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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and evaluation of curcumin analogues as cytotoxic agents

Ahmed A. Fadda · Farid A. Badria · Khaled M. El-Attar

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Abstract Seventeen curcumin analogues were prepared and evaluated for in vitro and in vivo cytotoxicity against an Ehrlich ascites carcinoma (EAC). In vitro results revealed that compounds **10**, **7**, and **12** were the most potent analogues against EAC respectively. However, in vivo evaluation of compound **10** proved its capability to normalize the blood picture compared with 5-fluorouracil, a well-known anticancer drug.

Keywords Hydroxycurcuminoids · Synthesis · Ehrlich ascites in vitro · Antitumor

Introduction

Curcumin (**1**) is a natural yellow-orange dye extracted from the rhizomes of the plant *Curcuma longa* L. *(zingiberaceae)* (Jasim and Ali, 1988). A variety of pharmacological properties, such as anticancer (Kuttan *et al*., 1985; Soudamini and Kuttan, 1989) activities, have been reported. It also has been used as a photodynamic agent for the destruction of bacteria and tumor cells (Dahl *et al*., 1989; Pervaiz *et al*., 1990). From toxicological studies, curcumin is nontoxic even at high dosage (Tonnesen, 1986). Attempts to use it as an antioxidant additive for lubricants and motor oils, photoresists, and sunscreen compounds have been made (Martin and Rajaratnam, 1988; Nambudiry and Natraj, 1991; Sharma, 1976). Well-known anti-cancer drugs, e.g., tamoxifen, dexamethasone, cyclophosphamide, and

A. A. Fadda (✉) · K. M. El-Attar
Chemistry Department, Faculty of Science, Mansoura University, Mansoura 35516, Egypt
e-mail: afadda2@yahoo.com

F. A. Badria
Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

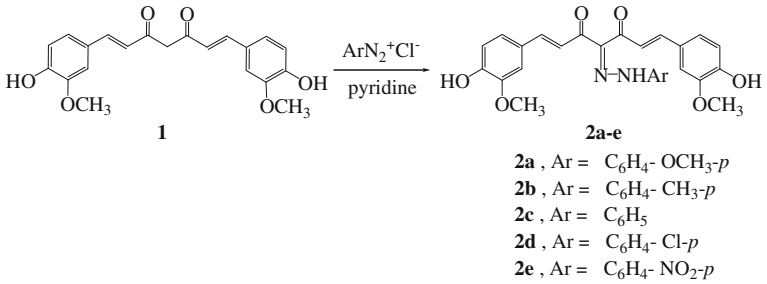
methylprednisolone, have been reported to induce leukopenia and other toxic manifestations to the host (Bergan *et al*., 1999; Steele, 2002; Varshavsky, 1998; Wolff *et al*., 1998). Curcumin (diferuloylmethane), the active component of turmeric (*Curcuma longa* L.), has been widely used as an anti-inflammatory, antioxidant, and chemotherapeutic agent (Ammon and Wahl, 1991).

It has been recognized for chemopreventive properties in breast, skin, and fore stomach carcinogenesis (Choudhuri *et al*., 2002; Kawamori *et al*., 1999). Interestingly, turmeric has been used in a large quantity as a condiment for thousands of years with no adverse reactions, thereby indicating its candidacy as probable nontoxic, anticancer agent (Choudhuri *et al*., 2002). This active phytochemical has an immunomodulatory property and stimulates macrophage phagocytic activity (Antony *et al*., 1999). Curcumin protects thymocytes from dexamethasone-induced apoptosis (Jaruga *et al*., 1998). It also is able to effectively quench singlet oxygen at a very low concentration in aqueous systems and is a powerful inhibitor of hydrogen peroxide damage in human keratinocytes and fibroblasts (Das and Das, 2002; Phan *et al*., 2001; Pal *et al*., 2005). In vivo and in vitro studies have demonstrated curcumin's ability to inhibit carcinogenesis at three stages: tumor promotion, angiogenesis, and tumor growth. Curcumin suppresses mitogen-induced proliferation of blood mononuclear cells, inhibits neutrophil activation and mixed lymphocyte reaction, and inhibits both serum-induced and platelet-derived growth factor (PDGF)-dependent mitogenesis of smooth muscle cells (Huang *et al*., 1992). It has been reported to be a partial inhibitor of protein kinase (Liu *et al*., 1993; Reddy and Aggarwal, 1994). The other salient feature of turmeric/curcumin is that despite being consumed daily for centuries in Asian countries, it has not been shown to cause any toxicity (Ammon and Wahl, 1991). Although a number of excellent reviews on curcumin are available, this short review specifically focuses on the antioxidant, wound healing, anti-angiogenic, and anti-cancer effects of turmeric/curcumin (Maheshwari *et al*., 2006). Recently numerous studies have demonstrated the remarkable cancer preventive properties of curcumin. The chemopreventive effects of curcumin have been attributed to various biological properties, including neutralization of carcinogenic free radicals and anti-angiogenesis action, which limits the blood supply to rapidly growing malignant cells (Chandru *et al*., 2007; and references cited therein). In a continuing search for potent and selective cytotoxic antitumor agents, we synthesized **17** curcumin analogues (**1**–**17**) and evaluated their cytotoxic effects against an Ehrlich ascites cells.

Chemistry

Results and discussion

The designed target compounds are depicted in Schemes (1, 2, 3, 4). Derivatives of hydroxy arylazobenzene are well known as dyes (Daris and Muchowski, 1975; Gilman and Stern, 1957; Gilman and Stern, 1960). Consequently, the authors decided to couple the diazonium salt of different aromatic amines with curcumin (**1**) with a view to synthesis new azodisperse dyes to explore the possibility of finding



Scheme 1

some new azodyes capable of dyeing different types of fibers and have expected wide spectrum of biological activity. Diazonium salts undergoes a coupling reaction with curcumin (**1**) to give the corresponding 4-arylazo derivatives (**2a–e**). In accordance with the nomenclature of arylazo curcumin, the general structure formula for 1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-4-arylazo-3,5-dione (**2a–e**) should be as follows:

From the infrared measurements of the prepared arylazo derivatives (**2a–e**), presence of strong bands in the region 1550–1580 cm^{-1} indicating the (–N=N–) stretching frequency and the (NH) absorption was recorded at 3250 cm^{-1} besides the (OH) stretching at 3400 cm^{-1}, and in a tautomeric mixture (Daris and Muchowski, 1975) as well. Furthermore, the α,β-unsaturated carbonyl function appeared at 1690 cm^{-1}.

Generally, variation in color of these dyes results from the alternation in the diazonium components. The UV spectra of the diazonium coupling products of (**1**) provide additional evidence that such compounds have the tautomeric relation with azo-hydrazo system. Most of the dyes show four absorption bands in the region 196–438 nm. The relatively small differences in λ_{max} may be due to the polarity change of the absorbing system caused by solvent interactions due to the general solvent effect (Gilman and Stern, 1957). It has been reported that UV spectra of monophenyl azo compounds differ from those of monophenyl hydrazones. The azo compounds generally show two absorption bands at 400–410 and 290–300 nm corresponding to n–π^* and π–π^* transitions respectively (Gilman and Stern, 1960). On the other hand, monophenyl hydrazones show three intense bands at 220–230, 250–280, and 330–390 nm regions (Gilman and Stern, 1957). The UV spectra of compounds (**2a–e**) investigated can be interpreted in terms of the tautomeric mixture as well. It is clear that these dyes exhibit four bands; of these, the medium and high wavelength bands seem to be affected by the nature of the polar substituents in the arylazo group, and the low wavelength bands is unaffected. This indicates the presence also of hydrazone structure where the resonance interactions with the substituents in the diazo component are minimal due to steric factors.

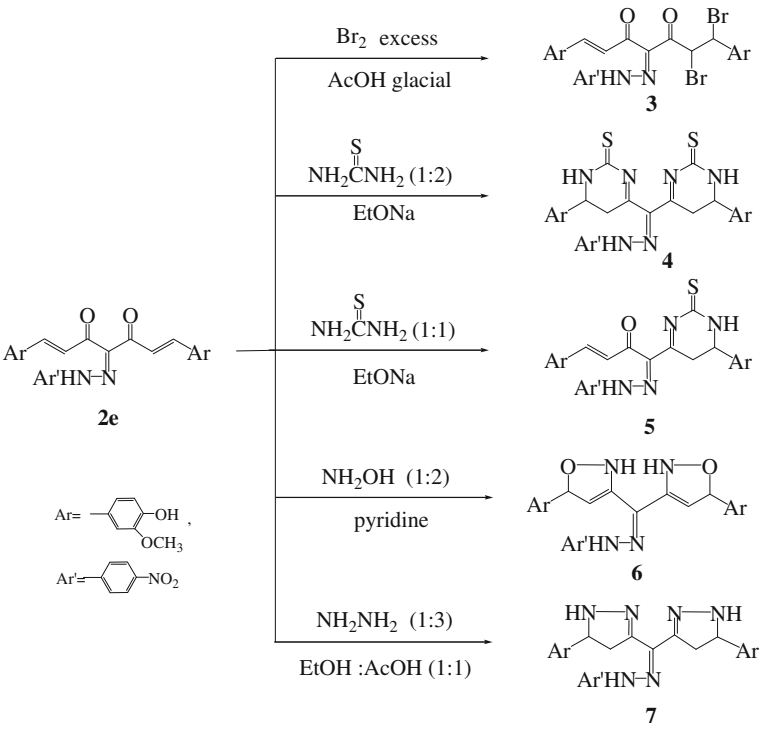
Treatment of 1,7-bis-(4-hydroxy-3-methoxy-phenyl)-4-[(4-nitro-phenyl)-hydrazono]-hepta-1,6-diene-3,5-dione (**2e**) with bromine in the presence of glacial acetic acid afforded the corresponding 6,7-dibromo-1,7-bis-[3-(4-nitrophenylazo)-4-hydroxy-5-methoxy]hepta-1-ene-3,5-dione (**3**).

Condensation of (**2e**) with thiourea was performed in molar ratio (1:2), respectively, in boiling ethanolic sodium ethoxide to give the corresponding (*Z*)-4-(4-hydroxy-3-methoxy-phenyl)-6-[{6-(4-hydroxy-3-methoxy-phenyl)-2-thioxo-1,2,5,6-tetrahydro-pyrimidine-4-yl}(2-(4-nitrophenyl)hydrazono)methyl]-5,6-dihydro-pyrimidine-2*(1H)*-thione (**4**). Structure (**4**) was confirmed by its IR spectrum, which showed the absence of spectrum band in the region of 1600 and 1690 cm^{-1}, i.e., α,β-unsaturated ketone was involved in the cyclocondensation process, and in the same time the IR spectrum showed new bands (cf. experimental part).

Moreover, (1*Z*,3*E*)-4-(4-hydroxy-3-methoxy-phenyl)-1-[6-(4-hydroxy-3-methoxy-phenyl)-2-thioxo-2,3,4,5-tetrahydro-pyrimidine-4-yl]-1-[2-(4-nitrophenyl)hydrazono]-but-3-ene-2-one (**5**) was prepared by refluxing compound (**2e**) with thiourea (1:1) molar ratio in boiling ethanolic sodium ethoxide. Structure (**5**) was deduced from both correct analytical and spectral data (cf. experimental part).

Similarly, it has been found that *N*-[bis-(5-(4-hydroxy-3-methoxy-phenyl)-2,5-dihydro-isoxazol-3-yl)-methylene]-*N′*-(4-nitro-phenyl)-hydrazine (**6**) has been prepared by reaction of (**2e**) with hydroxylamine hydrochloride in refluxing pyridine.

As an extension of our interest in the synthesis of new heterocycles incorporating a pyrazole nucleus (Metwally *et al*., 1985), we report the behavior of (**2e**) toward hydrazine hydrate and/or its derivatives as a facile and convenient route to some heterocyclic derivatives containing a pyrazole moiety. Therefore, the reaction of



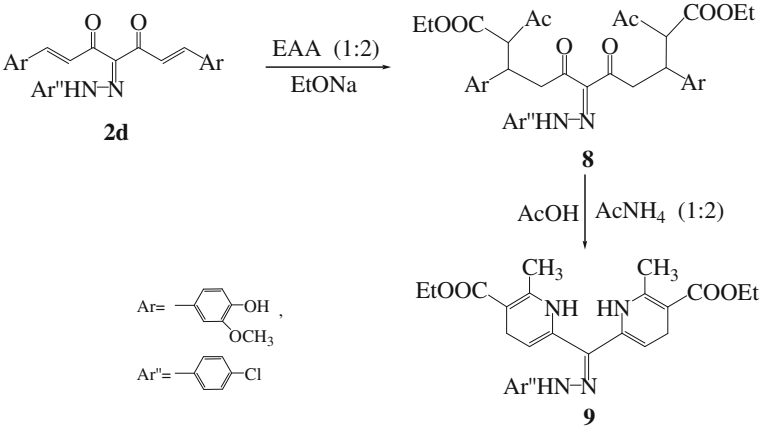
Scheme 2

diferuloyl-(4-nitrophenyl)methane (**2e**) with hydrazine hydrate in (1:3) molar ratio in boiling mixture of ethanol-glacial acetic acid afforded the bis pyrazolyl derivative (**7**). The structure of (**7**) was established by both elemental and spectral data. The H^1-NMR spectrum revealed the absence of signals at δ 6.5 and 7.5 of olefinic protons and instead appeared signal at δ 3.9 due to the methylene protons of pyrazole ring. The mass spectrum showed m/z 545 (M^+).

In connection of our search toward the design and synthesis of heterocyclic analogues of curcumin as potential chemotherapeutic agents, it was decided to take 4-[(4-chlorophenyl)-hydrazono]-1,7-bis-(4-hydroxy-3-methoxy-phenyl)hepta-1,6-diene-3,5-dione (**2d**) as adaptable starting material for the synthesis of new heterocyclic binary system with the hope that the new compounds may exert more potent effect. We report the synthesis of a new bis pyridyl derivative. Thus, it was found that compound (**2d**) reacted with ethyl acetoacetate in boiling ethanolic sodium ethoxide in (1:2) molar ratio, respectively, to afford the Michael adduct 2,10-diacetyl-6-[(4-chloro-phenyl)-hydrazono]-3,9-bis-(4-hydroxy-3-methoxy-phenyl)-5,7-dioxo-undecanedioic acid diethyl ester (**8**).

The Michael adduct (**8**) was then subjected to react with excess ammonium acetate in boiling glacial acetic acid to give the corresponding (*Z*)-ethyl-6-{2-(4-chloro-phenyl)hydrazono}[4-(4-hydroxy-3-methoxy-phenyl)-6-1,4-dihydropyridin-2-yl]-4-(4-hydroxy-3-methoxy-phenyl)-2-methyl-1,4-dihydropyridine-3-carboxylate (**9**) (cf. experimental part).

In addition, it was found that diketone moieties of curcumin were replaced with hydrazine hydrate in refluxing absolute methanol; triethylamine and catalytic amounts of glacial acetic acid were added. One step coupling of (**1**) with hydrazinium dihydrochloride gave compound (**10**) (Joong *et al*., 2002; Ishida *et al*., 2002; Flynn *et al*., 1991). The compound was purified by crystallization from ethanol to give dark red crystals, which was analyzed for $C_{21}H_{20}N_2O_4$ by elemental analysis. 4,4′-(1*E*,1′*E*)-2,2′-(1*H*-Pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-[2-(4-chloro-phenyl)]diazenyl-6-methoxy-phenol (**10**) was proven on the basis of both analytical and spectral data.



Scheme 3

In continuation of our study, we report the use of compound (**10**) as a key intermediate for the synthesis of new series of curcumin derivatives, which have not been investigated so far. Selective *N*-bromination of (**10**) using *N*-bromosuccinimide in chloroform gave a gummy bromopyrazoline derivative (**11**), which was converted directly into the corresponding 4,4′-(1*E*,1′*E*)-2,2′-(1-hydrazinyl-1*H*-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-(2-methoxy-phenol) (**12**). Structure of compound (**11**) was elucidated by the formation of compound (**12**). Structure (**12**) was established on the basis of both analytical and spectral data. The IR spectrum of compound (**11**) showed the absence of band at 3230 cm^{-1} due to the absorption frequency of (NH) and instead appeared new band at 720 cm^{-1} due to (N-Br) absorption.

N-Nitrosopyrazoline derivative (**13**) also was obtained on treating (**10**) with sodium nitrite and hydrochloric acid—the structure that was confirmed from IR, H^1-NMR, and mass spectra. The absence of absorption due to (NH) group and the appearance of new absorption band at δ (1580 cm^{-1}) in the IR spectrum of (**13**) substantiate the assigned structure of this product.

Moreover, it has been found that fusion of (**10**) with chloroacetyl chloride at 95°C for 2 h afforded the corresponding chloroacetyl derivative (**14**). The IR spectrum of (**14**) showed absorption at 2900, 1685, and 760 cm^{-1} due to (CH_2), (CO), and (C–Cl) functions. The H^1-NMR showed singlet signal at δ 3.5 ppm of (CH_2) protons, in addition to the other expected signals (cf. experimental part).

It was found that the pyrazole derivative (**10**) was subjected to coupling reaction with *p*-chlorophenyl diazonium chloride in (1:2) molar ratio afforded the corresponding 3,5-bis-[β-(5-{4-chloro-phenylazo}-4-hydroxy-3-methoxy-phenyl)-ethynyl]-1*H*-pyrazole (**15**). Structure of compound (**15**) was established on the basis of both elemental and spectral analysis.

Reaction of 3,5-bis-[β-(5-{4-Chloro-phenylazo}-4-hydroxy-3-methoxy-phenyl)-ethynyl]-1*H*-pyrazole (**15**) with formamide in methanol gave 3,5-bis-{β-[3-(4-chloro-phenylazo)-4-hydroxy-5-methoxy-phenyl]-ethynyl}-pyrazole-1-formyl (**16**) (cf. experimental part).

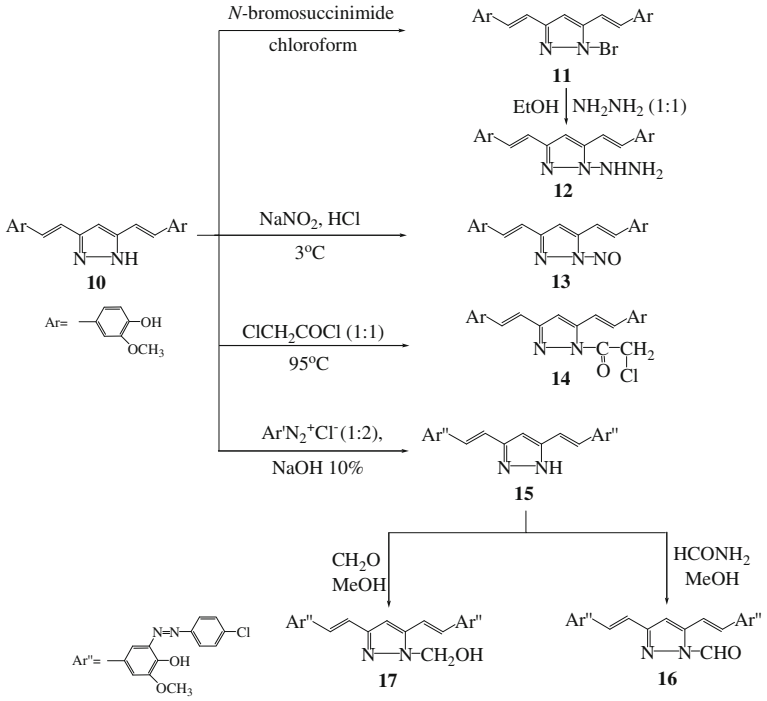
Treatment of 3,5-bis-[β-(5-{4-Chloro-phenylazo}-4-hydroxy-3-methoxy-phenyl)-ethynyl]-1*H*-pyrazole (**15**) with formaldehyde in methanol afforded 3,5-bis-[β-(5-{4-Chloro-phenylazo}-4-hydroxy-3-methoxy-phenyl)-ethynyl]-2-hydroxy-methyl-pyrazole (**17**) (cf. experimental part).

Biological activity

Discussion

Effect of drugs on the viability of Ehrlich ascites cells in vitro

To examine whether these substances have a direct cytotoxic effect on Ehrlich ascites cells (EAC) viability, the percentage of viable cells was estimated by the trypan blue (Sheeja *et al*., 1997) exclusion test. The desired concentration of tumor cells (2×10^6 cells per 0.2 ml) was obtained by dilution with saline (0.9% sodium



Scheme 4

chloride solution). Viability of tumor cells obtained and used in this experiment was always greater than 90%. Below this percentage, the cells were discarded and the entire procedure was repeated. Seventeen 1,3-diketo-propane and related analogues (**1**–**17**) were tested for cytotoxicity against EAC in vitro. Results for the ED_{50}, ED_{25} and ED_{10} values of the active compounds are summarized in Table 1. Compounds **7** and **12** displayed moderate cytotoxicity, whereas (**10**) was the most potent with ED_{25}. The other rest of compounds showed weak to no activity. Thus, it would appear that introducing pyrazole moiety enhances the cytotoxic properties. By comparing the cytotoxicity results in Table 1, the following structure-activity relationships (SARs) were drawn: (a) Converting the keto-enol moiety to the corresponding pyrazole (**10**, **7**, and **12**) led to increase cytotoxicity against EAC. Compound 10 was the most active, whereas compounds **7** and **12** exhibited good activity. Thus, the ring substituents affected the activity in the pyrazole derivatives. (b) All halogenated derivatives (**2d**, **3**, **8**, **14**–**17**) were very weak or completely inactive at (ED_{25}). Thus, the position and nature of substituents on the structure of curcumin seem to modulate antitumor activity. The reliable criteria for judging the value of any anticancer drug are prolongation of life span and decrease of WBC from blood (Clarkson and Burchenal, 1965; Oberling and Guerin, 1954). The results of the present study showed an antitumor effect of compounds (**1–17**) against EAC in Swiss albino mice (Table 2).

Table 1 In vitro cytotoxicity of curcumin analogues using EAC assay

Compound	Dead (%)		
	$ED_{50} \times 10^3$ μM	$ED_{25} \times 10^3$ μM	$ED_{10} \times 10^3$ μM
5-flu	96.3	0	0
1	100	4.8	3.4
2a	100	3.4	0
2b	100	0	2.4
2c	94.1	0	0
2d	100	3.2	0
2e	100	2.8	2.5
3	96.1	3.2	0
4	100	4.8	0
5	100	0	2.9
6	100	5.3	0
7	100	38.9	0
8	100	0	0
10	100	66.7	0
12	100	22.2	0
13	28.6	6.7	0
14	100	0	0
15	100	0	0
16	100	0	0
17	100	3.6	0

ED is the concentration of compounds in μM

Table 2 In vivo cytotoxicity of compound 10 using EAC assay

Test group	Hb 12–16 g/dl	HCT (hematocrit) 35–50%	WBCs 4,000–11,000/cmm	Ehrlich cells count mil/ml	MST/day
Normal[a]	13.9	53.6	8.4	–	–
Control[b]	8.7	35.5	38.6	220	9.1
5-fluorouracil	10	42.3	13.8	123	16.7
Compound (**10**)	9.4	41	13.8	169.6	14.6

[a] Normal test: without EAC

[b] Control test: with EAC

Effect of compound 10 on survival time

The mean survival time (MST) of each group consisting of seven mice was noted (Sur and Ganguly, 1994). The antitumor efficacy was compared with that of 5-fluorouracil (5-FU) for 9 days. The MST of the treated groups was compared with that of the control group using the following calculation: % Increase in life span

over control = (MST of treated group−MST of control group) × 100−100, where MST = survival time (days of each mouse in a group)/Total no. of mice (Table 2).

*Effect of compounds (**1–17**) on hematological parameters*

To examine the influence of (**1–17**) on the hematological status of EAC-bearing mice, a comparison was made among the following groups ($n = 7$) of mice on the 14th day after inoculation. The groups were comprised of 1) tumor-bearing mice, 2) tumor-bearing mice treated for the first 9 days, and 3) control mice (normal). Blood was drawn from each mouse by the retro orbital plexus method and the white blood cell count (WBC), red blood cell count (RBC), and hemoglobin was determined. From each group, 100-μl sample of Ehrlich ascites cells (from three mice) was taken and 20-fold dilutions in saline were made. The cells were stained by Giemsa stain and the number of viable cells was measured under a microscope. The viable cells, those that did not take up the stain, i.e., the dead cells (stained cells).

From Table 2, it was noticed that: (1) Mice received compound (**10**) and 5-FU were more protected against ascites and increase in weight than control mice; (2) Percentage increase of life span compared with control was high in mice treated with total extracts; (3) Mice that received compound (**10**) showed comparable results to 5-FU; (4) Hematological parameters of tumor-bearing mice on day 14 showed significant changes compared with normal mice; (5) Total WBC count increased with a reduction in the hemoglobin content and increase of RBC; (6) Compound (**10**) and 5-FU showed minimal ascites and slight increase in body weight unlike control group.

Conclusions

Modification of curcumin produced compounds with potential for further development as anticancer agents. Based on these preliminary screening results, compounds (**10**), (**7**), and (**12**) showed significant activity in certain cancer cells and have been targeted for further studies. Additional research, including mode of action studies, is planned to accurately establish relative activity for (SAR) and rational design. Recently, the cancer chemopreventive effects of curcumin have been intensively investigated. Curcumin exhibited pronounced antitumor activity by triggering apoptosis inhuman tumor cells (Ishida *et al*., 2002). Studies are underway to investigate the apoptosis-inducing activity of compounds found to be cytotoxic in this study.

Experimental

General

All melting points (uncorrected) are in degree centigrade and were determined on Gallenkamp electric melting point apparatus. FTIR spectra (KBr disk) were

recorded on a Nicolet Magna. IR model 550 spectrophotometers, ^{1}H-NMR spectra in DMSO, were determined on Brucker Wpsy 200 MHz spectrometer with TMS as internal standard and the chemical shifts are in δ ppm. Mass spectra were recorded at 70 ev with a varian MAT 311. C, H and N elemental analyses are satisfactory for all synthesized compounds (**1–17**).

Synthesis of curcumin derivatives

*Synthesis of 1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-4-arylazo-3,5-dione; (**2a–e**)*

General procedure Preparation of the diazonium salt: A solution of sodium nitrite (0.2 g in 2 ml water) was gradually added to a well-cooled solution of different aromatic amines (0.01 mol) in concentrated HCl (3.0 ml). The diazonium salt solution was added drop wise with continuous stirring to cold solution of curcumin (**1**) in pyridine (20 ml). The reaction mixture was stirred at 0–5°C for 2 h and left to stand at room temperature. The solid product that obtained was filtered off, dried, and recrystallized from ethanol to give compounds (**2a–e**).

*4-(4-methoxy-phenylazo)-1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione (**2a**)*

Yield 58%; m.p. 153°C; IR (KBr): ν/cm^{-1} = 3400 (OH), 3250 (NH), 1688 (C=O), 1618 (C=C), 1550 (N=N). H^1-NMR (DMSO-d_6): δ/ppm = 3.73 (s, 3H, OCH_3), 3.95 (s, 6H, 2 × OCH_3), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–8.02 (m, 10H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH), 11.03 (s, 1H, NH). MS: m/z (%) = 505 (M^++1, 0.81), Anal. for $C_{28}H_{26}N_2O_7$ (502.52): Calcd. C, 66.92; H, 5.22; N, 5.57%. Found: C, 66.88; H, 5.20; N, 5.62%.

*4-(p-tolylazo)-1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione (**2b**)*

Yield 68%; m.p. 142°C; IR (KBr): ν/cm^{-1} = 3400 (OH), 3250 (NH), 1690 (C=O), 1620 (C=C), 1560 (N=N). H^1-NMR (DMSO-d_6): δ/ppm = 2.35 (s, 3H, CH_3), 3.95 (s, 6H, 2 × OCH_3), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–7.94 (m, 11H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH), 11.03 (s, 1H, NH). MS: m/z (%) = 486 (M^+, 7.31), Anal. for $C_{28}H_{26}N_2O_6$ (486.52); Calcd. C, 69.12; H, 5.39; N, 5.76%; Found: C, 69.20; H, 5.29; N, 5.50%.

*4-(phenylazo)-1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione (**2c**)*

Yield 93%; m.p. 132°C;IR (KBr): ν/cm^{-1} = 3400 (OH), 3250 (NH), 1690 (C=O), 1605 (C=C), 1580 (N = N). H^1-NMR (DMSO-d_6): δ/ppm = 3.95 (s, 6H, 2 × OCH_3), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–8.12 (m, 11H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH),

11.03 (s, 1H, NH). MS: m/z (%) = 472 (M^+, 0.32), Anal. for $C_{27}H_{24}N_2O_6$ (472.49): Calcd. C, 68.63; H, 5.12; N, 5.93%; Found: C, 68.58; H, 5.15; N, 5.98%.

*4-(4-Chloro-phenylazo)-1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione (**2d**)*

Yield 90%; m.p. 150°C; IR (KBr): ν/cm^{-1} = 3400 (OH), 3250 (NH), 1690 (C=O), 1615 (C=C), 1575 (N=N). H^1-NMR (DMSO-d_6): δ/ppm = 3.95 (s, 6H, 2 × OCH_3), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–7.93 (m, 10H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH), 11.03 (s, 1H, NH). MS: m/z (%) = 507 (M^+, 8.85), Anal. for $C_{27}H_{23}ClN_2O_6$ (506.93): Calcd. C, 63.97; H, 4.57; N, 5.53%; Found: C, 63.90; H, 4.63; N, 5.57%.

*1,7-Bis-(4-hydroxy-3-methoxy-phenyl)-4-[(4-nitro-phenyl)-hydrazono]-hepta-1,6-diene-3,5-dione (**2e**)*

Yield 49%; m.p. 152°C; IR (KBr): ν/cm^{-1} = 3400 (OH), 3250 (NH), 1690 (C=O), 1600 (C=C), 1565 (N=N). H^1-NMR (DMSO-d_6): δ/ppm = 3.95 (s, 6H, 2 × OCH_3), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–7.98 (m, 10H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH), 11.03 (s, 1H, NH). MS: m/z (%) = 517 (M^+, 15.2), Anal. for $C_{27}H_{23}N_3O_8$ (517.49): Calcd. C, 62.67; H, 4.48; N, 8.12%; Found: C, 62.60; H, 4.40; N, 8.18%.

*Synthesis of 6,7-dibromo-1,7-bis-[3-(4-nitrophenylazo)-4-hydroxy-5-methoxy]hepta-1-ene-3,5-dione; (**3**)*

To 4-(4-nitro-phenylazo)-1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione (**2e**), (0.01 mol) in acetic acid (22 ml) was added slowly. With stirring a solution of bromine (0.02 mol) in acetic acid (20 ml). The reaction mixture was left overnight and crude product filtered, washed with water, dried, and recrystallized from ethanol to give compound (**3**). Yield 65%; m.p. 140°C; IR (KBr): ν/cm^{-1} = 3425 (OH), 1665 (C=O), 1581 (N=N), 700 (C–Br). MS: m/z (%) = 678 (M^++1, 1.50), 671 (2.06), 578 (0.37), 368 (16.76), 356 (9.64), 79 (100.0), Anal. for $C_{27}H_{23}Br_2N_3O_8$ (677.29): Calcd. C, 47.88; H, 3.42; N, 6.20%; Found: C, 47.95; H, 3.48; N, 6.15%.

*Synthesis of (Z)-4-(4-hydroxy-3-methoxy-phenyl)-6-[{6-(4-hydroxy-3-methoxy-phenyl)-2-thioxo-1,2,5,6-tetrahydro-pyrimidine-4-yl}(2-(4-nitrophenyl)hydrazono)methyl]-5,6-dihydropyrimidine-2(1H)-thione; (**4**)*

A mixture of compound (**2e**) (0.01 mol) thiourea (0.02 mol) and sodium ethoxide (0.02 mol sodium, in 50 ml ethanol) was refluxed for 6 h, left to cool, dilute, and acidified with dilute acetic acid. The precipitated solids were filtered and crystallized from ethanol to give product (**4**). Yield 35%; m.p. 225°C; IR (KBr): ν/cm^{-1} = 3420 (OH), 3220 (NH), 1620 (C=N), 1575 (N=N), 1273 (C=S). H^1-NMR (DMSO-d_6): δ/ppm = 1.5, 1.7 (m, 2H, CH_2), 1.8, 2.0 (m, 2H, CH_2), 2.6 (t, 1H, CH),

3.8 (s, 6H, 2 × OCH_3), 3.9 (t, 1H, CH), 6.68–8.0 (m, 10H, Ar–H), 9.8 (s, 2H, 2 × OH), 10.6 (s, 1H, $NH_{hydrazo}$). MS: m/z (%) = 632 (M^+-1, 4.13), Anal. for $C_{29}H_{27}N_7O_6S_2$ (633.7): Calcd. C, 54.96; H, 4.29; N, 15.47%; Found: C, 54.85; H, 4.30; N, 15.49%.

*Synthesis of (1Z,3E)-4-(4-hydroxy-3-methoxy-phenyl)-1-[6-(4-hydroxy-3-methoxy-phenyl)-2-thioxo-2,3,4,5-tetrahydro-pyrimidine-4-yl]-1-[2-(4-nitrophenyl)hydrazono]-but-3-ene-2-one; (**5**)*

A mixture of compound (**2e**) (0.01 mol) thiourea (0.01 mol) and sodium ethoxide (0.02 mol sodium, in 50 ml ethanol) was refluxed for 4 h, left to cool, dilute, and acidified with dilute acetic acid. The precipitated solids were filtered and crystallized from ethanol to give product (**5**). Yield 54%; m.p. 235°C; IR (KBr): ν/cm^{-1} = 3400 (OH), 3230 (NH), 3210 (NH), 2930 (CH_2), 1690 (CO), 1620 (C=N), 1580 (N=N), 1275 (C=S). H^1-NMR (DMSO-d_6): δ/ppm = 1.5, 1.7 (m, 2H, CH_2), 2.0 (s, 1H, NH), 2.6 (t, 1H, CH), 3.8 (s, 6H, 2 × OCH_3), 6.43 (d, j = 13 Hz, 1H, CH=CH), 6.68–8.0 (m, 10H, Ar–H), 7.55 (d, j = 13 Hz, 1H, CH=CH), 9.8 (s, 2H, 2 × OH), 10.6 (s, 1H, $NH_{hydrazo}$). MS: m/z (%) = 575 (M^+, 3.93), Anal. for $C_{28}H_{25}N_5O_7S$ (575.59): Calcd. C, 58.43; H, 4.38; N, 12.17%; Found: C, 58.55; H, 4.44; N, 12.20%.

*Synthesis of N-[bis-(5-(4-hydroxy-3-methoxy-phenyl)-2,5-dihydro-isoxazol-3-yl)-methylene]-N′-(4-nitro-phenyl)-hydrazine (**6**)*

A mixture of compound (**2e**) (0.01 mol) hydroxyl amine hydrochloride (0.02 mol) in pyridine (30 ml) was refluxed for 6 h, left to cool, diluted with water, and the obtained solid products were crystallized from ethanol to give product (**6**). Yield 47%; m.p. 185°C; IR (KBr): ν/cm^{-1} = 3400 (OH), 3240 (NH), 3220 (NH), 2929 (CH), 1594 (N=N). MS: m/z (%) = 546 (M^+-1, 1.34), Anal. for $C_{27}H_{25}N_5O_8$ (547.52): Calcd. C, 59.23; H, 4.60; N, 12.79%; Found: C, 59.30; H, 4.52; N, 12.84%.

*Synthesis of 5-(4-hydroxy-3-methoxy-phenyl)-3-{[5-(4-hydroxy-3-methoxy-phenyl)-4,5-dihydro-1H-pyrazol-3-yl](4-nitro-phenylazo) methyl}-4,5-dihydro-1H-pyrazole; (**7**)*

A mixture of compound (**2e**) (0.01 mol) hydrazine hydrate (0.03 mol) in ethanol, acetic acid (1:1), was refluxed for 4 h, left to cool, diluted with water, and the obtained solid products were crystallized from ethanol to give product (**7**). Yield 54%; m.p. 220°C; IR (KBr): ν/cm^{-1} = 3400 (OH), 3230 (NH), 3210 (NH), 2900 (CH), 1690 (CO), 1620 (C=N), 1600 (C=C). H^1-NMR (DMSO-d_6): δ/ppm = 1.5, 1.7 (m, 2H, CH_2), 1.8, 2.0 (m, 2H, CH_2), 2.6 (t, 1H, CH), 3.8 (s, 6H, 2 × OCH_3), 3.9 (t, 1H, CH), 6.68–8.0 (m, 10H, Ar–H), 9.8 (s, 2H, 2 × OH), 10.6 (s, 1H, $NH_{hydrazo}$). MS: m/z (%) = 545 (M^+, 3.03), Anal. for $C_{27}H_{27}N_7O_6$ (545.51): Calcd. C, 59.44; H, 4.99; N, 17.97%; Found: C, 59.35; H, 4.94; N, 17.92%.

*Synthesis of 2,10-diacetyl-6-[(4-chloro-phenyl)-hydrazono]-3,9-bis-(4-hydroxy-3-methoxy-phenyl)-5,7-dioxo-undecanedioic acid diethyl ester; (**8**)*

A mixture of 4-(4-Chloro-phenylazo)-1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione (**2d**) (0.01 mol), ethyl acetoacetate (0.022 mol), was refluxed in absolute ethanol for 6 h in the presence of sodium ethoxide (3%). The reaction mixture was allowed to stand overnight and then acidified by dilute HCl. The solid products obtained was crystallized from ethanol to give the corresponding Michael adduct (**8**). Yield 48%; m.p. 142°C; IR (KBr): $\nu/cm^{-1} = 3424$ (OH), 3220 (NH), 3100 (CH_3), 2900 (CH_2), 1735 (CH), 1730 (carbonyl acetyl), 1710 (carbonyl ester), 1513 (N=N). MS: m/z (%) = 768 (M^++1, 3.47), Anal. for $C_{39}H_{43}ClN_2O_{12}$ (767.22): Calcd. C, 61.05; H, 5.65; N, 3.65%; Found: C, 61.15; H, 5.60; N, 3.71%.

*Synthesis of (Z)-ethyl-6-{2-(4-chloro-phenyl)hydrazono}[4-(4-hydroxy-3-methoxy-phenyl)-6-1,4-dihydropyridin-2-yl]-4-(4-hydroxy -3-methoxy-phenyl)-2-methyl-1,4-dihydropyridine-3-carboxylate; (**9**)*

A mixture of 2,10-diacetyl-6-[(4-chloro-phenyl)-hydrazono]-3,9-bis-(4-hydroxy-3-methoxy-phenyl)-5,7-dioxo-undecanedioic acid diethyl ester (**8**) (0.01 mol) and ammonium acetate (0.022 mol) was refluxed in boiling acetic acid (30 ml) for 4–6 h. The reaction mixture was cooled and diluted with water to give product (**9**). Yield 39%; m.p. 235°C; IR (KBr): $\nu/cm^{-1} = 3400$ (OH), 3300 (NH), 3000 (CH), 2900 (CH_3), 1720 (carbonyl ester), 1595 (C=C), 1580 (N=N). H^1-NMR (DMSO-d_6): δ/ppm = 1.3 (t, 6H, 2 × CH_3CH_2), 2.26 (s, 6H, 2 × CH_3), 3.83 (s, 6H, 2 × OCH_3), 4.2 (q, 4H, CH_2CH_3), 4.4 (s, 2H, 2 × CH), 6.68–8.0 (m, 10H, Ar–H), 9.83 (s, 2H, 2 × OH), 13.2 (s, 1H, $NH_{hydrazo}$). MS: m/z (%) = 729 (M^+, 24.00), Anal. for $C_{39}H_{41}ClN_4O_8$ (728.5): Calcd. C, 64.24; H, 5.67; N, 7.68%; Found: C, 64.31; H, 5.70; N, 7.62%.

*Synthesis of 4,4′-(1E,1′E)-2,2′-(1H-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-[2-(4-chloro-phenyl)]diazenyl-6-methoxy-phenol; (**10**)*

Purified curcumin (**1**) (10 mg, 0.027 mol) was dissolved in methanol (2 ml), and hydraziniumdihydrochloride (14 mg, 0.135 mol) and triethylamine (18.8 ml, 0.135 mol) were added to the solution. In the presence of catalytic amount of glacial acetic acid, the reaction mixture was refluxed for 4 h, left to cool, the solvent was evaporated in vacuo, and the solid products obtained were crystallized from ethanol to give product (**10**). Yield 83%; m.p. 218°C; IR (KBr): $\nu/cm^{-1} = 3400$ (OH), 3230 (NH), 1590 (C=C), 1620 (C=N). H^1-NMR (DMSO-d_6): δ/ppm = 3.95 (s, 6H, 2 × OCH_3), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–8.12 (m, 6H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH), 9.6 (s, 1H, NH). MS: m/z (%) = 364 (M^+, 70.46), Anal. for $C_{21}H_{20}N_2O_4$ (364.39): Calcd. C, 69.22; H, 5.53; N, 7.69%; Found: C, 69.31; H, 5.56; N, 7.60%.

*Synthesis of 4,4′-(1E,1′E)-2,2′-(1-bromo-1H-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-(2-methoxy-phenol); (**11**)*

A mixture of 4,4′-(1*E*,1′*E*)-2,2′-(1*H*-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-[2-(4-chloro-phenyl)]diazenyl-6-methoxy-phenol (**10**) 0.005 mol and *N*-bromosuccinimide 0.005 mol in chloroform. The mixture was stirred in direct sun light for 2 h. The solvent was evaporated to give product (**11**). Yield 63%; m.p. 250°C; IR (KBr): $\nu/cm^{-1} = 3400$ (OH), 3230 (NH), 1590 (C=C), 1620 (C=N), 720 (N–Br). H^1-NMR (DMSO-d_6): δ/ppm = 3.95 (s, 6H, 2 × OCH_3), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–8.12 (m, 6H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH), Anal. for $C_{21}H_{19}BrN_2O_4$ (443.29): Calcd. C, 56.90; H, 4.32; N, 6.32%; Found: C, 55.70; H, 4.90; N, 6.74%.

*Synthesis of 4,4′-(1E,1′E)-2,2′-(1-hydrazinyl-1H-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-(2-methoxy-phenol); (**12**)*

An equimolar mixture (0.005 mol) of 4,4′-(1*E*,1′*E*)-2,2′-(1*H*-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-[2-(4-chloro-phenyl)]diazenyl-6-methoxy-phenol (**11**) and hydrazine hydrate in ethanol (30 ml) was heated at reflux for 2 h, and cooled. The resulting solid was collected, washed with dilute ammonia, air-dried, and recrystallized from ethanol afforded the corresponding product (**12**). Yield 90%; m.p. 200°C; IR (KBr): $\nu/cm^{-1} = 3450$ (NH_2), 3420 (OH), 3200 (NH), 1590 (C=C), 1620 (C=N). H^1-NMR (DMSO-d_6): δ/ppm = 3.95 (s, 6H, 2 × OCH_3), 4.6 (s, 1H, NH), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.2 (s, 1H, NH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–8.12 (m, 6H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH). MS: m/z (%) = 395 (M^++1, 1.01), Anal. for $C_{21}H_{22}N_4O_4$ (394.42): Calcd. C, 63.95; H, 5.62; N, 14.20%; Found: C, 64.05; H, 5.68; N, 14.10%.

*Synthesis of 4,4′-(1E,1′E)-2,2′-(1-nitrosyl-1H-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-(2-methoxy-phenol); (**13**)*

A mixture of (**10**, 0.005 mol) in 1:1 hydrochloric acid (20 ml) was treated at 3°C with a cooled concentrated solution of sodium nitrite (0.02 mol in 10 ml water). The mixture was stirred in the cold for 1 hour and left in the refrigerator for 12 h. The separated solid was recrystallized from ethanol to give product (**13**). Yield 75%; m.p. over 280°C; IR (KBr): $\nu/cm^{-1} = 3425$ (OH), 3200 (NH), 1580 (N=O). MS: m/z (%) = 393 (M^+, 4.24), 368 (10.10), 313 (27.7), 264 (25.92), 236 (24.69), 185 (19.73), 135 (38.78), 97 (89.48), 69 (100.00); Anal. for $C_{21}H_{19}N_3O_5$ (393.39): Calcd. C, 64.12; H, 4.87; N, 10.68%; Found: C, 64.22; H, 4.90; N, 10.74%.

*Synthesis of 4,4′-(1E,1′E)-2,2′-(1-chloroacetyl-1H-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-(2-methoxy-phenol); (**14**)*

To a solution of 4,4′-(1*E*,1′*E*)-2,2′-(1*H*-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-[2-(4-chloro-phenyl)]diazenyl-6-methoxy-phenol (**10**), 0.01 mol was added to

chloroacetyl chloride (0.012 mol).The reaction mixture was subjected to fusion at 95°C for 2 h. The solid products obtained recrystallized from ethanol and washed by pet-ether. Yield 83%; m.p. 190°C; IR (KBr): ν/cm^{-1} = 3420 (OH), 2900 (CH_2), 1685 (C=O), 1620 (C=N), 760 (C–Cl). H^1-NMR (DMSO-d_6): δ/ppm = 3.5 (s, 2H, CH_2), 3.95 (s, 6H, 2 × OCH_3), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–8.12 (m, 6H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH). MS: m/z (%) = 440 (M^+, 2.46); Anal. for $C_{23}H_{21}ClN_2O_5$ (440.88): Calcd. C, 62.66; H, 4.80; N, 6.35%; Found: C, 62.49; H, 4.85; N, 6.30%.

*Synthesis of 3,5-bis-[β-(5-{4-chloro-phenylazo}-4-hydroxy-3-methoxy-phenyl)-ethynyl]-1H-pyrazole; (**15**)*

General procedure Preparation of the diazonium salt: A solution of sodium nitrite (0.2 gm in 2 ml water) was gradually added to a well-cooled solution of *p*-chloro aniline (aromatic amine) (0.01 mol) in concentrated HCl (3.0 ml). The diazonium salt solution was added drop wise with continuous stirring to (0.005 mol) cold solution of 4,4′-(1*E*,1′*E*)-2,2′-(1*H*-pyrazole-3,5-diyl)-bis-(ethane-2,1-diyl)-bis-[2-(4-chloro-phenyl)]diazenyl-6-methoxy-phenol (**10**) in sodium hydroxide 10% (20 ml). The reaction mixture was stirred at 0–5°C for 2 h and left to stand at room temperature. The solid product that was obtained was filtered off, dried, and recrystallized from ethanol to give product (**15**). Yield 63%; m.p. 270°C; IR (KBr): ν/cm^{-1} = 3423 (OH), 3230 (NH), 1598 (C=C), 1585 (N=N). MS: m/z (%) = 641 (M^+, 11.49), 633 (17.24), 616 (13.79), 583 (33.33), 553 (36.78), 474 (88.51), 415 (64.37), 342 (60.92), 207 (36.78), 126 (100.00), 74 (70.11), Anal. for $C_{33}H_{26}Cl_2N_6O_4$ (641.50): Calcd. C, 61.79; H, 4.09; N, 13.10%; Found: C, 61.85; H, 4.15; N, 13.02%.

*Synthesis of 3,5-bis-{β-[3-(4-chloro-phenylazo)-4-hydroxy-5-methoxy-phenyl]-ethynyl}-pyrazole-1-formyl; (**16**)*

Compound (**15**, 0.005 mol) was suspended in mixture of formamide (30 ml) and methanol (20 ml), stirred under reflux for 30 min, cooled to 0°C, and the desired product (**16**) filtered off and recrystallized from methanol. Yield 65%; m.p. 240°C; IR (KBr): ν/cm^{-1} = 3423 (OH), 1690 (C=O), 1595 (C=C), 1580 (N = N). H^1-NMR (DMSO-d_6): δ/ppm = 3.95 (s, 6H, 2 × OCH_3), 5.8 (s, 1H, CH), 5.86 (s, 2H, 2 × OH), 6.43 (d, j = 13 Hz, 2H, 2 × CH=CH), 6.91–8.12 (m, 12H, Ar–H), 7.55 (d, j = 13 Hz, 2H, 2 × CH=CH), 8.8 (s, 1H, CH). MS: m/z (%) = 670 (M^++2, 2.01), Anal. for $C_{34}H_{26}Cl_2N_6O_5$ (669.51): Calcd. C, 60.99; H, 3.91; N, 12.55%; Found: C, 61.09; H, 3.87; N, 12.61%.

*Synthesis of 3,5-bis-[β-(5-{4-Chloro-phenylazo}-4-hydroxy-3-methoxy-phenyl)-ethynyl]-2-hydroxy-methyl-pyrazole; (**17**)*

A mixture of (**15**) (0.005 mol) and formaldehyde (40%; 2 ml) in methanol (30 ml) was stirred at room temperature for 1 hour, cooled (0°C) for 12 h, and the precipitated solid filtered and recrystallized from methanol to yield product (**17**).

Yield 61%; m.p. 255°C; IR (KBr): ν/cm^{-1} = 3425 (OH), 1600 (C=N), 1582 (N=N). H^1-NMR (DMSO-d_6): δ/ppm = 2.0 (s, 1H, OH), 3.83 (s, 6H, 2 × OCH_3), 6.2 (s, 1H, CH), 5.97 (s, 2H, CH_2), 6.9 (d, j = 13 Hz, 2H, 2 × CH=CH), 7.06–7.94 (m, 12H, Ar–H), 7.5 (d, j = 13 Hz, 2H, 2 × CH = CH), 9.8 (s, 2H, 2 × OH), MS: m/z (%) = 671 (M^+, 4.67), Anal. for $C_{34}H_{28}Cl_2N_6O_5$ (671.53): Calcd. C, 60.81; H, 4.20; N, 12.51%; Found: C, 60.95; H, 4.28; N, 12.44%.

Antitumor activity

Cells of Ehrlich ascites tumor were obtained from National Cancer Institute, Cairo, Egypt. Animals, adult Swiss male albino mice (20–25 g) were procured from Pharmacology Faculty, Mansoura University, Egypt, and used throughout this study. They were housed in microlon boxes in a controlled environment (temperature 25 + 2°C and 12-h dark/light cycle) with standard laboratory diet and water ad libitum (5-fluorouracil → 20 mg/kg).

Animals were divided into seven groups as follows: All animals were inoculated with 2 × 10^6 cells/mouse on day 0 except normal group and treatment started 24 h after inoculation, at 2 doses of 50 and 250 mg/kg/day, i.p. The control group was treated with the same volume of 0.9% sodium chloride solution. All the treatments were given for 9 days.

Tumor-reducing activity

Ehrlich ascites tumor cells (0.2 ml) were injected into the mice peritoneal cavity and different concentrations (1 mg/ml to 0.1 ml/mice on day) of the drug (5 mice/group) were injected from day 1 to day 9 every day. Animals were observed for the development as ascites tumor and death due to tumor burden. Life span % I LS = (T−C)/C × 100, where T is the number of days the treated animals survived and C is the number of days control animals survived. % I LS > 25% was considered significant (Kuttan *et al*., 1985).

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