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In vitro and in vivo studies on the anticancer potential of curcumin and nanocurcumin

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ABSTRACT

Purpose: Curcumin, a polyphenolic compound that obtained from the herb of Curcuma longa, has many anticancer effects. But, its effect is low due to poor water solubility. In order to improve its solubility and drug delivery, we have utilized a nano-curcumin. Methods: In vitro cytotoxicity and anti-tumor promoting effects of nanocurcumin and normal curcumin were investigated. Results revealed that nanocurcumin is able to inhibit the growth of two human cancer cell lines Hep-G2 and **HCT116** with IC50 values of 5.68 and 6.53 µg ml−1, respectively,while free curcumin expresses the activity with IC50 values of 8.28 and 9.64µg ml−1. At the concentration of 40 µg ml−1. Nanocurcumin showed anti-tumor promoting effects in reducing tumor size by 59.8 % , while the percentages caused by curcumin was 41.4%, respectively. Mice were treated with equal concentration of nanocurcumin and curcumin showed an improvement in the antioxidant and anti-inflammatory cytokines. The level of imprement in EC bearing mice treated with curcumin was lower than that of cells treated with nano curcumin (P=0.001). Conclusion:Results are suggesting that Nano curcumin can be more effective than free curcumin in inhibition of Elrich ascites carcinoma cell lines.

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1. INTRODUCTION

Curcumin (diferuloylmethane), is a nontoxic yellow poly phenolic compound occurring naturally in rhizomes of turmeric plant(Curcuma longaL.). This compound consists of 3 components:(i)curcumin,(ii) demethoxycurcumin and(iii)bisdemethoxycurcuminoidswith ratio 77:17:3. Curcumin(1, 7-bis(4-hydroxy-3-methoxyphenyl)-1, 6 heptadiene-3, 5-dione)is widely known for abilities to promote wound healing,enhance blood circulation and traditionally used as a coloringagent. In recent years, curcumin has been found to have interesting pharmacological and biological activities such as anti-oxidation ***[1],*** anti-inflammation, anti-regression andanti-ischemia, anti-cancer and anti-viral. In addition, very recently, curcumin is demonstrated as a potentialfluorescent probe for monitoring the biodistribution of drug delivery nanosystem in cancer cell and tumor. However,natural curcumin has low water solubility(0.6μgml−1) ***[2].*** Therefore, it is introduced into the body at only low con-centration, which is quickly broken down by enzymes and the liver, thus reduce bioavailability. Recently, there have beenmany researches on increasing solubility and bioavailabilityof curcumin. Notably, natural polymers such as starch,chitosan, casein, cellulose are being investigated as deliverin gmaterials for curcumin which can enhance efficiency of curcumin usage ***[2]*** . In this paper, effects of polymeric nanoparticles curcumin and curcumin in vitro anti-cancer activities (cytotoxicity and anti-tumor promoting effects) were investigated and evaluated. Results of our current study may provide evidences for applying the new nature-originated-nanomaterial in health caring as well as in treating acute diseases.

2. MATERIALS AND METHODS

2.1.Materials

2.1.1. Animals: Outbred female Swiss pale skinned person mice (20-25g) taken from National Cancer Institute (NCI) that were used as exploratory creatures. Creatures were housed in plastic enclosures and kept up under standard states of brightening, ventilation, temperature, mugginess and a 12 light/dull cycle along the trial time frame. Mice were offered with a pellet diet containing all the vital nutritive elementsand water promotion libltum all through the exploratory period. Mice were left for adjustment around multi week before beginning the trial time frame. The creatures were kept up as per the creature morals and the guide for the consideration and utilization of lab creatures (National Research Council, 1996).

2.1.2. Ehrlich Ascites Carcinoma Cell Line(EAC): Ehrlich Ascites Carcinoma were taken from National Cancer Institute (NCI), Cairo college. The cells were proliferated as ascite in female Swiss pale skinned person mice weekly intraperitoneal vaccination of 2.5 x 10^6 cells/mouse ***[3]***

2.1.3. Cell lines: Cell lines were provided by National Cancer Institute (NCI), Cairo university.including human hepatocellularcarcinoma(Hep-G2) and human colon malignant growth (HCT-116).

2.1.4.Nano curcumin: Nano curcumin and curcumin used in this study were purchased from India . Other chemicals and kits were purchased from Randox company, Egypt.

2.2.Methods

2.2.1. Cell culture study:

Cytotoxic movement of curcumin and nanocurcumin was performed on a board of human tumor cell line HEPG2 (hepatocellular carcinoma), HCT 116 (human colon carcinoma cell line) at various fixations. The strategy for Philp et al was utilized to complete the cytotoxicity as sulphorhodamine-B(SRB) test ***[4].*** SRB is a protein recolor in mellow acidic conditions. This stain is utilized to give a delicate record of cell protein content. It is

a splendid pink ammoxanthrene color with two sulphonic gatherings

2.2.2.Cell viability assay:
The antitumor impact of curcumin and nano curcumin was surveyed by perception of changes as for reasonable just as nonviable tumor cell tally ***[5].*** To recognize the cytotoxicity of nano-curcumin, EACs were treated with nanocurcumin or curcumin at the centralizations of 5,10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml. The EACs were gotten by needle goal of ascites liquid from the preinoculated mice under aseptic condition utilizing ultra violet laminar wind stream framework. The rates of non-feasible cells were estimated by considering reasonable well as dead EACs. To separate among practical and dead EAC cells, trypan blue stain was utilized. At that point the rates of non-suitable cells (NVC) were estimated by the accompanying condition % NVC= C/T X 100, Where(C) is the quantity of non-reasonable cells and (T) is the all out number of feasible cells.

2.3. Experimental Design:

Mice were permitted 7 days for adjustment. 40 mice were then arbitrarily dispersed into 4 equivalent gatherings, 10 mice for each gathering. The creature bunches were partitioned as follows:G1: Normal control gathering (neither infused nor treated) . G2: Ehrlich carcinoma (EC) bearing gathering. Mice were intramuscularly infused with 0.2ml of 2.5×10^6/ml/mouse suitable Ehrlich ascite carcinoma cells in left thigh.G3: EC bearing curcumin treated gathering. G4: EC bearing treated with nanocurcumin particles. Mice of gathering 3 and 4 were infused intramuscularly with 0.2ml of 2.5×10^{6} Ehrlich ascite carcinoma cells in left thigh, at that point following one day of tumor vaccination curcumin or nanocurcumin was given at adose dimension of 50 mg/kg body weight orally consistently for 4weeks.

2.3.1. Checking the Tumor Size:

Tumor estimate was estimated utilizing Vernier calipers each week amid test. The tumor measure was assessed utilizing the accompanying recipe: Tumor estimate (mm3) = 4 (A/2) (B/2)2 = 0.25 A.B2, where An is the significant pivot and B is the minor hub ***[6].***

2.3.2. Sample Preparation:

Toward the finish of the test time frame, all mice were relinquished utilizing anesthetized diethyl ether. Blood and tumor from mice of each gathering were gathered and utilized for the proposed investigations. The blood tests were gathered legitimately from gateway vein into heparinized and non-heparinized rotator tubes. Plasma and serum were isolated by centrifugation at 3000 r.p.m for 15 minutes and were solidified at - 20 °C for consequent biochemical investigation. Heparinized blood tests were utilized for the assurance of decreased glutathione. The erythrocytes were washed twice with cold saline and kept at - 20 °C for Zn, Cu SOD and GPX estimation. Following yielding rodents, tumor tissues were extracted from the mice, washed in cool saline and homogenated to give 10% homogenates which put away at - 20 °C for biochemical examine.

2.3.3. Biochemical assays:

The dimensions of tumor rot factor-alpha, interferon-gamma, granzyme-B and caspase-3 were tested in serum by the standard sandwich compound connected safe sorbent (ELISA) test system . Lipid peroxidation (LPO) was assessed in plasma and tumor tissues homogenates by estimating the malondialdehyde (MDA) generation framed in the thiobarbituric corrosive response ***[7]*** .Glutathione focus was dictated by the strategy for **[8].** Entire blood and tumor homogenates were deproteinated with trichloroacetic corrosive by centrifugation and GSH discharged in the supernatant were derivatized with 5, 5' dithiobis-2-nitrobenzoic corrosive (DTNB). The improvement of shading was estimated at 412 nm. Nitric oxide was resolved by **[9]**

Glutathione peroxidase (GPX) movement was examined in erythrocytes and tumor homogenates by a coupled test framework, in which glutathione reductase is utilized for recovery of GSH and butylhydroperoxide utilized as the acceptor substrate. The lessening in NADPH focus was enrolled photometrically at 340 nm **[10].** The assurance of Cu, Zn-SOD action in erythrocytes and tumor homogenates was by **[11].** Catalase action was assessed by following the disintegration of H_2O_2 legitimately by the lessening in termination of hydrogen peroxide at 240 nm **[12]**

2.4. Statistical Analysis:

The got information were communicated as mean ± standard deviation (SD). All information were examined measurably utilizing single direction investigation of fluctuation (ANOVA) trailed by Student's t-test. Measurable importance was considered at $P < 0.05$. Measurable Package for Social Sciences (SPSS) for Windows form 17.0 programming was utilized for this investigation **[13].**

3. Results

3.1. In vitro Studies

3.1.1. Cytotoxicity

The in vitro cytotoxic exercises of nanocurcumin and curcumin were appeared in Table1. The base inhibitory focus (IC50) of nanocurcumin was observed to be 6.53 µg/ml and 5.68 µg/ml against HCT116 and HEPG2 cell lines, separately. Moreove rthe colorimetric cytotoxicity tests demonstrated that the curcumin had in vitro cytotoxic action against the analyzed destructive cell lines with IC50 estimations of 9.64 µg/ml and 8.28 µg/ml against HCT116 and HEPG2 cell lines, separately. The present outcomes uncovered that nanocurcumin had a lower IC50 lower than that of curcumin.

3.1.1.Effect of nanocurcumin and curcumin on Ehrlich ascite carcinoma cells . The tumoricidal effect of varying concentrations of the tested materials on Ehrlich cell viability is presented in table 2. The low concentration (5 µg/ml) of nanocurcumin and curcumin reduces the tumor cell viability by 15% and 10% respectively.The median lethal concentrations of nanocurcumin and curcumin were (40 µg/ml vs. 60 µg/ml for Ehrlich carcinoma cells. The concentration which causes the death of most Ehrlich carcinoma cell lines was 80 µg/ml (the death of 95% and 70% for nanocurcumin and curcumin respectively).

3.3. In vivo Studies

3.3.1. Ehrlich carcinoma tumor size monitoring:

The extent of strong Ehrlich carcinoma (EC) in left thigh of mice was estimated multiple times along one month beginning from EC tumor cells vaccination and start of tumor development in control EC bearing mice. The postponement of hindrance in tumor measure in mice treated with nanocurcumin The mean size of left thigh of sound, ordinary mice is 13.65 mm3and the vaccination of 2.5 million of EC cells in 0.2 ml physiological saline in the left thigh of sound, typical mice delivered a strong tumor with a mean size of 93.59±0.92 mm^3on the 7thday after tumor immunization . The expansion of EC tumor estimate continues by days achieving 2306.43± 6.76 mm3 on the 4^{th} week after tumor inoculation.The information acquired uncovered the lesser tumor measure through the perception time frame in gatherings of test creatures treated with nanocurcumin remove on the following day after tumor immunization for 4 weeks. The tumor size of mice treated with curcumin is also decreased at the end of study as shown in table (3)

3.4 Tumor Markers Responses:

3.4.1Caspase-3, Granzyme-B, Serum tumor corruption factor-alpha (TNF-α) and Serum Interferon gamma (IFN-γ) location:

The information uncovered in table (4) illustrated that female mice immunized with EC and treated with nanocurcumin every day for one month recorded an increase in caspase-3 and Granzyme-B

levels, a reduction in TNF-α Level and a lessening in IFN-γ Level in contrasted with EC gathering. While, every day treatment of female mice immunized with EC and treated with Trigonella foenum-graceum separate joined with SeNPs for one month predicts an expansion in caspase-3 action, an expansion in Granzyme-B movement, a diminishing in TNF-α level and an abatement in IFN-γ level contrasted with EC gathering.

3.5 Oxidative Stress and Antioxidant Markers:

From the results presented in tables 5 and 6, it is evident that inculation of female mice with EC promoted lipid peroxidation by increasing the levels of MDA and NO in both plasma and tumor tissue by (126.54% and 38.75%) and (59.92% and 38%), repectively, as compared to negative control ($p<0.001$).Inoculation with EC impairs the endogenous antioxidant defense system by inhibiting the main antioxidant enzymes (super oxide dismutase [SOD], glutathione peroxidase [GPX] and catalase[CAT]. It also depletes reduced glutathione (GSH),the most important intracellular non-enzymatic antioxidan. The oral gavages of female mice bearing EC by nanocurcumin for one month recorded reduction in plasma and tumor MDA and NO levels in contrast with the EC bearing gathering. There was statistically significant increase inthe GSH levels and SOD, CAT, and GPx activities in the nanocurcumin and curcumin treated groups compared to the EC-treated group ($p<0.05$).

DISCUSSION

Curcumin has a long history of utilization as a conventional prescription since it is nontoxic and has an assortment of helpful properties, for example, cancer prevention agent, pain relieving, calming, clean and anticarcinogenic action . The present investigation demonstrates that nanocurcumin diminishes harmful effects incited by Elrich ascites carcinoma cell lines in mice through the decrease of inflammatory and biochemical (MDA and NO) parameters just as the hindrance of the overexpression of incendiary arbiter TNF-α and interferon γ. Nanocurcumin is more potent than curcumin in inhibiting the cancer cell lines in vitro. This may be explained by the increase in solubility of curcumin.The result suggests that curcumin nano-particles possess increased effectiveness in activity of curcumin in vitro.

Cytotoxicity is an important component of the immune system and is a highly regulated, multi-factorial process carried out by different cytotoxic cells of the immune system. Cytotoxic CD8+T cells, NK and NK-like T cells mount immune responses to cancer via pathways that include the cytotoxic granzyme B/perforin pathway and by release of cytokines including IFN-γand TNF-α. The decreased IFN-γ is likely to contribute to the suppressive pro-tumour microenvironment and the sur-vival of cancer cells, as this cytokine inhibits angiogenesis and cellular proliferation and promotes apoptosis of the cancer cells. In addition, it activates the adaptive immune system, and thus contributes to effective antigen processingand presentation ***[14].***

Curcumin has been shown to suppress inflammation in some diseases and promote immune response to acute inflammation **[15].** Some studies suggested that curcumin attenuates inflammation by modulation of antioxidant enzymes and downregulation of IL-1β, IL-6 and TNF-α **[16].**

cell culture studies revealed that curcumin inhibits the transcriptional factor NFκB and increased caspase 3 activity **[17].** Due to these effects, curcumin also suppresses the paclitaxel-induced NFκB pathway in cancer cells and shows anti-apoptotic properties. The results of the present experiment confirmed the anti-inflammatory effects of curcumin and are in agreement with the previously published data in which curcumin led to a reduction in the formation of pro-inflammatory cytokines. Similarly, an experimental

model of LPS-stimulated production of TNF-α showed a dose-dependent reduction of the TNF-α secretion by cells exposed to curcumin. TNF-α is a major cytokine involved in inflammation **[18].** In addition to TNF-α curcumin supplementation also resulted in inhibition of LPS-induced IL-10 and IFN-γ and in stimulation of IL-4 secretion, suggesting that the mechanism of action for curcumin contains a component for differential regulation of cellular immune response during inflammation similar to the effects found for lactoferrin **[19].**

The results of our investigation revealed that daily oral supplementation with nanocurcumin and curcumin helped to reduce the levels of MDA and NO and an increase in the antioxidant defence system (SOD, GPX, GSH and catalase) in the plasma and tumor of tested mice. It is possible that the protective effects are caused by strong potentiation of antioxidant protective system, supported by protection of the GSH levels depressed due to the damage induced by EC. Many authors have demonstrated that lipid peroxidation is closely associated with liver pathogenesis. MDA is a byproduct of oxidant-induced liver protein and lipid oxidation, GSH is a component of the antioxidant system. SOD represents important endogenous antioxidant and acts via dysmutation of superoxide anions. The efficacy of any anticarcinogenic molecule depends on its ability to suppress damaging effects.

The result of the present study support the findings of the previous studies as there was an increase in lipid peroxidation (MDA) in both plasma and tumor tissue, depletion of blood and tumor GSH and decrease in the measured antioxidant enzymes like GPX, SOD and catalase. The observed increase in MDA in the current study could be because Ec induced formation of free radicals and also through exhaustion of antioxidants leading to oxidative stress ***[20]***

Curcumin (CUR) is a dietary antioxidant derived from turmeric (Curcuma longa, Zingiberaceae) and has been known since ancient times to possess therapeutic properties. It has been reported to scavenge oxygen free radicals, to inhibit lipid peroxidation, and has anticarcinogenic activities in experimental models **[20-23].**It has been reported that curcumin is a bifunctional antioxidant **[24]** because of its abilityto react directly with reactive species and to induce an up-regulation of various cytoprotective and antioxidant proteins.Curcumin is ableto scavenge superoxide anion($O\dot{2}^{-}$) **[25, 26],** hydroxylradicals(OH·) and hydrogen peroxide (H2O2)**[27, 28],** singletoxygen **[28],** nitricoxide **[29],** peroxynitrite **[30]** and peroxyl radicals(ROO·) **[27].** Together,these mechanisms might explain, at least in part,some of the cytoprotective effects of this compound. As the presence of phenolic groups in the structure of curcumin explains its ability to react with reactive oxygen species (ROS) and reactive nitrogen species (RNS) and might probably be one of the mechanisms through which curcumin treatment protects the epithelial cells of renal tubules (LLC-PK1) from oxidative damage induced by H2O2 **[31].**The indirect antioxidant capacity of curcumin is defined by its ability to induce the expression of cytoprotective proteins such as hemeoxygenase1 (HO-1) **[31,32],** glutathione-S-transferase (GST),NAD(P)H:quinine oxidoreductase1(NQO1) **[33].** In summary nanocurcumin and curcumin have proved to be an effective free radical quencher.In addition, nanocurcumin in oral route might be a promising antioxidant alternative to prevent cancer.Nanocurcumin is more effective than curcumin.

In this study, Curcumin and curcumin nanoparticles exerted amelioration in treated rats. This antioxidant and ROS scavenging effects of curcumin is only due to its phenolic (-OH) group, which would inhibit the -SH group

oxidation and block thiol depletion and protects the oxidation of protein **[34].** Further it also enhances the activities of some antioxidant enzymes such as SOD, catalase and GPx. It is clear from various studies that curcumin, like many natural products, has many biological activities and is relatively safe and well-tolerated. The therapeutic effects of curcumin are mediated partially through its antioxidant and anti-inflammatory properties. Thus a safe toxicological profile of curcumin nanoparticles, indicate their potential for evaluation in vivo efficacy models and further in human trials to establish their clinical benefits as an effective therapy against pathologies **[34].**

Conclusion : Our study showed that nanocurcumin and curcumin suppresses the production of TNF-α and (IFN-γ) while increase caspase-3 and Granzyme B. Moreover, curcumin had cytotoxic effect on tested cancer cells. It is suggested that curcumin induced inhibition of cytokines may be useful in treatment of inflammatory diseases.

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Table 1. Minimum inhibitory concentration of nanocurcumin and curcumin against HCT116 and HEPG2 cell lines

	HCT116	HEPG2
Nanocurcumin	6.53 µg/ml	5.68 µg/ml
Curcumin	9.64 µg/ml	8.28 µg/ml

Table 2. The effect of nanocurcumin and curcumin on the viability of Ehrlich ascites carcinoma cells

Concentration µg/ml	***Nanocurcumin***		***Curcumin***	
	% of viable cells	***% of dead cells***	***% of viable cells***	***% of dead cells***
5	85	15	90	10
10	74	26	82	18
20	65	35	75	25
30	58	42	70	30
40	40.2	59.8	58.6	41.4
50	39	61	53	47
60	30	70	50	50
70	19	81	40	60
80	5	95	30	70
90	1	99	22	78
100	0	100	10	90

Table 3. The effect of nanocurcumin and curcumin on the tumor size of Ehrlich ascites carcinoma cells

Time / **Groups**	**1 Week** mm^3	**2 Weeks** mm^3	**3 Weeks** mm^3	**4 Weeks** mm^3
G1	**0**	**0**	**0**	**0**
G2	93.57±0.92	394.6±2.84	1639.67±5.57	2306.43±6.76
G3	58.6±0.99 *	75.73±1.77 *	978.93±4.57 *	1407.77±17.82 *
G4	81.68±1.27 *	112.22±1.77 *	1102.99±2.51 *	1636.52±2.95 *

*: significant against G1 at $P \leq 0.001$

Table (4) : The effect of nanocurcumin and curcumin on tumor markers responses of Ehrlich ascites carcinoma cells

parameters / Groups	**Caspase-3** (µmol pNA/min/ml)	**Granzyme-B** (pg/ml)	**TNF- α** (pg/ml)	**IFN-γ** (pg/ml)
G1	2.07±0.04 *	71.49±0.43 *	29.23±0.31 *	19.77±0.65 *
G2	2.93±0.06	29.29±0.5	78.34±0.5	81.28±1.08
G3	4.3±0.23 *	41.72±0.4 *	47.6±0.64 *	23.84±0.44 *
G4	3.45±0.18	47.49±1.06 *	51.5±0.78 *	32.89±0.63 *

*: very highly significant against G2 at P ≤ 0.001

Table (5): The effect of nanocurcumin and curcumin on plasma oxidative and antioxidant markers of Ehrlich ascites carcinoma cells

parameters / Groups	**Plasma MDA (mg/dl)**	**Plasma NO (mg/dl)**	**Blood GSH (mg/dl)**	**Blood GPX(µg NADPH/min/mg protein**	**Erthrocytes SOD**	**Plasma catalase**
G1	3.09±0.06 *	39.66±0.3 *	43.88±0.84 *	17.19±0.24 *	379.05±4.22 *	18.32±0.21 *
G2	7±0.08	24.29±0.3	31.49±0.17	12.46±0.28	285.27±3.32	14.15±0.22
G3	4.78±0.14 *	33.36±0.67 *	39.61±0.32 *	14.32±0.19 *	340.87±5.46 *	16±0.07 *
G4	5.76±0.09 *	31.21±0.35 *	36.95±0.22 *	14.65±0.14 *	310.85±2.53 *	15.14±0.06 *

*: very highly significant against G2 at P ≤ 0.001

Table (6): The effect of nanocurcumin and curcumin on tumor oxidative and antioxidant markers of Ehrlich ascites carcinoma cells

parameters / Groups	**Tumor MDA** ***µM/gm tissue)***	**tumor NO** ***µM/gm tissue***	**tumor GSH** ***(mg GSH/gm tissue)***	**tumor GPX (µg NADPH/min/gm tissue**	**tumor SOD (u/mg tissue)**	**Tumor Catalase** ***(µM Catalase/gm tissue)***
G1	59.63±0.28 *	50.17±0.17 *	15.48±0.3 *	5.11±0.06 *	57.64±0.7 *	42.92±0.57 *
G2	95.36±0.99	31.1±0.45	11.1±0.23	2.16±0.07	23.22±0.59	28.5±0.55
G3	63.94±0.44 *	42±0.56 *	14.04±0.1 *	4.42±0.1 *	34.69±0.4 *	36.34±0.62 *
G4	74.12±0.97 *	38.97±0.76 *	13.26±0.24 *	3.68±0.1 *	39.8±0.29 *	31.34±0.34 **

*: very highly significant against G2 at P ≤ 0.001

**: highlysignificant P ≤ 0.01