mg/kg body weight) three days a week by gavage for eighteen weeks. Group IV rats received DEN as group II and treated with Nano-Cur as group III for six weeks. Group V received Nano-Cur as protector, for six weeks before DEN administration with continuous administration with Nano-Cur till the experiment end. Blood and tissue samples were collected and removed for determination of biochemical, antioxidant, cytokine and apoptotic marker p53 examination at the end of experiment. Based on the findings, DEN group indicated significantly increased serum liver function enzymes ALT, AST, ALP this was accompanied with significant decrease in liver antioxidant enzyme activities of (SOD and CAT) along with significant elevation in lipid peroxidation. DEN significantly elevated IL-6 and reduced apoptotic marker p53. The findings of this research indicated that Nano-cur has a anemaelioration effect against DEN carcinogenic effect.

1. INTRODUCTION

The sixth most common cancer in the world is hepatocellular carcinoma (HCC) (Forner et al., 2018,Rashed et al., 2020). In Egypt, it represents the fourth common cancer (Akinyemiju et al., 2017). Many hospital based cases (Abd-Elsalam et al., 2018) recorded increasing the incidence of HCC. HCC is the world's fourth leading cause of cancer-related death (Villanueva, 2019). HCC is the most common cause of cancer-related mortality and morbidity in Egypt. The majority of HCC cases are caused by infections with the hepatitis B and C viruses (Schutte et al., 2009). There is currently no confirmed successful systemic chemotherapy for HCC. Given the restricted treatment options for liver cancer and the poor prognosis, chemoprevention is the best method for minimizing existing hepatocarcinogenesis morbidity and mortality. (Yates and Kensler, 2007).

Diethylnitrosamine (DEN) is a potent nitrosamine causing gene toxicity that is known to destroy nuclear enzymes involved in DNA repair and replication of DNA and often used experimentally to induce hepatocarcinogenisis (Sadik et al., 2008). Also, DEN active metabolites generated by cytochrome isoform 2E1 (CYP 2E1) cytotoxicity because of the increased level of oxidative stress. (Mandal et al., 2008).

Curcumin is one of the polyphenol compound derivatives that have strong anticancer effect, which is derived from the Curcuma longa herb, also known as turmeric. Curcumin increased importance because of its astonishing exceptional anti-inflammatory, anti-angiogenic, anti-carcinogenic, antioxidant effects without any observed toxicity (Hassan et al., 2014). Gathered experimental evidence suggests that curcumin had the ability to interfere with a lot of molecular targets and involved processes in carcinogenesis and inhibit such processes (Hasima and Aggarwal, 2012). However, curcumin therapeutic applications in humans are well known and limited by its well-known metabolic instability, insufficient absorption and poor bioavailability (Yang et al., 2018). Curcumin changing into nano-particles represent one of the strategies used to increase therapeutic application benefits. Curcumin's solubility increased by using nanoparticles "10–100" nm size and improves its bioavailability inside human body (Yallapu et al. 2012, Birgani et al. 2015). As compared to the same oral dose of free curcumin, the synthesized nanoparticles increase the absorption rate 10–14-fold. (Yallapu et al. 2012).

As a result, this study was conducted to see whether (CUR NPs) could reduce the hepatocarcinogenic effect of DEN in white albino rats.

2. MATERIAL AND METHODS

Fifty white male albino rats (10-12) weeksold, weighing (100 - 120 g) were used in this study. Rats were obtained from the Research Center of Laboratories Animal, Faculty of Veterinary Medicine, Moshtohor, Benha University. Rats were housed in normal light and temperature conditions and were supplied with adlibitum, free access to the standard pellet diet and tape water. Before beginning the experiment

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the animals were left to adjust for 10 days.
The experimental protocols were approved by the Animal Care and Use committee at Benha University and are in accordance with the National Institute of Health Guide for the Care and Use

1 .Chemicals:

The chemicals used in the present study were:
Diethylnitrosamine (DEN)and curcuminwere broughtfrom Sigma Chemical Co. (St. Louis, MO, USA.) .
Preparation of Diethylnitrosamine (DEN) :
Diethylnitrosamine was diluted using physiological saline to be administrated at a dose of 20 mg/kg of rats body weight (El-Shahat, et al., 2012).

Synthesis of curcumin nanoparticles:

To make curcumin nanoparticles, 1M low-solubility curcumin was mixed with 4M sodium bicarbonate buffer, then ground for 8 hours in a mechanical ball mill (350 rounds per second).Synthesized nanoparticles were characterized by Transmission electron microscope (TEM). Images of TEM showed that the synthesized nanoparticles is spherical in shape and has a diameter in range of 15-62.7 nm (Hassan et al., 2014).

Preparation and dosage of nano-curcumin- :

Nano-Cur was dissolved freshly in distilled water .

2.3.Experimental Design:

Five Groups were formed at random, each group had ten rats, as following:
Group (1): Negative control:
Rats provided physiological saline 1ml orally by gavage daily during the experimental period .
Group (2): DEN group "Positive group:"
Rats received DEN at a dose of (20 mg/kg body weight) daily orally via gavage for 12 weeks, then observed to the experiment end (Hassan et al., 2014).
Group (3): Nano-curcumin group:
Rats administrated Nano-Cur (20 mg/kg body weight) orally three times per week for 18 weeks.
Group (4): DEN + nano-curcumin (treatment):
Rats received DEN as group (2) for 12 weeks, followed by receiving Nano-Cur as group (3) for 6 weeks.
Group (5): Nano-curcumin + DEN group (prophylactic):
Rats received Nano-Cur as group (3) for 6 weeks followed by, receiving DEN as group (2) for 12 weeks with continuous administration of Nano-Cur.

2.4.Sampling:

After 18 weeks, rats of each group were fasted overnight and then euthanized. Blood and liver tissue samples were obtained from all rats groups at the end of the experimental period .

Blood samples :

The blood samples were collected from retro-orbital plexus of eyes puncture. Blood was allowed to clot then centrifuged for 15 minutes at 3,000 r.p.m. Sera were separated in dry sterile tubes by automatic pipette, and then stored at -20 °C in a deep freezer until determination of biochemical parameters.

2.4.2 Tissue sample:

The liver was quickly removed, washed with ice-cold saline, snap-frozen directly in liquid nitrogen and stored at -80ºC. Briefly, one gram of liver tissue was cut and cut into very small pieces, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH7.4) to make 10% homogenates, then centrifuged for 15 minutes at 6000 r.p.m at 4°C. The supernatant used directly for measuring of antioxidants activities.

2.5 .Analysis:

2.5.1Biochemical analysis:

Biochemical parameters to evaluate liver functions: liver enzymes activity alanine aminotransferase (ALT), aspartate amino transferase (AST), Alkaline Phosphatase (ALP) were determined in the serum using human diagnostics worldwide kits (Germany), according to Fischbach, et al., (1992) and Klin, 1970 respectively.

2.5.2 Antioxidant enzymes:

Moreover, the supernatant of hepatic tissue homogenate (10%) were used for the determination of Catalase (CAT), Superoxide dismutase (SOD) activities, using Biodiagnostic kit (Cairo, Egypt), according to Fossati.et al.,(1980) , Nishikimi et al., (1972) respectively.

2.5.3IL-6 level:

Serum Il-6 level was estimated usingQuantikine Elisa kit for rat IL-6 Immunoassay, R&D Systems, Inc, USA according to (Hirano, T. 1998).

2.5.4Molecular analysis:

Real-time quantitative polymerase chain reaction analysis (real-time qPCR) was used to determine the mRNA expression contents of p53 in rat liver. We used the QIAamp RNA Blood Mini Kit to extract total RNA from homogenised liver samples (Qiagen, USA). A NanoDrop 2000 spectrophotometer was used to check the conc. and purity of the extracted RNA (Thermo Scientific, USA). The QuantiTect Reverse Transcription kit was used to make reverse transcripts from the extracted RNA (Qiagen, USA). A QuantiTect SYBR Green PCR kit was used to measure gene expression in a relative manner (Qiagen, USAThe primer was pre-made and issued by the company (Invitrogen USA). P53's relative quantification was measured using GAPDH as a reference gene. 2 l of primer, 12.5 l of 2x QuantiTect SYBR Green PCR Master Mix, 8.5 l of RNase-free water, and 2 l of cDNA were used in PCR reactions. Cycling started at 95°C for 15 minutes, accompanied by 40 cycles of 94°C denaturation for 15 seconds, 55°C annealing for 30 seconds, and 72°C extension for 30 seconds, all followed by a melt curve (Livak and Schmittgen, 2001).

Gene	Primer sequence	***Excision*** no.
P53	Forward primer :5'- CGCAAAAGAAGAAGCCACTA-3 Reverse primer:5′-TCCACTCTGGGCATCCTT-3	XM_008767773.3
GAPDH	Forward primer :5'- TGATTCTACCCACGGCAAGTT-3 Reverse primer:5′-TGATGGGTTTCCCATTGATGA-3	NM_017008.4

Ref. for target genes and ref. gene

2.6. Statistical analysis:

SPSS software was used to analyse all of the results (version 19). One-way analysis of variance (ANOVA) was used to evaluate hypotheses, preceded by the least significant difference (LSD) test to find discrepancies between group means. The minimum degree of significance was set at P-values of 0.05.

3. RESULTS

3.1. Liver enzymes:

Data obtained from our study showed that rats received DEN revealed an elevation in ALT, AST, and ALP levels compared to the normal control group. However rats administrated DEN then received nano-Cur as a treatment recordeda significant decline in ALT, AST and ALP activities compared to the DEN group. As well as the nano-cur protected rats showed a significant decrease in ALT,

AST and ALP activities compared to DEN group and Nano-cur treated group, these data shown in (fig.1, 2).

3.2. Antioxidant enzymes:

Rats received DEN exhibited a significantdecrease SOD and CAT activity compared to negative control group, while DEN administrated group with Nano-Cur, showed a significant increase in SOD and CAT activity compared to the DEN group. Data also documented that protection with Nano- Cur significantly increased the activity of SOD and CAT in comparison to the DEN-treated group and Nano-Cur treated group, as shown in (fig.3,4).

3.3. IL-6 level:

IL-6, as pro-inflammatory cytokine, significantly elevated in DEN treated group when compared with the negative control group, this elevation was significantly decreased in nano-Cur treated group when compared to DEN treated group, also nano-cur protected group showed a significant decrease in IL-6 level when compared to DEN group and nano-cur treated group, these data were documented in fig (5).

3.4. P53 gene expression:

In DEN treated group, relative gene expression of tumor suppressor P53 level was up regulated significantly compared to the negative control. Nano-cur treated and protective groups showed significant increase in P53 expression compared to DEN-treated group. (Fig 6).

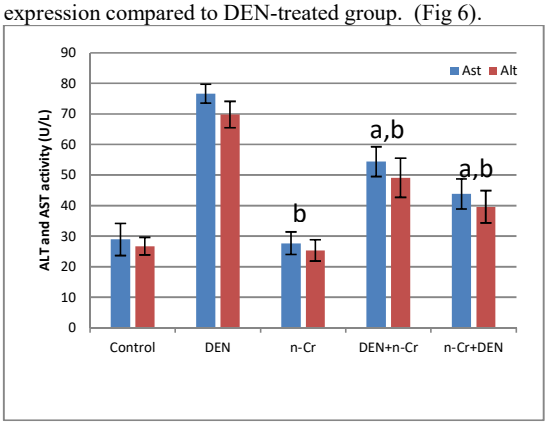

Fig (1): Serum ALT, AST activity (U/L) in control, DEN group and Nano-Cur treated groups of male albino rats.

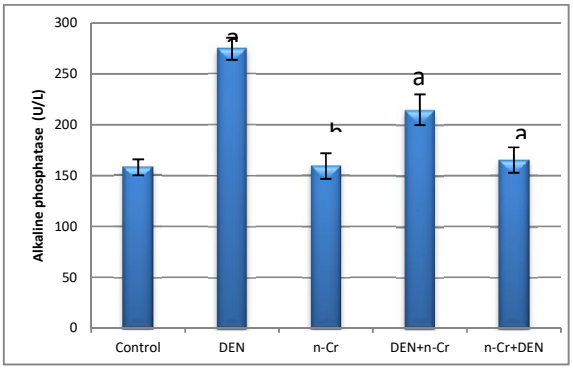

Fig (2): Serum ALP activity (U/L) in control, DEN group and Nano-Cur treated groups of male albino rats.

4. DISCUSSION

HCC, the foremost dominant sort of liver cancer, is that the third leading explanation for cancer mortality around the world, with quite 500,000 people affected every year (El-Ahwany et al., 2019, Mohammed et al., 2021). HCC can be treated in a variety of ways. The recurrence rates, on the other hand, are as high as 50%.

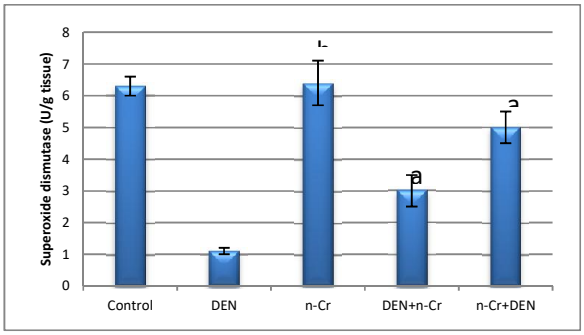

Fig.(3): Hepatic SOD activity (U/g) in control, DEN group and Nano-Cur treated groups of male albino rats.

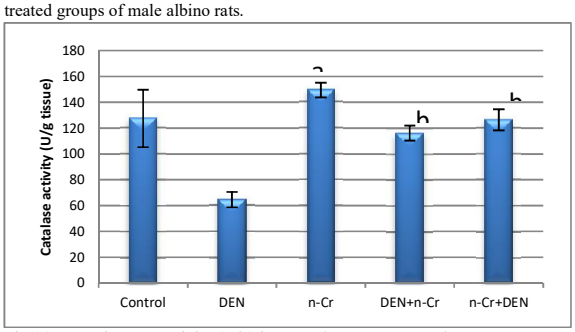

Fig.(4): Hepatic CAT activity (U/g) in control, DEN group and Nano-Cur treated groups of male albino rats.

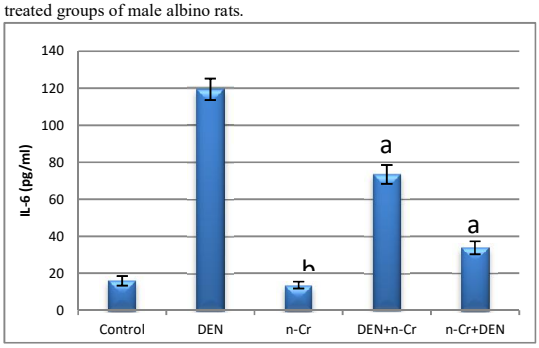

Fig.(5):IL-6 activity (pg/ml) in control, DEN group and Nano-Cur treated groups of male albino rats.

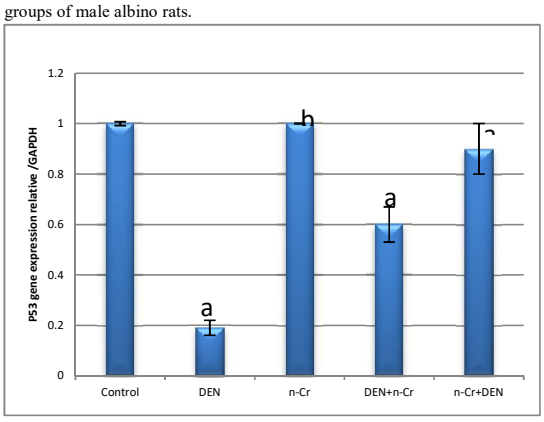

Fig.(6):P 53 gene expression in control, DEN group and Nano-Cur treated groups of male albino rats.

Furthermore, HCC is commonly recognized as a chemotherapy-resistant cancer. As a result, chemoprevention has been proposed as the most important strategy for reducing HCC incidence and mortality. (Horng et al., 2017).

It has been documented that curcumin has anti-tumor effects on many varieties affecting malignancies of the digestive,

nervous, urinary, pulmonary, and reproductive systems (Heger et al., 2014). However, the curcumin therapeutic applications are limited because of its unstable metabolic state also poor absorption and bioavailability (Yang et al., 2018). As a result, nanoparticles were used in such research to address these issues because they're known to accumulate mainly at tumor cells due to macromolecules poor lymphatic drainage in solid tumors and cancer cells' retention strength. (Lammers et al., 2008). To clarify the role of Nano-Cur within the treatment and HCC prevention, study established of hepatocarcinogenis induced is in male Albino rats by using DEN model. It is well documented that, the prolonged DEN oral administration is highly effective in causing hepatic cancer in rodents when given at high doses. (Magee and Lee 1963).Reactive intermediates were the final degradation of DEN which leads to macromolecules that have been methylated. N7-methylguanine and O6-methylguanine formation in DNA are particularly important. The pathogenesis of hepatocarcinogenesis is considered to be assisted by oxidative stress caused by DEN. (Kolaja and Klaunig 1997). The liver enzymes ALT, AST, and ALP increase in response to a hepatotoxic agent and are indicative of hepatic insufficiency. In comparison to the HCC positive group, using Nano-Cur as a treatment or protector significantly reduced the activities of AST, ALT, and ALP enzymes in the hepatocyte. This decrease may also be linked to a decrease in hepatocellular injury, implying that curcumin could protect against DEN-induced liver damage. DEN-treated groups showed a substantial reduction in the activities of SOD and CAT relative to the usual control group, suggesting increased generation of free radicals as a result of DEN administration, disrupting antioxidant protection systems and increasing reactive oxygen species. (Mohammed et al., 2020). In contrast to the HCC group, Nano-Cur administration resulted in a substantial increase in SOD and CAT activity, which reduced the oxidative stress caused by DEN.

Curcumin may be a potent scavenger of free radicals like superoxide anions, nitrogen dioxides, and hydroxyl radicals (Motterlini et al., 2000). The Nano-Cur reversal effects against DEN toxicity is possibly because of reduced or complete inhibition of the oxidative stress as well as an increase in the antioxidant status. This is confirmed by the findings of the current research, which showed that antioxidant enzymes SOD and CAT were increased whereas LPO was decreased. Nano-Cur has been shown to restore the antioxidant system and enhance the structure and function of the liver in previous studies.

Cytokines are immune system pleiotropic hormones that play a key role in cancer initiation, maintenance, and progression. (Abdelaziz and Ali, 2014). Tumor-associated macrophages and neoplastic cells secrete angiogenic and lymphangiogenic growth factors as IL-6, IL-1α, IL-10, TGF-β and TNF-α that initiate and promote tumor development (Dranoff, 2004). IL 6 is one of the most well-known inflammatory cytokines and tumorigenic factor that promotes the development of HCC. (Nakagawa et al., 2014, Taniguchi and Karin,2014).

There was an elevation in IL-6 observed in cirrhosis and hepatocarcinogenesis (Xiang et al., 2018). Though resident immune cells secrete the majority of IL 6, hepatocytes contribute to the total expression of IL 6 in the liver microenvironment. (Park et al., 2010)As a result, IL 6 accelerates compensatory hepatocyte proliferation, primarily as a result of tumour progression. (Gosain et al., 2019). Within the present study, hepatic pro-inflammatory biomarker, IL- 6, was significantly increased at DEN group due to the inflammation induced within the hepatic tissue because of DEN inflammatory known effect. While IL- 6 reduced in rats treated or protected with Nano-Cur particles. DEN treated rats exhibited a significant decrease in p53 concentration, which may be due to P53, a tumour suppressor gene, was found to be up-regulated. This could be linked to the formation of various types of DNA adducts, each of which has a different effect on DNA helix damage, DNA replication, and gene mutation induction, which leads to the expression of the P53 gene. Our findings are based on the findings of (Hassan et al., 2014), who found that P53 is clastogenic in the rat liver when induced by DEN due to the development of different forms of DNA–adducts, each of which has a particular impact on DNA–helix distortion, DNA–replication, and the induction of gene mutations. On the other hand rats treated or protected with Nano-Cur showed low mutation in P53 which may be due to the ability of Nano-Cur to promotes de novo synthesis of p53 protein or another proteins types which help in stabilization of p53, indicating that Nano-Cur can induce cancer cell killing by using p53-associated signaling pathway. One of the major factors regulating cell proliferation, growth suppression, and transformation is the p53 gene, which functions as a genome guardian.. P53 tumor suppressor gene inactivation could be an essential in tumor induction. Mutations in the p53 gene have been shown to occur at various stages of malignant cell transformation, signaling, tumor development, growth, aggressiveness, and metastasis in a number of ways. (Van Gijssel et al., 1997).Moreover, (Levine et al., 1991) documented that changes in the p53 gene tend to be essential for the occurrence of hepatocarcinogenesis.

5. CONCLUSION

This research showed that Nano-Cur has strong anti-oxidant, anti-inflammatory, and anti-carcinogenic properties, and that it was successful in ameliorating liver condition of DEN-induced liver carcinoma. Furthermore, nanoparticles enhance the physical properties, absorption, and bioavailability of curcumin.

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