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Anti-oxidant and anti-apoptotic role of curcumin against *in vivo* induced breast cancer

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ABSTRACT

Curcumin's chemo-preventive efficacy in almost all stages of carcinogenesis has received even more attention because of curcumin's nontoxic nature. 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; against animal carcinogenesis (Ehrlich carcinoma). Results: We found that administrating of curcumin/BSA (200 mg/kg I.P.) results in a significant inhibitory effect on tumor *in vivo* represented in the reduction in the volume of the EAC and in the count of EAC cells in both preventive and therapeutic groups. An antioxidant effect of curcumin *in vivo* was observed; our results investigate a significant decrease in malodialdehyde and Nitric Oxide serum levels. Caspase-3 is an attractive therapeutic target for treatment of cancers. Overall, our results suggest that curcumin can induce apoptosis by multiple mechanisms, theses mechanism are negatively regulated by anti-apoptotic proteins Bcl-2 and caspase-3 activation. Conclusion: Curcumin has a strong inhibitory activity against tumors. The anti-tumor mechanism may be mediated by preventing oxidative damage and induction of apoptosis improved animal chances of survival and they became healthier. The results of clinical trials will be needed to spur the development of curcumin as cancer preventive and therapeutic.

KEYWORDS

Curcumin;
Ehrlich ascites carcinoma cells;
Apoptosis;
Oxidative stress;
Antioxidants.

INTRODUCTION

Cancer is considered one of the major causes of mortality in the world. Despite the recent advances in science, cancer has not been cured yet. It is estimated that by 2020 there will be 16 million new cancer cases every year[1]. 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 surgery, immunotherapy and radiotherapy[2,3]. 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]. The search for new chemo-preventive and anti-tumor agents that are more effective and less toxic has kindled great interest in phytochemicals[5].

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. We have shown previously that curcumin inhibits tumor metastasis, invasion, and angiogenesis[6-8]. Therefore, it is regarded as a high potential to develop into modern drug. Curcumin in its free form is poorly absorbed in the gastrointestinal tract and therefore may be limited in its clinical efficacy[9]. In the pre- research it had been further confirmed that curcumin has antioxidant activity *in vitro* and it can enlarge strength of mice[10]. 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. Preclinical studies have revealed the chemo-preventive potential of curcumin in several different animal tumor bioassay systems, including colon[11], duodenal, stomach[12], prostate[13], and breast[14] carcinogenesis, both *in vitro* and *in vivo*.

Curcumin acts as a scavenger of oxygen species, such as hydroxyl radical, superoxide anion, and singlet oxygen, and it interferes with lipid peroxidation. Curcumin inhibits the induction of nitric oxide synthase in activated macrophages and has been shown to be a potent scavenger of free radicals like nitric oxide. Curcumin treatment showed antitumorigenic potential by significantly reducing the levels of inducible nitric oxide synthase (NOS)[15].

Curcumin's chemo-preventive activity in animal model systems has led investigators to study its potential impact upon tumor cell growth and apoptosis. Curcumin suppresses a number of key elements in cellular signal transduction pathways pertinent to growth, differentiation, and malignant transformation[16]. Much of its beneficial effect is found to be due to its inhibition of the transcription factor nuclear factor kappa B (NF-kappa B) and subsequent inhibition of pro-inflammatory pathways. Curcumin prevents phosphorylation and degradation of inhibitor kappaBalpha, thereby blocking NF-kappaB activation, which results in down-regulation of iNOS gene transcription[17]. Curcumin activated caspase-8, induced BID cleavage, caused mitochondrial cytochrome *c* release and induced caspase-3 activation. Also, the ectopic expression of Bcl-2 inhibited both upstream and down-stream steps involved in curcuim-induced apoptosis[5].

MATERIALS AND METHODS

Animals

Female Swiss albino mice of 8 weeks of age, weighed 22 to 25 g body weight 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.

Tumors

Ehrlich ascites carcinoma (EAC) was initially supplied by the National Cancer Institute, Cairo, Egypt, and maintained in female Swiss albino mice through serial intraperitoneal (I.P) inoculation at 8 or 10 day intervals in our laboratory in an ascites form.

Curcumin

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

Experimental design

Mice were divided into four groups, each group includes 10 mice. Group I, were received a saline solution by I.P. injection represented as negative control group, Group II, were received EAC cells (2×10^6 cells/mouse) by I.P. injection for 10 days served as positive control group. Group III, were injected I.P. with curcumin one day before EAC (tumor inoculation) represented as preventive group, Group IV, were injected I.P. with EAC (2×10^6 cells/mouse), represented as therapeutic group, then curcumin was injected at 1,3,5,7,9 days of EAC injection for 10 days to the last two groups.. The mice of four groups were maintained

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under the same conditions and were carefully observed to the end of experiment.

After the experiment, the blood samples and EAC cells were collected from mice for determination anti-oxidants assays, Caspase-3 activity, Bcl-2 percentage, and cytological study.

Cell viability and counting of EAC cells

The viability of EAC cells was determined by the Trypan Blue Exclusion Method[18], where the total and viable cells (non-stained) were counted in thomacytometer at magnification ×40; as the number of cells/ml was determined in the studied groups.

Estimation of nitric oxide in serum

The Biodiagnostic Nitrite Assay Kit provides an accurate and convenient method[19] for measurement of endogenous nitrite concentration as indicator of nitric oxide production in biological fluids. 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.

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[20].

Caspase-3 colorimetric assay

The activity of caspase-3 was determined by the colorimetric caspase-3 kit according to the manufacturer's instructions (R& D system, Inc.)[21]. This assay is based on spectrophotometrically detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate7-amino-4- trifluoromethyl coumarin conjugated *p*NA (DEVD-*p*NA) in equal amount of cells protein lysates. Briefly, 1×10^6 cells were collected and lysed with 50 µl of chilled lysis buffer and incubated on ice for 10 min. Cell lysates were centrifuged at maximum speed for 5 min at 4 °C, after which 50 µl of 2× reaction buffer/dithiothreitol (DTT) mix and 5 µl of 1 mM caspase-3 substrate (DEVD-pNA) were added to each reaction and incubated at 37 °C for 1 hr. The *p*NA light emission was quantified using a microplate reader at 400- or 405- nm.

Determination of Bcl-2 percentage in cells by flowcytometry

The apoptosis rate was measured by flowcytometry assay (Santa Cruz Biotechnology, inc.) through determination of Bcl-2 expression in cells[22]. Immunohistochemical staining was adopted to test the protein expression of bcl-2 in the hepatic tissues of the rats and EAC cells of mice. After bcl-2 protein expression of hepatic tissues and EAC cells was observed under light microscope, the comprehensive judgment was carried out based on the percentage of positive cells.

Cytological study of EAC cells

The EAC cells obtained from the ascitis fluid by centrifugation were smeared on 3 glass slides. Then the air-dried smears were fixed in 70% ethanol and covered by Giemsa solution (0.1%). After exactly 20 minutes, the slides were removed quickly and washed under running tap water, and examined under an electric microscope.

Statistical analysis

All results were analyzed by SPSS software (version 14)[23]. 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 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.

RESULTS

Effect of curcumin on the viability and counting of EAC cells

TABLE 1 summarizes the effect of curcumin as chemo-preventive natural product; where Curcumin administration results in a significant reduction of EAC growth *in vivo*; indicating decrease in the volume of EAC by 51.44% and 58.85% in the therapeutic and

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preventive groups, respectively; compared to positive control group; as shown in Figure (1a); and a significant decrease in the count of EAC by 51.85% and 56.28 % in the therapeutic and preventive groups, respectively; compared to positive control ($p<0.05$); as shown in Figure (1b).

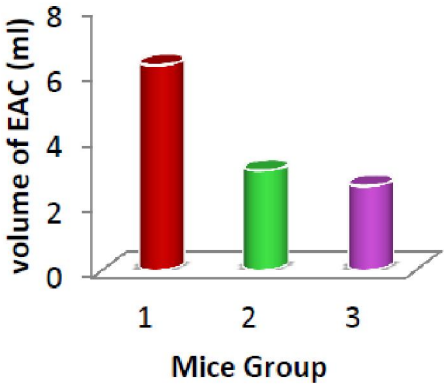

Figure 1a : Effect of curcumin on the volume of EAC in mice groups. (1: positive control, 2: therapeutic, 3: preventive).

TABLE 1 : Effect of curcumin on the volume and count of EAC in the studied groups.

Parameter	Group II	Group III	Group IV
Volume of Ascitis Fluid (ml)	6.26 ± 1.24	3.04±0.49**	2.57±1.05**
Count of EAC cells ($\times 10^6$)	126.52±12.13	60.92±4.31**	55.31±19.63**

The significant difference: $P^{**} < 0.01$ → high significant $P^{*} < 0.05$ → significant

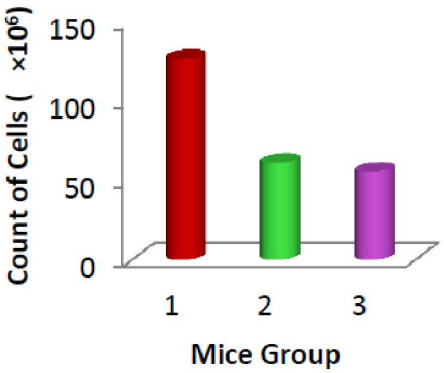

Figure 1b : Effect of curcumin on the count of EAC cells in mice groups. (1: positive control, 2: therapeutic, 3: preventive).

Effect of curcumin on serum concentrations of NO and MDA

TABLE 2 summarize the results of NO and MDA in serum among the four groups of animals. In mice groups, there was a highly significant increase in NO and MDA levels in group II compared to group I [59.46±5.83 (µmol/l) vs. 14.07±1.75 (µmol/l) and 14.61±0.58 (nmol/ml) vs. 7.31±0.70 (nmol/ml); respectively] ($P<0.05$). Curcumin has been shown to exhibit antioxidant; NO level showed a significant decrease in group III & group IV by 74.92% and 75.38%; respectively. Also there was a significant decrease in MDA levels in group III & group IV by 49.69% and 55.44%; respectively, compared to group II; as represented in Figure 2.

TABLE 2 : Effect of curcumin on antioxidants activity of curcumin in serum in the studied groups.

Parameter	Group I	Group II	Group III	Group IV
Nitric Oxide (NO) (µmol/l)	14.07±1.75	59.64±5.83	14.91±3.57**	14.64±2.23**
Malondialdehyde (MDA) (nmol/ml)	7.31±0.70	14.61±0.58	7.35±0.46**	6.51±0.86**

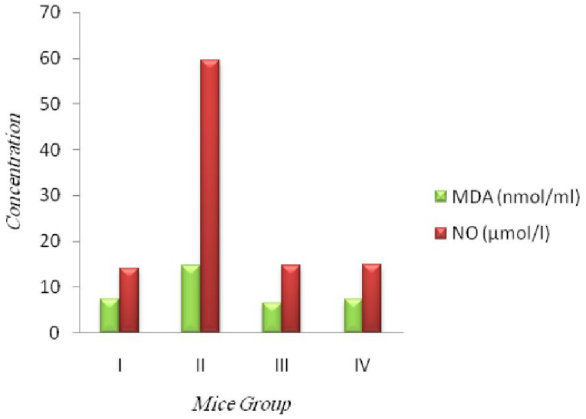

Figure 2 : Antioxidants activity in mice groups.

Effect of curcumin on caspase- 3 activation

Caspase-3 activity was increased by -105.2% and -106.94% in the therapeutic and preventive groups, respectively compared to its activity in the positive control group; as illustrated in the Figure 3, and TABLE 3.

TABLE 3 : Caspase-3 activity and Bcl-2 percentage expression in the studied different studied groups.

Parameter	Group II	Group III	Group IV
Caspase-3 (ng/ml)	1.73±0.32	3.55±0.32	3.57±0.31
Bcl2 %	78.85 ± 5.98	56.0±1.63	45.3±2.71

The significant difference: $P^{**} < 0.01$ → high significant $P^{*} < 0.05$ → significant

Percentage of Bcl-2 in cells by flowcytometry

TABLE 3 summarize flowcytometric studies show that, curcumin down-regulates the expression of bcl-2

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significantly by 28.95% and 42.51 % in the therapeutic and preventive groups, respectively ($p < 0.01$) compared to the positive control group, as illustrated in EAC cells (Figure 4).

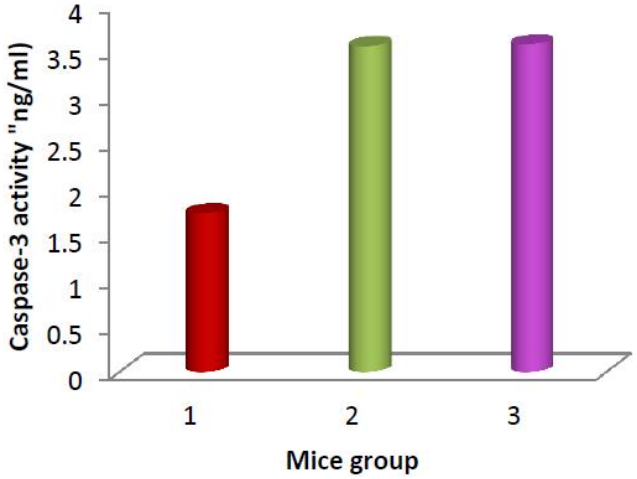

Figure 3 : Effect of curcumin on the caspase-3 activity in EAC in mice groups. (1: positive control, 2: therapeutic, 3: preventive).

Cytological studies of EAC in different groups

Cytological studies using Giemsa staining methods revealed that, Curcumin induces a significant decrease in mitotic cells in EAC compared to the increase in number of mitotic cells in positive control. The number of apoptotic cells was high in the groups injected with Curcumin "preventive and therapeutic groups"; as illustrated in Figure (5 a, b, and c), respectively.

Correlations between different studied parameters among different groups

In studied groups, there were significant positive correlations between volume and count, MDA, NO, and Bcl-2; ($r = 0.864$, $r = 0.862$, $r = 0.868$, $r = 0.825$; respectively) ($p<0.01$). Also, there were positive correlations between count and MDA, NO, and BCl2; ($r = 0.919$, $r = 0.918$, $r = 0.886$; respectively) ($p<0.01$). In the studied groups, there was a positive correlation between serum MDA and NO ($r=0.968$, $p<0.01$). Also, there was a positive correlation between MDA and Bcl-2 ($r = 0.949$, $p < 0.01$). There was a positive correlation between NO and BCl2 levels in the studied groups ($r = 0.930$, $p<0.01$). While there were significant negative correlations between Caspase-3 and other parameters volume, count, MDA, NO, and Bcl-2; ($r = -0.785$, $r = -0.909$, $r = -0.918$, $r = -0.933$, and $r = -0.904$, respectively; $p<0.01$).

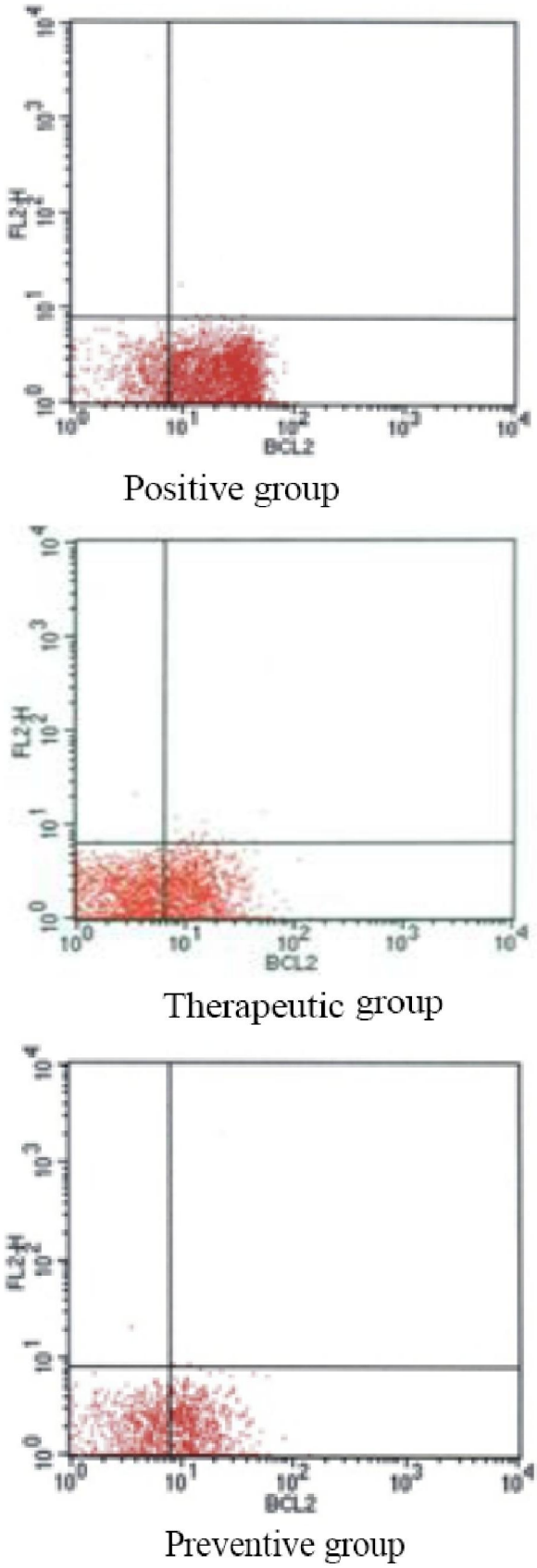

Figure 4 : Dot plot display effect of curcumin on the BCl2 expression in mice in tumor and treated groups.

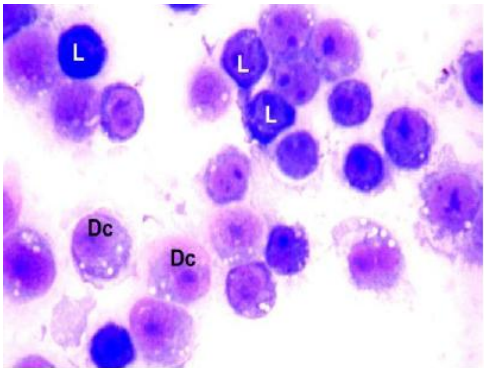

Figure 5a : Photomicrographs of untreated EAC cells treated (Positive control). (L= life cell; Ac= apoptotic cell; Dc= degenerative cell).

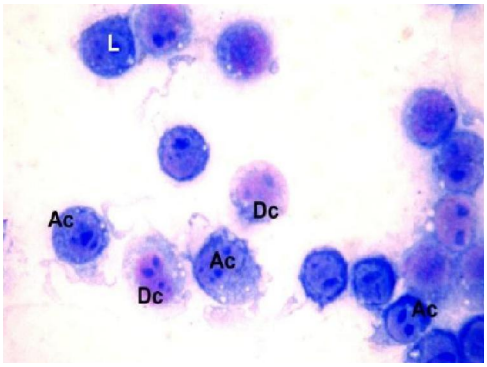

Figure 5b : Photomicrographs of EAC cells in therapeutic group. (L= life cell; Ac= apoptotic cell; Dc= degenerative cell).

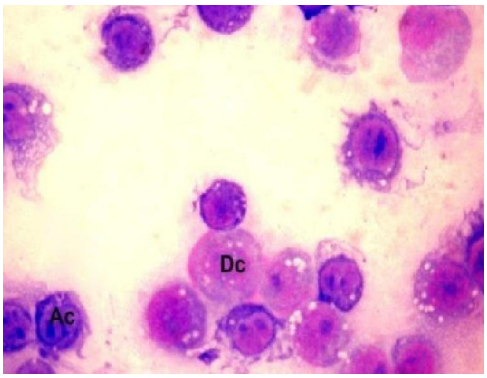

Figure 5c : Photomicrographs of EAC cells in preventive group. (L= life cell; Ac= apoptotic cell; Dc= degenerative cell).

DISCUSSION

Cancer is considered one of the most common causes of morbidity and mortality worldwide. The target of much research has been on the discovery of natural and synthetic compounds that can be used in the prevention and/or treatment of cancer. Natural products of either plant or animal origin that exhibited antitumor activities have been discovered[24]. Curcumin is the active constituent of *CURCUMA LONGA* (turmeric). It is used as a spice, food preservative and herbal medicine. 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[25]. This restricts their systemic availability in orally administered formulations and limits their therapeutic potential. Solubility of curcumin is very important and its incensement of its solubility using bovine serum albumin. Curcuminoids were highly soluble in solutions of purified albumin, a major component of serum[26]. Also, several studies recently confirm the high affinity nature of curcumin binding to BSA[27]. Our results revealed that, the curcumin decrease the volume of EAC significantly in the therapeutic and preventive groups by 51.44% and 58.85 %, respectively. Also, it reduces the count of EAC cells significantly in both groups by 51.85% and 56.28 %, respectively compared to positive control group (bearing EAC), as shown in Figure (1 a, b); TABLE 1. 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*[28]. Curcumin may also operate through the suppression of NF-›B activation, where this factor required for the expression of genes involved in cell proliferation, cell invasion, metastasis, angiogenesis, and resistance to chemotherapy[29]. Johann *et al.*,[30] who demonstrated that curcumin *in vivo* administration of curcumin (40–80 mg/kg b.w) can inhibit tumor growth, tumor metastasis on EAT cells. Also, Thippeswamy and Salimath[31], study the effect of *Curcuma aromatica* extract to mice transplanted with EAT cells, effect on *in vivo* growth and proliferation of EAT cells is more evident in the cell number and ascites volume obtained from both control and treated animals.

Anti-oxidant effect of Curcumin/BSA showed a significant decrease in MDA by (49.69% and 55.44%; respectively, $p<0.01$); and NO levels by (74.92% and 75.38%; respectively, $p<0.01$) in the therapeutic and preventive groups compared to EAC group, as shown in Figure 2, and TABLE 2. Curcumin was found to be a very potent anti-oxidant. As, the phenolic (-OH) plays a major role in the anti-oxidant activity of curcumin[32].

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Curcumin has also been shown to quench reactive oxygen species and scavenge superoxide anion radicals and hydroxyl radicals and strongly inhibits NO production by down-regulating inducible nitric oxide synthase gene expression[33]. Curcumin inhibit free radical generation and act as free radical scavengers and antioxidants, inhibiting lipid per-oxidation and oxidative DNA damage, with abilities to inhibit activation of NF-›B[34]. By performing detailed *in vitro* antioxidant assays the authors also demonstrated effective radical scavenging properties of curcumin[35]. Also, our results agree with Giselle *et al.*,[36] who suggest that, Curcumin is several times more potent than vitamin E as a free radical scavenger, protects the brain from lipid per-oxidation, and scavenges NO-based radicals. Our study has shown that curcumin was effective in imparting growth inhibition, cell cycle deregulation and apoptosis in EAC cells. It is now well recognized that whether a cell becomes committed to apoptosis partly depends upon the balance between proteins that mediate growth arrest and cell death. Enhancement of the apoptotic potential of tumor cells increases tumor responses to chemotherapy. TABLE 3 showed the apoptotic and anti-apoptotic effects of curcumin, where the preclinical and clinical I.P. injections of curcumin in EAC inoculation result in a significant increase in Caspase-3 activity in the therapeutic and preventive groups by (-105.2% and -106.94%; respectively, $p<0.01$) compared to group II, as shown in Figure 3. While, the flowcytometric results of EAC revealed the levels of Bcl-2 expression was significantly increased (78.90±1.66 %). Interestingly, the level of Bcl-2 decreased upon curcumin treatment by 28.95 % and 42.51 % in the therapeutic and preventive groups, respectively compared to positive control group (II) as illustrated in Figure 4. As Curcumin can induce apoptosis by different mechanisms, such as, by inhibiting the expression of the anti-apoptotic genes bcl-2 and bcl-xL, by inhibiting AP-1 and NF-›B transcription factors[37]. Physiologically bcl-2 protein blocks the apoptotic process by inhibiting the release of cytochrome C from mitochondria whereas it locates at the cytoplasmic surface of the mitochondrial membrane[38]. Recently, ROS has been shown to down-regulate bcl-2 expression, thereby sensitizing the cells to apoptotic death[39]. Apoptotic signals provoke cytochrome c release from the mitochondria into the cytoplasm where it associates with Apaf-1 (apoptosis activating factor 1) that recognizes the inactive pro-caspase 9 and forms the apoptosome, which triggers autocatalytic processing of pro-caspase 9. In turn, active caspase 9 activates downstream executioner caspases. Caspase 3 is the ultimate executioner caspase that is essential for the nuclear changes associated with apoptosis, including chromatin condensation[40]. Caspase-3 is a downstream effector cysteine protease in the apoptotic pathway. Our data were in a line with Woo et al.[41] who suggested that the induction of Caki (human kidney carcinoma cells) programmed cell death is activated by Akt dephosphorylation, Bcl-2, Bcl-XL and inhibitor of apoptosis (IAP) protein inhibition, as well as cytochrome c release and caspase 3 activation.

Cytogenetic study of EAC in mice groups indicates that, curcumin induces apoptosis. Staining methods revealed a significant increase in number of mitotic cells in EAC untreated group (positive control) (Figure 5 a). Curcumin induces significant decrease in mitotic cells in EAC (treated groups) as illustrated in Figure (5 b and c). Holy[42] reported disruption of mitotic spindle structure and induction of micro-nucleation in human breast cancer cells by curcumin "yellow pigment".

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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