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New antitumoral agents I: In vitro anticancer activity and in vivo acute toxicity of synthetic 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one and derivatives

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Dedicated to Professor Dr. Klaus Peseke on his 70th birthday.

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ABSTRACT

This paper describes a new method for the preparation of 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one **1** and its derivatives **2–5**. This set of synthetic compounds exhibited high antitumoral activities regarding in vitro screening against several human tumor cell lines as lung carcinoma NCI-460, melanoma UACC-62, breast MCF-7, colon HT-29, renal 786-O, ovarian OVCAR-03 and ovarian expressing the resistance phenotype for adriamycin NCI-ADR/RES, prostate PC-3, and leukemia K-562. Compounds were also tested against murine tumor cell line B16F10 melanoma and lymphocytic leukemia L1210 as well as to their effect toward normal macrophages. Specific activity against colon cancer cells HT-29 was observed for all tested compounds and suggests further studies with models of colon cancer. Compounds **1**, **2**, and **4** showed significant cytotoxic activity with IC_{50} values $\leqslant$2.3 μM for all human cancer cell lines. Intraperitoneal acute administration of compound **1** and **2** showed very low toxicity rate.

1. Introduction

Compounds chemically known as 1,5-diaryl-1,4-pentadien-3-ones have been widely explored as promising pharmacological agents, exhibiting potent anti-oxidative,[1] anti-inflammatory,[1,2] anti-HIV,[3,4] and insecticide[5] activities.

These are structural analogs of the natural product curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) which is found as a major pigment in the Indian spice turmeric *Curcuma longa*, *Zingiberaceae*.[6] They share not only similar chemical structures but also similar biological properties as antioxidant and anticancer promising agents. The antitumoral activity is noteworthy since it can be expressed in terms of prophylactic and growth inhibition effects.[7]

Organic synthesis provides a cheaper and more efficient approach to obtain large amounts of 'natural inspired' chemicals to therapeutics than by extracting few milligrams from the nature.

In 1927, Glaser and Tramer[8] reported the first synthesis of 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one with 60% of yield by starting from the vanillin and acetone in the presence of concentrated hydrochloric acid as catalyst.

Further Ramanan and Rao[9] synthesized this product in 1989 by starting from 4-*O*-methoxymethylvanillin and acetone in alkaline medium, obtaining a yield of 42% after purification by thin-layer chromatography.

In 1997, Sardjiman et al.[10] developed a new variant synthetic approach using equimolar quantities of vanillin and acetone in the presence of concentrated hydrochloric acid, reporting a yield of raw product of 89%. For this reason the melting point indicated

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in this procedure was 58 °C less than the one reported by Glaser and Tramer.[8]

Artico et al.[3] also obtained this substance 1 year later, but with a low yield of 18%. But they reported a melting temperature range (114–116 °C) lower than the one reported by Glaser and Tramer, what suggests that this compound was not obtained pure despite of purification by column chromatography.

Only in 1998, compound 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one **1** was isolated and characterized in the rhizomes of *Curcuma domestica* by Masuda et al.[1] The authors also reported the anti-oxidative and anti-inflammatory activity of this pentadienone.

The method for the preparation of 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one[11] **1**, by Claisen condensation, starting from vanillin or vanillin derivatives, respectively, and acetone in acid medium under ultrasonic irradiation conditions with good yield and purity, was patented in 2002.

Ohori et al.[12] development in 2006 a very important work about curcumin analogs. These authors included compound **4** in this study and reported their potent antitumoral activity, but they did not cite our published patent in this paper.

Liang et al.[13] synthesized in 2009 compound **1** and found a significative cytotoxic activity toward a diverse panel of cultured human tumor cell lines, but they did not cite our published patent in their paper.

Two series of curcumin analogs, a total of 24 compounds, were synthesized and evaluated as new antitumoral agents by Fuchs et al.[14] in 2009. They included in this list compounds **1** and **4** too and found that compound **1** was 5–8 times more potent than curcumin. Our published patent was not present in the list of cited references.

More than 85% of the modern therapeutic drugs, including the anticancer agents, are obtained from synthetic or semi-synthetic approaches.

Cancer or neoplasia represents the collective name for more than 100 diseases characterized by the uncontrolled growth of abnormal cells that may affect almost any tissue of the body. It affects more than 11 million people every year and it is responsible for 7 million deaths per year, which may be translated, from a statistical point of view, as 12.5% of world deaths.[15]

Unfortunately, despite of all knowledge and advances in the cancer research, it is still urgent the need for new and effective agents capable of bringing this disease under control, mainly in consequence of drug toxicity and development of resistance to the actual chemotherapeutic agents.

It is preconized that effective novel anticancer drugs require not only disease specificity, but also minimal toxic side-effects. Drug capability for precise cancer therapy without consequence to the function of normal cells has yet to be developed.

This paper reports the in vitro cytotoxic effects produced by five diarylpentadienone derivatives, which were synthesized under ultrasonic reaction conditions, against a panel of relevant human tumor cell lines as well as against peritoneal normal macrophages to determine the selectivity of compounds. It also reports the in vitro antitumoral assays against the murine tumoral cell lines melanoma B16F10 and lymphocytic leukemia L1210. Finally, intraperitoneal LD_{50} values for the most promising analogs were also obtained in mice.

2. Chemistry

The synthesis of 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one, **1**, can be accomplished by Claisen condensation starting from vanillin and acetone in a molar ratio of 2:1 at 5 °C, in the presence of concentrated hydrochloric acid under ultrasonic irradiation conditions (40 kHz) for 1 h[11] (Scheme 1).

Compound **2**, 1,5-bis(4-acetoxy-3-methoxyphenyl)-1,4-pentadien-3-one, was obtained as described earlier by our group.[2]

The prenylated derivative **3** was synthesized from **1** and prenyl bromide in the presence of potassium carbonate in dry *N,N*-dimethylformamide. The reaction mixture was stirred for 8 h at 40 °C and was completed after 8 h.

Veratraldehyde, acetone and concentrated hydrochloric acid reacted at 40 °C and under ultrasonic irradiation (40 kHz) to obtain compound **4** with good yield.

Finally, compound **5** was obtained starting from **1** and malononitrile in the presence of ammonium acetate and acetic acid under Knoevenagel reaction conditions.

3. Results and discussion

The chemical structure of the 1,5-diaryl-1,4-pentadien-3-ones and derivatives was determined by spectroscopic methods and elemental analysis, thus excluding any other alternative structures (Section 5).

Table 1 exhibits the cytotoxic effects of compounds **1–5** toward nine different human cancer cell lines.

Compounds **1**, **2**, and **4** exhibited a better potency than the analogs **3** and **5**, since their IC_{50} values were less than 2.29 μM, which can be considered to show very promising activities. Indeed, compound **1** was found to be the most potent one with selectivity for melanoma and breast cancer cells lines. Compound **2** had a low selectivity for renal cell line and compound **4** did not show any selectivity at all.

Compounds **3** and **5** presented higher selectivity for some cancer cells lines. This was observed for compound **3**, to leukemia and colon cell lines, and for compound **5**, to ovarian expressing the resistance phenotype for adriamycin NCI-ADR/RES, melanoma and colon.

These differences in potency found for compounds **3** and **5**, compared to the other analogs, have inspired us to design preliminary steric studies using computer-aided tools.

These methodologies could arise some hypothesis about the reasons behind the different biological behaviors presented by those compounds.

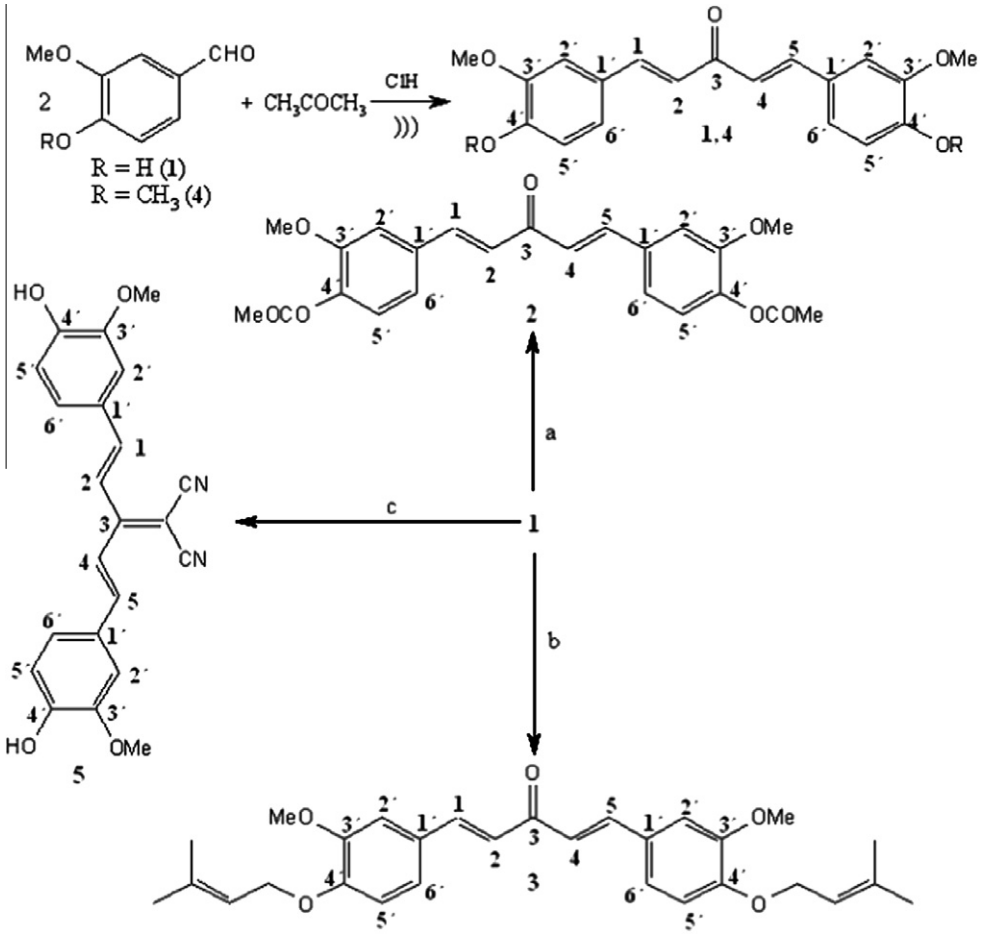
Figure 1 shows the electrostatic potential maps calculated onto surfaces obtained for all five compounds.

Prenyl group at the 4-position of both aromatic rings creates important increase in the overall volume of the compound **3** and could be related to the low cytotoxic activity obtained for this analog. This result could also be related to lipophilic factors since the prenyl side chain is known as a high apolar group.

Minor differences can be observed in the electrostatic potential map of this compound when compared to the maps obtained to the other analogs. Actually, no relationship between activity and the electrostatic potential can be established to the series. This does not imply that this parameter is not important to the cytotoxic activity of the compounds and a larger series could shed light on this point.

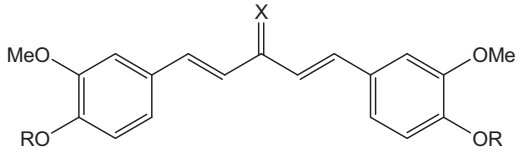
Compound **5** presents major differences of shape and volume characteristics. The cyano groups, which substitute the carbonyl group of the other structures, lead one of the aromatic ring to an 'out-of-plane' conformation as well as induce a different distribution of the electrostatic potential of the molecule. These differences, however, do not seem to be as important as the steric hindrance at 4-position of the aromatic rings since compound **5** presented better activity than compound **3**.

It is noteworthy, however, that these results are preliminary ones and more specific structure activity studies have to be accomplished in order to understand the efficiency of this series of compounds as promising antitumoral agents.



Scheme 1. Synthesis of 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one and derivatives. Reagents and condition: (a) acetic acid anhydride/sodium acetate, 1 h, reflux; (b) prenyl bromide/K_2CO_3/DMF; (c) malononitrile/NaAc/HAc/Knoevenagel,))) = ultrasonic irradiation.

Table 1
Antitumoral activities of diarylpentadienones, by means of IC_{50} values (μM), toward different human tumor cell lines



Compound	R	X
1	H	O
2	COMe	O
3	$CH_2CH{=}C(Me)_2$	O
4	Me	O
5	H	$C(CN)_2$

Human cell lines	Compounds				
	1	**2**	**3**	**4**	**5**
UACC-62	0.09	4.31	30.6	2.12	3.34
MCF-7	0.12	1.09	18.2	2.00	5.24
NCI-ADR	0.83	3.12	7.22	2.37	2.97
786-O	1.99	0.66	7.87	3.36	7.14
NCI-460	1.53	1.7	34.1	1.86	6.89
K-562	1.84	1.41	4.8	1.83	7.59
PC-03	1.25	0.95	60.5	2.32	4.25
OVCAR-03	2.2	1.39	30.3	2.32	115
HT-29	2.3	1.48	4.04	2.43	3.37

The line average indicated in Table 1 shows that HT-29 colon cells were the most sensible tumor line to the tested compounds, followed by the leukemia (K-562), ovarian expressing multidrug resistance phenotype (NCI-ADR/RES) and kidney (786-O).

Considering that compounds **1** and **2** were the most potent ones of the series, they were also assayed against murine tumoral cell lines (melanoma B16F10 and lymphocytic leukemia L1210) as well as against peritoneal normal macrophages (Table 2).

Table 2 shows that compound **1** was more potent than compound **2** for murine cancer cells lines but also exhibited higher cytotoxic profile against normal cells (macrophages) since the IC_{50} values for leukemia and macrophages were almost the same. This toxicity for macrophages may be related with an anti-inflammatory effect already observed and reported for compound **1** by Masuda et al.[1] and for compound **2** by Paulino et al.[2]

Park et al.[16] showed a reduction in Cox-2 expression after exposure of T24 bladder tumor cells to curcumin. More recently, Chadalapaka et al.[17] reported inhibition of 253JB-V and KU7 bladder cancer cell growth using 10–25 mM/L of curcumin, showing a decrease in the expression of NF-κB-dependent genes, Cyclin D1, survivin and Bcl-2. In agreement with these literature data, our results show that the strain of colon cancer (HT-29) is the most sensitive to the compounds studied and those with high expression of Cox-2.

The acute toxicity assay (LD_{50}) is also an important item in the development of new drugs, mainly against neoplasias, and also important for the subsequent studies. For this reason, this assay

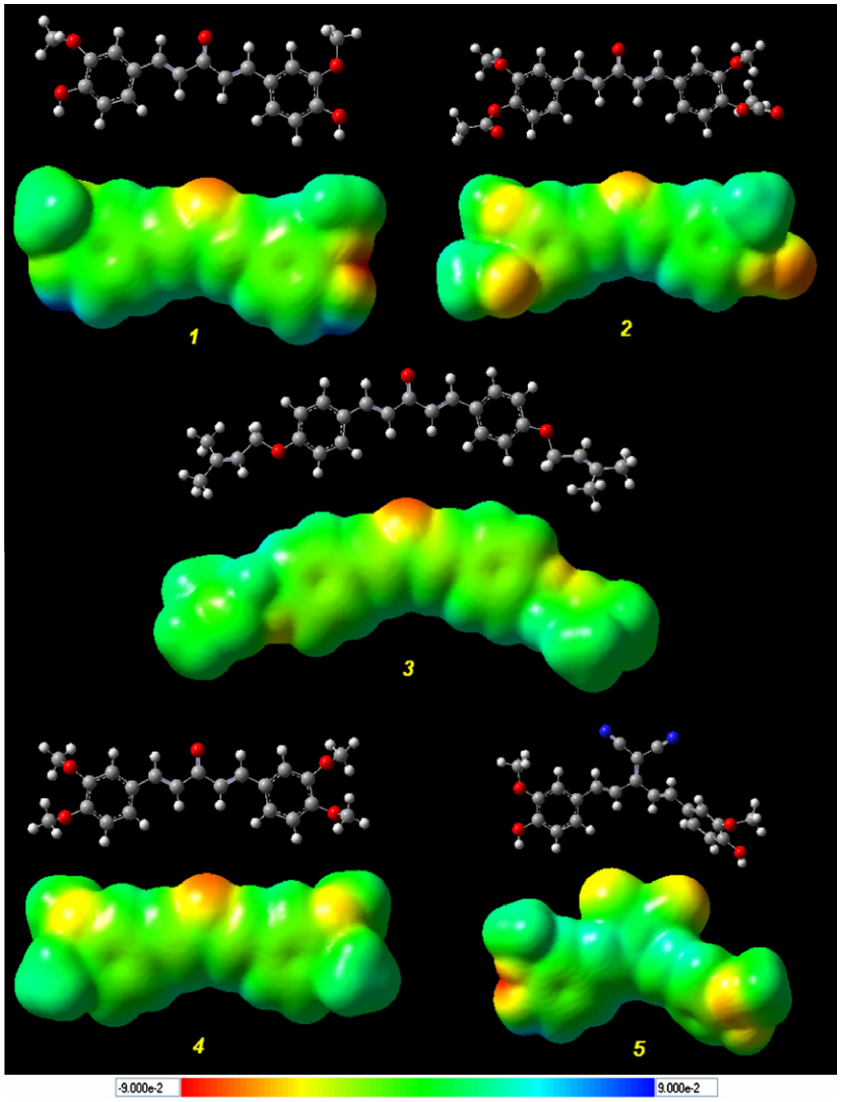


Figure 1. Electrostatic potential mapped onto the surfaces calculated to the five analogs. Negative electrostatic potential regions are represented in red color (high electronic density) while positive electrostatic potential areas are shown in dark blue color (low electronic density).

Table 2
Antiproliferative effects of compounds **1** and **2** on B16F10 melanoma cells, L1210 leukemia cells, and mice macrophages

Compounds	Cell line (μM)		
	B16F10	L1210	Macrophages
1	21.47 ± 2.7	56.1 ± 3.8	51.81 ± 1.92
2	220.2 ± 10.3	245.6 ± 12.4	274.7 ± 11.5

was also applied to compounds **1** and **2** which demonstrated the most promising antitumoral activities.

The animals treated with compound **2** (intraperitoneal) presented the first toxic symptoms with the dose of 2.5 g/kg of animal body weight. The LD_{50} value was determined by linear regression and was found to be 3.68 g/kg after 14 days of observation.

Compound **1** exhibited a much lower acute toxicity (8.54 g/kg) which is almost two fold lower compared to the acetylated derivative. This could be related to the higher liposolubility of compound **2** but further studies could clarify these results.

The animal treated with these compounds did not develop any clinical signs of toxicity either immediately or during the post-treatment period. No mortality occurred immediately or during the 14-day observation period; body weight dose was considered as the 'Limit test' as recommend by the acute toxicity testing procedures. Administration of further higher doses was considered physiologically unsound and, is not generally recommended. The treated animals did not show any significant alteration in water or food consumption or in body weight during the experimental period. Furthermore, the animals showed no symptoms of diarrhea, piloerection, convulsion, and sleepiness.

According to Loomis,[18] compounds with LD_{50} values ranging from 5 to 15 g/kg are considered to be practically non-toxic.

4. Conclusions

In the present work, we report the synthesis of new 1,5-diaryl-1,4-pentadien-3-one derivatives with good yield and purity under ultrasonic irradiation conditions. Compound **1** showed significant cytotoxic activity with IC_{50} values of 1.35 μM or lower for all human cancer cell lines. Lethal doses determination after intraperitoneal acute administration of this compound revealed very low toxicity rate (LD_{50} = 8.54 g/kg). For this reason, compound **1** could be applicable as a promising new antineoplastic agent with the

desirable profile of high efficiency and selectivity. Even compound **2**, by its antitumoral and toxicity characteristics, could be considered, at least, as a good lead to further drug design studies. With less potency, the other compounds also had anticancer activity, especially against colon cell lines. Apparently, colon cancer may be a target for diarylpentadienone derivatives.

5. Experimental

5.1. Chemical synthesis

TLC was carried out on silica gel 60 GF_{254} (Merck) with detection by UV light (λ = 254 nm) and/or by charring with iodine. IR spectra were recorded with a Nicolet 205 FT-IR spectrometer. ^{1}H NMR (300.13 MHz) and ^{13}C NMR (75.5 MHz) spectra were recorded on Bruker instrument ARX 300 with deuterochloroform or DMSO-d_6 as solvent. The mass spectra were recorded on an AMD 402/3 spectrometer (AMD Intectra GmbH). Elemental analyses were performed on a Leco CHNS-932 instrument.

5.1.1. 1,5-Bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one (1)

Vanillin (0.02 mol; 3.04 g) and acetone (0.01 mol; 0.73 ml) were contacted at a molar ratio of 2:1, at 40 °C, under ultrasonic irradiation (40 kHz) in the presence of concentrated hydrochloric acid for 2 h. Then, the mixture was poured onto water/ice. The crude product was dissolved in a sodium hydroxide solution, and the filtrate treated with hydrochloric acid. The formed product was filtered, and washed with distilled water until a neutral pH value was achieved. Yield obtained: 92% of the crude product (yellow-green powder).

For further purification, the crude product was recrystallized from acetonitrile/water. Purity = 100% (determined by HPLC), yellow powder, mp 155 °C, TLC R_f 0.82 in hexane/EtOAc 1:2; IR (KBr): 3415 (OH); 3070 (=CH); 2959 (CH_3); 2848 (–CH); 1588 (C=O); 1513 (C=C) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 9.68 (s, 2H, 2 OH); 7.65 (d, 2H, H-1/H-5, $^3J_{1,2=4,5}$ = 16.0 Hz); 7.37 (d, 2H, 2 H-2′, $^4J_{2',6'}$ = 1.9 Hz); 7.20 (dd, 2H, 2 H-6′, $^3J_{5',6'}$ = 8.0 Hz, $^4J_{2',6'}$ = 1.9 Hz); 7.16 (d, 2H, H-2/H-4, $^3J_{1,2=4,5}$ = 16.0 Hz); 6.85 (d, 2H, 2 H-5′, $^3J_{5',6'}$ = 8.0 Hz); 3.85 (s, 6H, 2 MeO). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 188.2 (C-3); 149.6 (C-4′); 148.2 (C-3′); 142.9 (C-1/C-5); 126.5 (C-1′); 123.5 (C-6′); 123.2 (C-2/C-4); 115.9 (C-5′); 111.6 (C-2′); 55.9 (MeO). Anal. Calcd for $C_{19}H_{18}O_5$: C, 69.94; H, 5.52. Found: C, 69.91; H, 5.48; EI-MS *m*/*e*: 326 (M^+).

5.1.2. 1,5-Bis(4-acetoxy-3-methoxyphenyl)-1,4-pentadien-3-one (2)

Compound **2** was obtained as described earlier by our group.[2]

5.1.3. 1,5-Bis[3-methoxy-4-(3-methyl-but-2-enyloxy)phenyl]-1,4-pentadien-3-one (3)

To a stirred solution of 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one (**1**) (0.652 g, 2 mmol) in dry DMF (10 ml), anhydrous potassium carbonate (0.828 g, 6 mmol) was added and the solution was stirred for 30 min at 40 °C under argon. Prenyl bromide (0.596 g, 4 mmol) was added drop-wise and the resulting mixture was heated and stirred at the same temperature for 8 h under argon. Then the mixture was poured into cold water (50 ml) and extracted with chloroform. The combined organic layers were washed with diluted sodium hydrogen sulfate solution, then with water, dried with Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel 60 (63–200 mesh, Merck) with hexane/ethyl acetate 2:1. Yield 0.49 g (53%); colorless oil, TLC R_f 0.48 in hexane/EtOAc 2:1; IR (KBr): 3035 (=CH); 2962, 2929 (CH_3); 2885 (–CH); 1664 (C=O); 1639, 1593 (C=C); 1250 (C–O) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.69 (d, 2H, H-1/H-5, $^3J_{1,2=4,5}$ = 15.8 Hz); 7.14 (d, 2H, 2 H-2′, $^4J_{2',6'}$ = 1.8 Hz); 7.12 (dd, 2H, 2 H-6′, $^3J_{5',6'}$ = 8.1 Hz, $^4J_{2',6'}$ = 1.8 Hz); 6.92 (d, 2H, H-2/H-4, $^3J_{1,2=4,5}$ = 15.8 Hz); 6.87 (d, 2H, 2 H-5′, $^3J_{5',6'}$ = 8.1 Hz); 5.51 (m, 2H, 2 $=CH_{prenyl}$); 4.60 (m, 4H, 2 $CH_{2prenyl}$); 3.90 (s, 6H, 2 MeO); 1.77 (s, 6H, 2 $CH_{3prenyl}$); 1.72 (s, 6H, 2 $CH_{3prenyl}$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 188.6 (C-3); 150.5 (C-4′); 149.4 (C-3′); 143.0 (C-1/C-5); 138.1 ($=C(CH_3)_2$); 127.5 (C-1′); 123.4, 122.6 (C-2/C-4, C-6′); 119.3 (C-5′); 112.5 (C-2′); 109.9 ($=CH_{prenyl}$); 65.4 ($CH_{2prenyl}$); 56.0 (MeO); 25.5, 18.0 ($CH_{3prenyl}$). Anal. Calcd for $C_{29}H_{34}O_5$: C, 75.32; H, 7.36. Found: C, 75.27; H, 7.41; EI-MS *m*/*e*: 462 (M^+).

5.1.4. 1,5-Bis(3,4-dimethoxyphenyl)-1,4-pentadien-3-one (4)

Veratraldehyde (7.8 mmol, 1.3 g) and acetone (3.9 mmol, 0.29 ml) were contacted at a molar ratio of 2:1 at 40 °C, under ultrasonic irradiation (40 kHz) in the presence of concentrated hydrochloric acid for 2 h. The mixture was poured onto water/ice and the crude product was extracted with ethyl acetate. The combined organic extracts were washed with sodium hydrogen carbonate solution, then with water, dried with anhydrous sodium sulfate and evaporated under reduced pressure to furnish, after recrystallization from methanol, yellow powder of **4** (2.4 g, 87% yield); mp 115–120 °C; IR (KBr): 3057 (=CH); 2999, 2956, 2935 (CH_3); 2835(–CH); 1645 (C=O); 1593, 1512 (C=C); 1261 (C–O) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.67 (d, 2H, H-1/H-5, $^3J_{1,2=4,5}$ = 16.0 Hz); 7.38 (d, 2H, 2 H-2′, $^4J_{2',6'}$ = 1.5 Hz); 7.30 (dd, 2H, 2 H-6′, $^3J_{5',6'}$ = 8.0 Hz, $^4J_{2',6'}$ = 1.5 Hz); 7.20 (d, 2H, H-2/H-4, $^3J_{1,2=4,5}$ = 16.0 Hz); 7.00 (d, 2H, 2 H-5′, $^3J_{5',6'}$ = 8.0 Hz); 3.81 (s, 6H, 2 MeO); 3.79 (s, 6H, 2 MeO). Anal. Calcd for $C_{21}H_{22}O_5$: C, 71.19; H, 6.22. Found: C, 71.15; H, 6.18.

5.1.5. 1,5-Bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-ylidenmalononitrile (5)

1,5-Bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one (**1**) (3.26 g, 0.01 mol), malononitrile (0.66 g, 0.01 mol), ammonium acetate (3.5 g, 0.045 mol), acetic acid (9.5 ml), and toluene (50 ml) are added following Cope's variant, heating under reflux for 8 h. Then the solution was concentrated under reduced pressure to a syrup. The residue was poured in water and extracted with ethyl acetate. The combined organic layers were washed with sodium hydrogen carbonate solution, then with water, dried with anhydrous sodium sulfate and evaporated until dryness. Further purification of the product was not necessary; orange powder of **5** (2.84 g, 76% yield); mp 216 °C; IR (Nujol): 3352, 3198 (OH), 2246, 2208 (CN); 1602, 1515 (C=C); 1273 (C–O) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.40 (br, 2H, 2 OH); 7.56 (d, 2H, H-1/H-5, $^3J_{1,2=4,5}$ = 15.8 Hz); 7.15 (d, 2H, 2 H-2′, $^4J_{2',6'}$ = 1.6 Hz); 7.12 (dd, 2H, 2 H-6′, $^3J_{5',6'}$ = 8.1 Hz, $^4J_{2',6'}$ = 1.6 Hz); 6.99 (d, 2H, 2 H-5′, $^3J_{5',6'}$ = 8.1 Hz); 6.73 (d, 2H, H-2/H-4, $^3J_{1,2=4,5}$ = 15.8 Hz); 3.76 (s, 6H, 2 MeO). Anal. Calcd for $C_{22}H_{18}N_2O_4$: C, 70.59; H, 4.82; N, 7.49. Found: C, 70.53; H, 4.79; N, 7.51; EI-MS *m*/*e*: 374 (M^+), 359 (M^+-CH_3), 357 (M^+-OH), 348 (M^+-CN), 343 (M^+-OCH_3).

5.2. Cytotoxic effects screening

5.2.1. Cytotoxic effects on human tumor cell lines

Since it is known that different cell lines display different sensitivities toward a cytotoxic compound, the use of more than one cell line is therefore considered to be necessary in the detection of cytotoxic compounds. Bearing this in mind, cell lines of different histological origin were used in the present study.

Human tumor cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-460 (lung, non-small cells), OVCAR-03 (ovarian), PC-03 (prostate), HT-29 (colon), 786-O (renal), and NCI-ADR/RES (ovarian

expressing phenotype multiple drugs resistance) were kindly provided by National Cancer Institute (NCI).

Stock cultures were grown in a medium containing 5 ml of RPMI-1640 (Gibco BRL, Life Technologies) and supplemented with 5% of fetal bovine serum. Gentamicin (50 μg/ml) was added to the experimental cultures. Cells in 96-well plates (100 μl cells/well) were exposed to four concentrations of samples in dimethyl sulfoxide at 37 °C, 5% of CO_2 in air for 48 h.

Then, a 50% of trichloroacetic acid solution was added and after incubation for 30 min at 4 °C, washing and drying, the cell proliferation was determined by spectrophotometric quantification (540 nm) of the cellular protein content using sulphorhodamine B assay.

Sulphorhodamine B (SRB) is an aminoxantine with a bright pink color that has two sulfonic groups that bind to protein's amino acids basic terminals of cells fixed with trichloroacetic acid. Therefore, this non-clonogenic methodology permits a high sensitive protein dosage with a straight relationship to cell culture.[19]

5.2.2. Cytotoxic and antiproliferative effects on B16F10 melanoma cells, L1210 leukemia cells, and mice macrophages

Analyses of cytotoxic and antiproliferative effects against B16F10 and L1210 tumor cell lines and mice macrophages were performed with compounds **1** and **2**. Solutions with different concentrations were obtained by diluting the substance with Miglyol 810®.

The colorimetric methodology for the respiratory route of mitochondria MTT (3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide) was used, as described by Mosmann.[20] The MTT assay is a colorimetric assay that relies on the enzymatic reduction of a yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which forms a purple formazan crystal in metabolically active cells. The formazan can then be solubilized producing a concentration dependent colorimetric signal at 540–570 nm proportional to the cell number and activity. This results in a sensitive assay that is readily adaptable to a 96-well microplate format to analyze a large numbers of samples using a microplate absorbance reader.

After growth and in vitro confluence of normal and tumoral cells, different concentrations of compounds **1** and **2** diluted in Miglyol 810® and the diluent-control (Miglyol 810®) were added on the adhering cells, incubated for 24 h. After the referred period, 10 μl of MTT (5 mg/ml) were added and incubated for 3 h at 5% CO_2 and 37 °C. After this period, the content of the culture plates was centrifuged for 2 min at 1800 rpm, at 4 °C.

The supernatant was removed and 100 μl of dimethyl sulfoxide were added to dissolve formazan crystals previously constituted and precipitated. The absorbances were obtained by an ELISA reader (TiterTek Multiskan) at 540 nm wavelength.

After growth and in vitro confluence of normal and tumoral cells, different concentrations of compounds **1** and **2** diluted in Miglyol 810® and the diluent-control (Miglyol 810®) have been added on the adhering cells, incubated for 24 h. After the referred period, 10 μl of MTT (5 mg/ml) were added and incubated for 3 h at 5% CO_2 and 37 °C. After this period, the content of the culture plates was centrifuged for 2 min at 1800 rpm, at 4 °C. The supernatant was removed and 100 μl of dimethyl sulfoxide were added to dissolve formazan crystals previously constituted and precipitated. The absorbances were obtained by an ELISA reader (TiterTek Multiskan) at 540 nm wavelength.

Commercial chemotherapics as paclitaxel and etoposide were considered as standard drugs.

5.2.3. Cytotoxic effect on peritoneal normal macrophages

Peritoneal macrophages were obtained from normal Balb-C mice by the following procedure: after anesthesia, abdominal cavities of the animals were opened under sterile conditions under a laminar flow and exposed. The acute toxicity tests were conducted some years ago and followed the OECD (Organization for Economic Co-operation and Development) protocol (OECD425).[20,21]

Two milliliters of the saline solution, containing 5000 U anticoagulant heparin (Liquemine-Roche), were injected into the cavity, followed by a massage and collection of the peritoneal wash irrigation liquid, which was centrifuged at 2000 rpm for 10 min at 4 °C.

The suspension was resuspended with RPMI-1640 culture medium supplemented with 10% bovine fetal serum, with the count cell number adjusted to 10^6/ml in a Neubauer chamber. Aliquots of the macrophages were cultivated on plates with 96 flat bottom wells, incubated with and without compounds **1** and **2** diluted in Miglyol 810®. After 24 h, the plates were submitted to the cytotoxic and antiproliferative effect assessment by the MTT colorimetric method.

5.2.4. Culture of leukemia murine L1210

L1210 lymphocyte murine leukemia cells were obtained from American Type Culture Collection (ATCC—clone CCL 219). These cells were grown in Dulbecco's modified Eagle's medium contained antibiotics, L-glutamine (70 mM), supplemented with 10% fetal bovine serum. Culture cell growth was measured by cell count using a Neubauer chamber. After treatment with drug, the cell viability was quantified by using an MTT [3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2*H*-tetrazolium bromide] standard spectrophotometric assay. After this treatment, the content of the plates was centrifuged for 2 min at 1800 rpm, at 4 °C. The supernatant was removed and 100 μl of dimethyl sulfoxide were added to dissolve formazan crystals. The absorbances were obtained by an ELISA reader (Titer-Tek Multiskan) at 540 nm wavelength and the inhibitory potencies of the compounds tested were expressed as IC_{50}.

5.3. Medium lethal dose assays (LD_{50})

Toxicological tests were performed using 20 male albino *Swiss* mice, weighting approximately 25 g each, divided equally in treated and control groups.

Animals were maintained in the test rooms at least for 7 days for adaptation. They were then submitted to fast for 12 h before administering the test substances by intraperitoneal administration after weight determination. The tested doses were of 1, 2.5, and 5 g/kg of animal body weight, respectively. They were observed for a minimum of 14 days for signals of toxicity and determination of number of deaths. The LD_{50} values were assessed by Litchfield and Wilcoxon's method.[22]

5.4. Computer-aided studies

The three-dimensional structures of each of the five pentadienones were constructed employing the *HyperChem* 7 software.[23] The crystallized structure of 1,5-bis(4-methylphenyl)-1,4-pentadien-3-one was retrieved from the Acta Crystallographica Section E: Structure Reports Online (entry code to cif file: bq2087, resolution at 1.04 Å)[24] and was applied as geometry references in the building up of all compounds.

The energy minimization was carried out employing the HyperChem 8 MM+ force field without any restriction, followed by calculating the partial atomic charges using RM1 semiempirical method, also implemented in HyperChem software.

Finally, geometry optimization and electrostatic partial atomic charges (Chelpg) were computed using the ab initio method HF/6-31G (Gaussian G03).[25] The electrostatic potential (EP) was mapped on a surface constructed to each of the five analogs. Negative electrostatic potential regions are represented in red color (high electronic density) while positive electrostatic potential areas are shown in dark blue color (low electronic density).

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