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ARTICLE

Antiproliferative Activity of Novel Isatinyl/Indanyl Nitrones (INs) as Potential *Spin Trapping* of Free Radical Intermediates

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Abstract A series of ketonitrones derived from isatin and indanone (INs) were synthesized and evaluated for their antiproliferative activities against several human cancer cell lines. Then, antioxidant properties of these substrates was measured by DPPH test to report the biological activity with their *spin trapping* action. In particular, one substrate has showed very high both biological and scavenging activity, probably due to strong correlation between *spin trapping* activity and structure.

Introduction

Small organic molecules have proven to be invaluable tools for investigating biological systems, but there is still much to learn from their use.[1-3]

Nitrones are small molecules that have general chemical formula X-CH=NO-Y. Their structural nature confers them their "*spin-trap*" ability for trapping free radical intermediates (R•), forming stable radical adducts (X-CHR=NO•-Y).[4] In fact, studies of spin trapping methods involved the reaction of nitrones with reactive free radicals such as hydroxyl (•OH), lipid alkoxy (•OL) or lipid hydroxyperoxyl (•OOL) radicals, observing the formation of more stable radicals that can be classified and quantified by Electron Spin Resonance (ESR) or Electron Paramagnetic Resonance (EPR) spectroscopy.[5-7]

Considering a) that the biological systems may actively produce reactive oxigen species (ROS) and reactive nitric oxide species (RNS), b) that specific oxidation products are produced from reaction between biological molecules and ROS and RNS, c) that ROS and RNS play an important role in many pathologic diseases, nitrones and, in particular, PBN-nitrones where X is a phenyl group and Y is a *tert*-butyl group were recently noted as therapeutics for their wide spread anti-cancer activity.[8-9] The mechanistic basis of their anti-cancer action is not known. It is probable that the ability to scavenge radical intermediates that are produced during disease processes, including cancer, is the basis for their anti-cancer activity.[4] Moreover, their action on important membrane enzymes and as anti-inflammatory agents seems to contribute to the magnification of their antioxidant activity.

Isatin and oxindole derivatives have found wide application in medicinal chemistry, including as potential anticonvulsant,[10] cyclin-dependent kinase 2-inhibitors,[11] poxvirus,[12] ectomelia,[13] rhinovirus,[14] HIV-1[15] and the corona-virus responsible for severe acute respiratory syndrome (SARS)[16]. Moreover, the insertion of an oxindole nucleus in spirocompounds has recently emerged because of their prevalence in various natural products and biologically active molecules.[17] In fact, the key of their activity seems to be combination of a spiro carbon and a variously substituted oxindole core. Therefore, the oxindole moiety could be considered as a useful tool in drug discovery.

It is generally been recognized that the incorporating of different bioactive scaffolds into one molecule is the powerful strategy to construct substrates with structural novelty and biological potential.[18] In an attempt to generate novel molecular entities with amplified spin trapping and antiproliferative activity on cancer cells, we synthesized some isatin and indanone ketonitrones, conjugating the simultaneous presence of an oxindole-like ring and a nitrone portion.

Results and discussion

In literature, few examples of synthetic procedures of nitrones of oxindole derivatives have been reported thus far.[19-22] Recently, we have realized an environmental-friendly approach to synthesize aldo- and ketonitrones by solvent-free condensation of alkyl- or arylhydroxylamines hydrochlorides with aromatic aldehydes under microwave irradiation.[23]

Herein, we report a practical and stereoselective synthesis of isatinyl and indanyl nitrones (INs) starting from substituted

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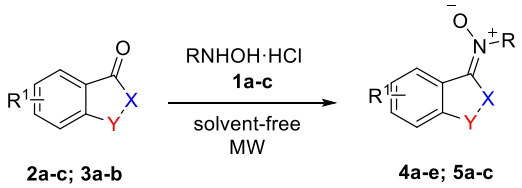
hydroylamines **1a-c** and isatin derivatives **2a-c** or indanone **3a-b**, as showed in table 1. Then, we investigated the antiproliferative activity of these substrates on MG63 and TE85 human osteosarcoma cell lines and K562 human erythroleukemic cell line, and we next sought to correlate it with their antioxidant properties measured by DPPH assay.

The target compounds **4a-e** and **5a-c** were prepared following the our previously reported methodology[23] that consists of the co-grinding of the ketone and hydroxylamine in a mortar, followed by transfer of the mixture without use of solvents in an appropriate vessel and further mixing of the solids in a vortex. Finally, the mixture is placed in a microwave oven without use of solvent and is irradiated at 600 W or 400 W for a variable time in the order of minutes.

Table 1. Synthesis of isatinyl and indanyl nitrones



Entry	R	R^1	Y	X	MW (W)	Time (min)	Product	Yield (%)
1	Me	5-NO_2	NH	CO	600	10	4a	95
2[a]	Ph	H	NH	CO	600	10	4b	92
3	Bn	5-NO_2	NH	CO	600	13	4c	92
4	Bn	H	NH	CO	600	12	4d	95
5	Bn	H	NMe	CO	600	12	4e	92
6	Me	H	CH_2	CH_2	400	30	5a	82
7	Me	5-F	CH_2	CH_2	400	28	5b	83
8	Bn	H	CH_2	CH_2	400	30	5c	85

[a] Ketone/alkylhydroxylamine ratio1:2 for all substrates except for entry 2 with ratio 1:3.

The variously substituted products **4a-e** and **5a-c** were obtained in high yields (83-95%). The minor reactivity of indanone cycle induced us both to reduce the MW power and to increase the reaction times. In all cases, nitrones were obtained as single isomers, *(E)* **4a-e** and *(Z)* **5a-c** respectively, as confirmed by NOESY experiments. In particular, for nitrone **4b** the formation of only a single product, *E*-isomer, is observed respect to *Z/E* mixture obtained by other synthetic methodologies.[24]

In an attempt to understand the molecular characteristics and the effects produced by the modifications introduced in our nitrones on tumour cell proliferation, nitrones prepared according to this procedure were evaluated for their antiproliferative activity against human osteosarcoma (MG63 and TE85)[25] and chronic myeloid leukemia (K562)[26] cell lines. Cells were cultured for three days in the absence or in the presence of increasing concentrations of nitrones and then their vitality was evaluated by dosing the activity of the oxidative metabolism by the 3-(4,5- dimethylthiazolyil-2)-2,5-diphenyltetrazolium bromide (MTT) assay.[27] In Figure 1 the proliferation of the treated cells was expressed as percentage compared to un-treated control cells.

The data indicated that not all the nitrones had antiproliferative activity, since the **5a-c** and **4b** compounds were completely inactive.

Compounds **4c-e** inhibited the proliferation of osteosarcoma MG63 cells compared to untreated cells (Fig. 1A, IC_{50} about 77 µM). In Figure 1B are shown the antiproliferative activities of **4a** and **4c-d** nitrones on TE85 cells (IC_{50} about 32 µM). The active compounds are less effective in inhibiting the proliferation of K562 cells, with the exception of **4e** nitrone with an IC_{50} of about 78 µM (Fig. 1C). These results together have shown that the indanyl derivatives do not possess antiproliferative activity on the tumour cells analyzed, as well as the derivative **4b** with the phenyl substituent. By contrast, isatinyl nitrones containing methyl or benzyl groups possess antiproliferative activity.

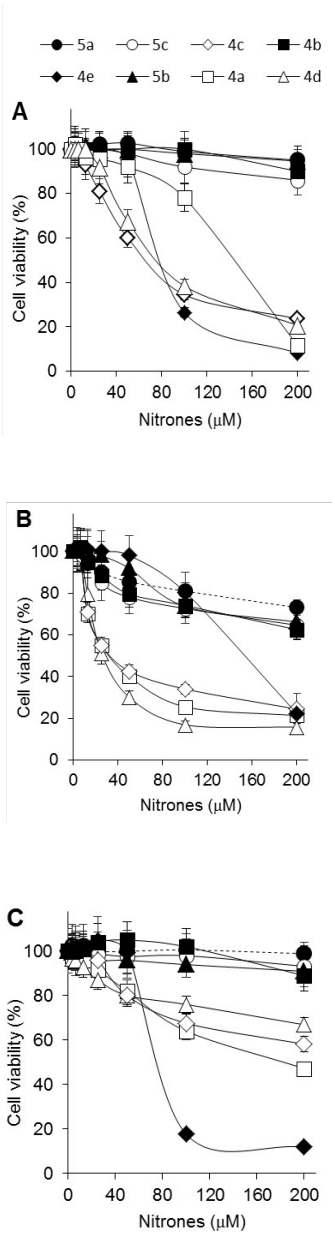
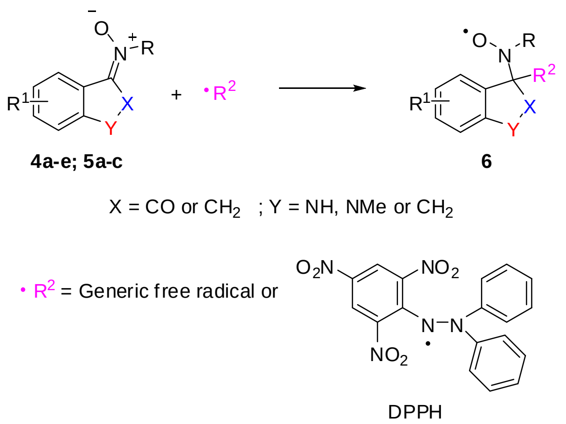


Figure 1. Effect of nitrones **4a-e** and **5a-c** on cell proliferation. MG63 (A), TE85 (B) and K562 (C) cells were incubated for three days in the presence of the indicated compounds. Viable cells were measured by MTT test and reported as % of untreated control. The arithmetic mean value ± standard deviation of three experiments performed in triplicates is shown.

The *in vitro* antioxidant activity of these ketooxindole nitrones **4a–e** and **5a-c** was evaluated by using the stable organic free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity.[28-31] In scheme 1 reaction mechanism for the *spin-trapping* action of nitrones **4a-e** and **5a-c** with a generic free radical (including DPPH radical) is illustrated, in according to literature.[32] It can be noted from the scheme below that the reaction produces the highly stable nitroxyl radical **6**.[33-34]



Scheme 1. Reaction mechanism of nitrones **4a-e** and **5a-c** with free radicals

When DPPH reacts with a radical scavenger its maximum absorbance decreases. A freshly prepared DPPH solution displayed a deep purple colour with an absorption maximum at 517 nm. The ethanolic solution of DPPH was added to the solution of the synthesized compounds in ethanol. After 10-min incubation in dark, the absorbance was measured by using absolute ethanol as a blank. BHT (butylated hydroxytoluene) was used as reference. In the presence of antioxidant the DPPH absorbance decreases. The antioxidant activity was calculated as radical scavenging activity (RSA%) as expressed in the equation 1:

$$RSA\% = [(A_0 - A_i) / A_0] \times 100 \qquad (1)$$

where A_0 and A_i are the DPPH absorbance at 517 nm in the absence and presence of the synthesized compounds, respectively. The RSA% for ketonitrones **4a–e** and **5a-c** at five different concentrations (*i.e.*, 1.15, 2.45, 4.00, 6.25 and 9.10, 10^{-6} mol/L) of the tested compounds with DPPH at 517 nm was reported in table 2.

Table 2. Percentage of *in vitro* radical scavenging activity of INs

Compounds	Concentrations (10^{-6} mol/L) 1.15	2.45	4.00	6.25	9.10	EC_{50}
4a	-	-	1.1±0.5	72±2	79±2	1.2±0.2
4b	1.1±0.5	1.2±0.5	1.2±0.5	69±2	80±2	1.3±0.2
4c	-	-	60±1	67±2	80±2	1.0±0.2
4d	1.1±0.5	1.1±0.5	1.2±0.5	1.2±0.5	78±2	1.8±0.1
4e	1.2±0.5	60±1	68±2	70±2	79±2	0.6±0.3
5a	-	-	1.1±0.5	60±1	78±2	1.3±0.2
5b	-	-	1.1±0.5	1.2±0.5	79±2	1.8±0.1
5c	1.1±0.5	1.2±0.5	1.3±0.5	70±2	79±2	1.2±0.2
BHT	-	4.6±0.5	11.7±0.5	23.0±0.5	27.0±0.5	3.8±0.5

As can be seen in the table 2, all the compounds exhibited antioxidant activity. The substances can be divided in three main groups. The first group is composed by two substances (**4c** and **4e**) with higher values of antioxidant activity than others. In particular, the greatest values were observed for **4e**, for which the RSA% is equal to 60% at a concentration of 2.45 10^{-6} mol/L.

Four substances compose the second group with intermediary antioxidant activity (**4a-b**, **5a** and **5c**). For all of these RSA% was major than 60% at a concentration equal to 6.25 10^{-6} mol/L. In the third group, only two compounds with lower less antioxidant activity were included: **4d** and **5b**. For these last two compounds RSA% was 80% at a concentration equal to 9.10 10^{-6} mol/L. As can be seen in the table 2 and in figure 2, all nitrones **4a-e** and **5a-c** exhibited a radical scavenging activity significantly major than those of the reference (BHT). We have not investigated concentrations higher than 9.10 10^{-6} mol/L as the RSA% trend reaches a plateau.

The RSA% of nitrones **4a-e** and **5a-c** may be compared with the value of archetypal (*Z*) α-phenyl-*N*-tert-butylnitrone (PBN) reported in literature.[35] Its RSA% is 1.4 ± 0.9 at concentration of 0.5 10^{-3} mol/L calculated through DPPH test, demonstrating an antioxidant property highly minor respect to our nitrones.

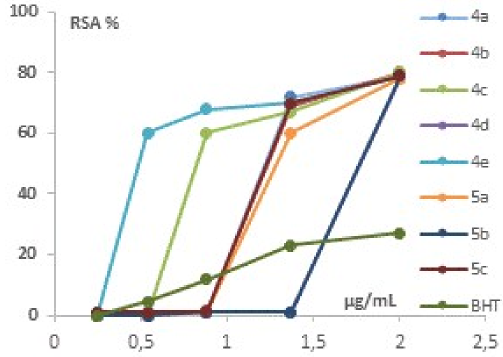


Figure 2. Graphical presentation of *in vitro* DPPH radical scavenging activity of compounds relative to the standard antioxidant BHT.

For all tested compounds the EC_{50} values were also calculated by using GraphPad Prism 5.01 program, as reported in literature (table 2).[36] The EC_{50} is the antioxidant concentration required to obtain a 50% radical inhibition. As can be seen in table 2 the value of EC_{50} of all eight synthesized nitrones are higher than reference substance (BHT).

The antioxidant properties of INs seems to converge quite well with the biological results, confirming the essentiality of isatinyl ring. In particular, the presence both of aromatic systems and electron-donating groups (e.g. methyl group) improve the performance, as can particularly be seen for **4e** substrate.

Experimental

Commercial starting materials were used without further purification. Reactions were monitored by TLC using silica plates 60-F264, commercially available from Merck. ^{1}H and ^{13}C NMR spectra were recorded at 300 MHz and 75 MHz, respectively, in $CDCl_3$ and DMSO-d_6 using tetramethylsilane (TMS) as internal standard (Bruker ACP 300 MHz). Chemical shifts are given in parts per million and coupling constants in Hertz. The stereochemistry were

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established by NOESY experiments. Purity was verified by NMR and HPLC spectra.

LC-MS analysis were carried using an Agilent 6540 UHD Accurate - Mass Q-TOF LC–MS (Agilent, Santa Clara, CA) fitted with a electrospray ionisation source (Dual AJS ESI) operating in positive ion mode. Chromatographic separation was achieved using a C18 RP analytical column (Poroshell 120, SB-C18, 50 × 2.1 mm, 2.7 µm) at 30 °C with a elution gradient from 5% to 95% of B over 13 min., A being H_2O (0.1% FA) and B CH_3CN (0.1% FA). Flow rate was 0.4 ml/min.

MW-assisted reactions were performed in Synthos 3000 instrument from Anton Paar, equipped with a 4x24MG5 rotor and an IR probe as external control of the temperature. 0.3–3 mL. Glass vials sealed with a dedicated PEEK screw-cup together with a reliable PTFE seal were used for all reactions. In synthesis of all derivatives the setting of temperature was always maintained to 180 °C in each experiment, except for **5a-c** where temperature was always maintained to 125 °C.

*General procedure for synthesis of nitrones **4a-e** and **5a-c***

The selected ketone (0.50 g) and appropriate hydroxylamine derivative (2 eq or 3 eq for *N*-phenylhydroxylamine) were grinded in a mortar, placed in apposite vessel and mixed by vortex. The mixture was transferred to a microwave oven and was irradiated with the opportune power. After the appropriate time the crude oil was recrystallized with ethyl acetate (**4a-e**) or cyclohexane (**5a-c**).

*(E)-N-(5-nitro-2-oxoindolin-3-ylidene)methanamine oxide **4a***

Yellow solid, 95% yield, (0.52 g). ^{1}H NMR (300 MHz, DMSO-d_6): 4.28 (s, 3H, CH_3), 7.02 (d, J = 8.73 Hz, 1H, Ar), 8.23 (d, J = 8.73 Hz, 1H, Ar), 8.75 (s, 1H, Ar), 11.53 (s, 1H, NH), ^{13}C NMR (75 MHz, DMSO-d_6): δ 51.8, 110.2, 118.7, 118.7, 128.0, 133.3, 142.4, 145.2, 161.9. ESI (+)-MS: m/z [M+H] calcd for $C_9H_8N_3O_4$ 222.0515, found: 222.0503.

*(E)-N-(2-oxoindolin-3-ylidene)aniline oxide **4b***

Orange red solid, 92% yield, (0.74 g). ^{1}H NMR (300 MHz, DMSO-d_6): 6.85 (d, J = 7.80 Hz, 1H, Ar), 7.15 (t, J = 7.65 Hz, 1H, Ar), 7.40 (t, J = 7.80 Hz, 1H, Ar), 7.45-7.65 (m, 5H, Ar), 8.27 (d, J = 7.65 Hz, 1H, Ar), 10.78 (s, 1H, NH), ^{13}C NMR (75 MHz, DMSO-d_6): δ 109.7, 118.4, 121.7, 123.9, 124.1, 128.7, 130.1, 132.2, 134.4, 140.9, 146.3, 159.9. ESI (+)-MS: m/z [M+H] calcd for $C_{14}H_{11}N_2O_2$ 239.0821, found: 239.0817.

*(E)-N-(5-nitro-2-oxoindolin-3-ylidene)-1-phenylmethanamine oxide **4c***

Yellow solid, 92% yield (0.64 g). ^{1}H NMR (300 MHz, DMSO-d_6): 5.91 (s, 2H, CH_2), 7.07 (d, J = 8.73 Hz, 1H, Ar), 7.31-7.58 (m, 5H, Ar), 8.28 (dd, J = 2.43 Hz, 8.73 Hz, 1H, Ar), 8.84 (d, J = 2.43 Hz, 1H, Ar), 11.70 (s, 1H, NH) ^{13}C NMR (75 MHz, DMSO-d_6): δ 65.6, 110.4, 118.8, 119.0, 128.3, 129.1, 129.1, 129.6, 133.2, 134.1, 142.6, 145.4, 161.9. ESI (+)-MS: m/z [M+H] calcd for for $C_{15}H_{12}N_3O_4$ 298.0828, found: 298.0816.

*(E)-N-(2-oxoindolin-3-ylidene)-1-phenylmethanamine oxide **4d***

Orange solid, 95% yield (0.80 g). ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.89 (s, 2H, CH_2Bn), 6.91 (d, J = 7.78 Hz, 1H, Ar), 7.10 (dt, J = 0.91, 7.65 Hz, 1H, Ar), 7.25-7.54 (m, 6H, Ar), 8.12 (d, J = 7.65 Hz, 1H, Ar), 11.01 (s, 1H, NH), ^{13}C NMR (75 MHz, DMSO-d_6): δ 64.3, 109.8, 118.1, 122.04, 123.9, 128.5, 129.6, 129.1, 131.8, 133.4, 134.2, 139.6, 161.2. ESI (+)-MS: m/z [M+H] calcd for $C_{15}H_{13}N_2O_2$ 253.0977, found: 253.0977.

*(E)-N-(1-methyl-2-oxoindolin-3-ylidene)-1-phenylmethanamine oxide **4e***

Yellow solid, 92% yield (0.76 g). ^{1}H NMR (300 MHz, DMSO-d_6): 3.25 (s, 3H, CH_3), 5.92 (s, 2H, CH_2), 7.08 (t, J = 8.26 Hz, 2H, Ar), 7.37-7.50 (m, 6H, Ar), 8.15 (d, J = 7.34 Hz, 1H, Ar), ^{13}C NMR (75 MHz DMSO-d_6): δ 26.6, 65.2, 109.2, 117.8, 123.1, 124.0, 129.0, 129.0, 129.59, 132.2, 134.6, 141.5, 160.4. ESI (+)-MS: m/z [M+H] calcd for $C_{16}H_{15}N_2O_2$ 267.1134, found: 267.1126.

*(Z)-N-(2,3-dihydro-1H-inden-1-ylidene)methanamine oxide **5a***

White solid, 82% yield (0.50 g). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.90-3.05 (m, 2H, CH_2), 3.11-3.22 (m, 2H, CH_2), 3.81 (s, 3H, CH_3), 7.29-7.44 (m, 3H, Ar), 8.84 (d, J = 7.81 Hz, 1H, Ar), ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.7, 29.4, 49.6, 124.5, 127.0, 131.0, 134.4, 147.8, 149.7. ESI (+)-MS: m/z [M+H] calcd for $C_{10}H_{12}NO$ 162.0919, found: 162.0912.

*(Z)-N-(5-fluoro-2,3-dihydro-1H-inden-1-ylidene)methanamine oxide **5b***

White solid, 83% yield (0.48 g). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.01 (dd, J = 5.43 Hz, 11.73 Hz, 2H, CH_2), 3.14 (dd, J = 5.55 Hz, 11.97 Hz, 2H, CH_2), 6.80-7.12 (m, 2H, Ar), 8.86 (dd, J = 5.79 Hz, 8.64 Hz, 1H, Ar), ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.8 (d, JCF = 2.28), 29.9, 49.6, 111.9 (d, J2CF = 23.02), 114.3 (d, J2CF = 22.86), 128.8 (d, J3CF = 9.03), 130.9 (d, J4CF = 2.02), 148.1, 150.5 (d, J3CF = 8.95), 164.43 (d, J1CF = 250.96). ESI (+)-MS: m/z [M+H] calcd for $C_{10}H_{11}FNO$ 180.0825, found: 180.0818.

*(Z)-N-(2,3-dihydro-1H-inden-1-ylidene)-1-phenylmethanamine oxide **5c***

White solid, 85% yield (0.76 g). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.91-3.09 (m, 2H, CH_2), 3.10-3.23 (m, 2H, CH_2), 5.11 (s, 2H, CH_2Bn), 7.21-7.45 (m, 7H, Ar), 7.51 (d, J = 7.16 Hz, 1H, Ar), 8.92 (d, J = 7.63 Hz, 1H, Ar), ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.0, 29.2, 66.6, 124.5, 127.1, 127.2, 128.2, 128.3, 128.9, 131.1, 133.4, 134.8, 147.7, 149.1. ESI (+)-MS: m/z [M+H] calcd for $C_{16}H_{16}NO$ 238.1232, found: 238.1230.

Antiproliferative activity evaluation

MG63 and TE85 human osteosarcoma cell lines were maintained in DMEM, while K562 human chronic myeloid leukemia cell line in RPMI 1640. Culture medium was supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 U/mL) and glutamine (2 mM); incubation was at 37 °C in a 5% CO_2 atmosphere. Compounds were solubilized in DMSO at 40 mM, kept at -80°C in the dark and diluted in complete medium immediately before use. The osteosarcoma cells were seeded at 4000 cells/well in 96-well plate 6 hours before the treatment. The leukemia cells were seeded at 20.000 cells/mL. Cells were treated with the test compounds at concentrations ranging from 3 to 200 µM. Untreated cells were placed in every plate as negative control. After 3 days of culture, the inhibitory effect on cell proliferation was analysed by addition of 25 µL MTT (thiazollyl blue) staining solution, in which metabolically active cells convert the yellow tetrazolium salt to purple formazan crystals providing a quantitative determination of viable cells. Two hours later, formazan crystals were solubilized in 100 µL of lysing buffer (50% DMF + 20% SDS, pH 4.7) for 16 hours, whereupon the spectrophotometric absorbance at 570 nm was measured. Half maximal inhibitory concentration (IC_{50}) was calculated using Scientist software. Three independent experiments were performed in triplicate.

Antioxidant evaluation

The free radical scavenging activity against DPPH was determined at five different concentrations according to a previous work.[28] Thus, EtOH solutions containing known amounts of the compounds (**4a-e** and **5a-c**) and of DPPH were prepared in a range of concentrations from 1.15 10^{-6} to 9.10 10^{-6} mol/L (table 2). The decrease in the absorbance of DPPH was measured at 517 nm, against ethanol as blank, by using a UV-Vis spectrophotometer (Varian Cary 50 Scan), after a period of 10 min since preparation. Experiments were carried out in triplicate and BHT (butylated hydroxytoluene) was used, in the same compounds range of concentrations, as the reference antioxidant.

Conclusions

In summary, a series of isatinyl/indanyl nitrones (INs) were synthesized and their antiproliferative activity on MG63, TE85 and K562 cells was determined. Derivatives with isatin ring showed highest activity, while the indanone nitrones were practically inactive. Then the antioxidant activities were evaluated through DPPH test. The results indicated that all compounds are markedly more active than reference compound BHT. Among them, nitrone **4e** exhibits the most potent antioxidant activity at low concentration and its *spin-trap* action matches perfectly with antiproliferative effect on cancer cells. Collectively, the current study may provide a new insight for the treatment of degenerative diseases such as cancer.

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