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RESEARCH ARTICLE

Antioxidant and cytotoxic properties of novel spirocyclic benzothiazolines

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ABSTRACT

In this work, we report the synthesis, structural characterization and evaluation of *in vitro* antioxidant and cytotoxic properties of novel spirobenzothiazolines (**1a**-**e**, **2a**-**e**). 5-nonsubstituted spirobenzothiazolines (**1a**-**e**) demonstrated notable inhibitory capacity on lipid peroxidation (LPO), reducing power and scavinging effects on diphenylpicryl hydrazine ($DPPH^{\bullet}$) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet +}$) radicals, that were similar to that of α-tocopherol. The most potent antioxidant was compound **1a** (incorporating an ethyl group on the cyclohexane ring) with an anti-LPO activity 2-fold higher than that of α-tocopherol. Compound **1a** exhibited anti-LPO and $DPPH^{\bullet}$ scavenging activities at concentrations lower than those cytotoxic for mouse normal fibroblast (NIH/3T3) cells and was also found to be slightly more selective for cancer cells (human prostat adenocarcinoma cell/PC-3) than normal mammalian cells.

Key words: Benzothiazole; spiroheterocycle; antioxidant activity; cytotoxic activity

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1. Introduction

Reactive oxygen species (ROS) are constantly formed in the body during normal cellular metabolism and eliminated by ROS scavenging mechanisms in order to maintain redox balance. The overproduction of ROS through endogenous and/or exogenous factors leads to cell-damaging oxidative stress [1]. Chronic and cumulative oxidative stress exerts detrimental effects on biomolecules such as DNA, proteins and lipids and contributes to the pathogenesis of cancer, rheumatoid arthritis, neurodegenerative diseases, atherosclerosis, diabetes mellitus and other diseases [2]. Some intracellular defences in mamalian cells (superoxide dismutase, glutathione peroxidase and catalase) can protect the cells against the extreme levels of free radicals. Also, exogenous antioxidants such as minerals (zinc, selenium), vitamins (A, C, E, β-karoten) or proteins (transferrin, albumin) have the ability to scavenge the ROS [3]. Thus, the search for new nontoxic compounds with antioxidant activity constitutes a very active search field.

Oxidative stress-induced DNA damage has been commonly accepted as a major cause of cancer. Actually, ROS have contradictory roles in carcinogenesis. They play a key role in tumor formation and progression by causing DNA mutations

and genomic instability [1]. On the other hand, many chemotherapeutics and radiotherapy exert cytotoxicity by generating reactive oxygen species since the excessive levels of ROS lead to apoptosis and cell death in cancer cells [4, 5]. Consuming antioxidants in the diet and supplements to prevent and fight diseases, especially cancer, has become very popular among the general public [6]. Antioxidants are also thought to be beneficial in alleviating the adverse effects of chemotherapy and/or radiotherapy [7]. However, the precise mechanism(s) of action of antioxidants in cancer is not yet fully known and clinical trials have still been reporting inconsistent outcomes [1, 4-9].

Due to their wide existance in bioactive natural products [10], spiroheterocycles have been focused on by researchers in chemistry and biology in the past decades. Indole phytoalexins are natural antimicrobial compounds produced by crucifers in response to biotic or abiotic stress [11]. Low-molecular spiro-phytoalexins such as spirobrassinin (**I**), 1-methoxyspirobrassinin and 1-methoxyspirobrassinol (**II**) (Figure 1) have been found to possess significant cytostatic/cytotoxic effects against various cancer cells and chemopreventive activity in models of mammary and skin carcinogenesis [12-15]. In two early reports, spiroindolinones incorporating a benzothiazole or 5-chlorobenzothiazole moiety (**III**) (Figure 1) have been described as promising antioxidants with potent scavenging activities against DPPH$^{\bullet}$ and (ABTS$^{\bullet+}$) radicals and reducing powers and significant inhibitory capacity on lipid peroxidation. Compounds have also been found to be cytotoxic against some cancer cell lines in anticancer screening [16, 17]. Likewise, the antioxidant properties of several small spiroheterocyclic molecules have been reported in different papers [18-21].

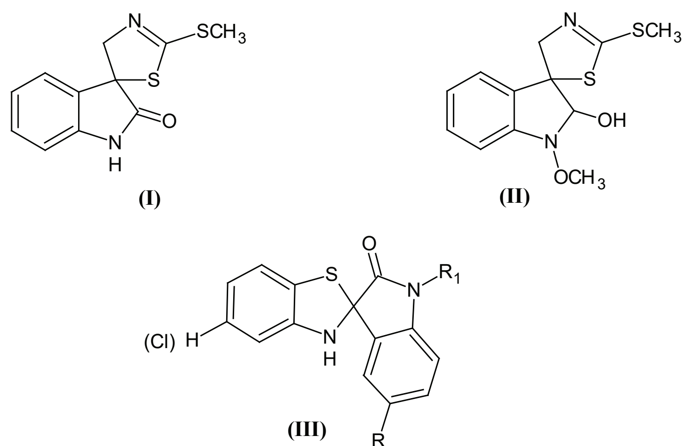


Figure 1. Structures of some bioactive spiroheterocyclic compounds

In this work, we report a series of spirobenzothiazolines that were obtained by condensation of 2-aminothiophenol/2-amino-4-chlorothiophenol and an appropriate ketone. We also report on their *in vitro* antioxidant activities including; inhibition of lipid peroxidation in liposomes, induced by Fe^{3+}/ascorbate system, scavenging effect on DPPH$^{\bullet}$, trolox equivalent antioxidant capacity (TEAC) and ferric ion reducing antioxidant power (FRAP), and cytotoxic potentials *in vitro* against mouse fibroblast (NIH/3T3, CRL-1658) and human prostate adenocarcinoma (PC-3, CRL-1435) cell lines.

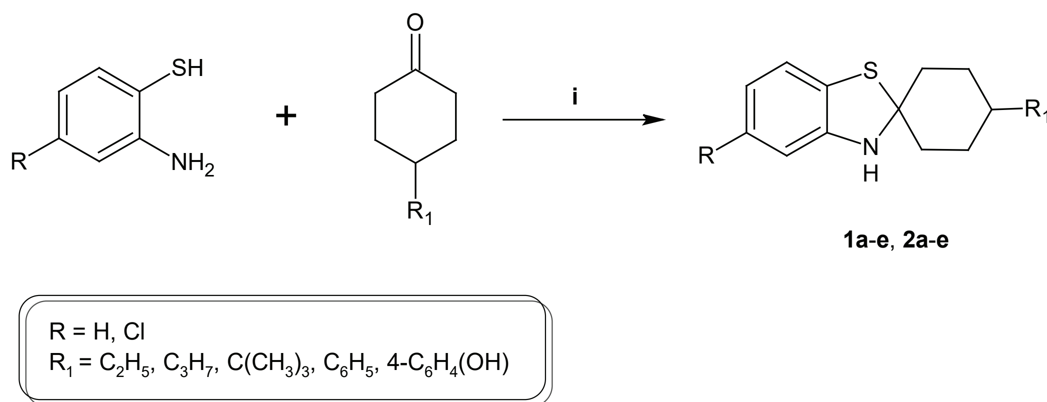
2. Results and Discussion

2.1. Chemistry and structural characterization

The spirocyclic benzothiazolines were synthesized by condensation of 2-aminothiophenol/2-amino-4-chlorothiophenol with appropriate ketone in absolute ethanol. Reactions occurred readily under mild temperatures (Scheme 1). Compound **1d** was previously reported by Coudert *et al.* as a potential analgesic [22]. Structures of compounds were established by microanalysis, IR, ^{1}H-NMR, ^{13}C-NMR (APT), 2D-NMR (HSQC, HMBC) and electrospray ionization mass spectrometry (ESI-MS). To assist in structural analysis, we have also studied the conformational properties of two compounds, **2a** and **2c**, by X-ray crystallography [23, 24].

The IR spectra of compounds **1** and **2** showed N-H stretching bands of the benzothiazole group at 3310-3389 cm^{-1}. Broad O-H stretching bands for compounds **1e** and **2e** were observed at 3343-3536 cm^{-1} region. Aliphatic and aromatic proton resonances of new compounds were found in the expected regions with appropriate coupling constants and integral values. ^{13}C-NMR experiments (APT) run on **1a**, **1c**, **1e** and **2b**-**e** and ^{1}H-^{13}C cross peaks observed in 2D-NMR experiments HSQC and HMBC run on **1b**, **1d** and **2a** allowed the complete assignment of the proton and carbon NMR signals. Benzothiazole N-H protons of **1a**-**e** and **2a**-**e** resonated at about 6.36-6.60 ppm and 6.73-7.17 ppm, respectively. Cross peaks observed between the benzothiazole NH proton and the bridgehead carbon in the HMBC spectrum of compound **2a** enabled definite assignment of N-H proton and bridgehead carbon resonances. The exchangeable N-H and O-H proton signals of compounds **1e** and **2a** were further confirmed by D_2O exchange experiments.

The proton spectra of both **1a**-**e** and **2a**-**e** displayed two sets of signals for most of the protons. Heteroatom protons N-H



Scheme 1. Synthesis of **1**-**2**. Reagent and condition: (i) abs. EtOH, reflux, 8 h.

and O-H (for compounds **1e** and **2e**) were observed as two separate singlets. Methyl protons of compounds **1a**, **1b** and **2a**, **2b** resonated as two triplets (*J=7.3 Hz*) and distorted multiplets, respectively. Many of the aromatic and aliphatic proton resonances were detected as duplicated signals or distorted multiplets. Similarly, two signal sets appeared for almost all carbon atoms in the ^{13}C-NMR spectra of compounds **1a**-**e** and **2a**-**e**. The multiplicity in the signals was thought to result from a conformational difference in spirocyclic ring system. In order to verify the purity of the products, solid structures of compounds **2a** and **2c** were investigated by X-ray crystallography [23, 24]. The X-ray studies have revealed a slight conformational difference between compounds **2a** and **2c**. In compound **2a**, the 2,3-dihydro-1,3-thiazole ring adopts an envelope with the S,N-bound C atom at the flap, whereas the nine-membered 2,3-dihydro-1,3-benzothiazole ring system is planar in compound **2c**. The cyclohexane ring has the expected chair conformation in both compounds. Interestingly, this conformational change leads to a clear difference in hydrogen bondings and crystal packings of two compounds. The crystal packing of **2a** is stabilized by intermolecular N-H---S hydrogen bonds, while molecules of **2c** are linked by C-H---π interactions. Based on the foregoing data, it can be concluded that compounds **1a**-**e** and **2a**-**e** exist as a mixture of two stable conformers in solution.

2.2. Biological activity

2.2.1. Antioxidant activity

In order to evaluate the antioxidant activities of ten spirobenzothiazoline derivatives, as an index of pharmacological usefulness, we decided upon the use of four model systems, namely, inhibition of Fe^{3+}-ascorbate induced phospholipid degradation, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging activities, and Fe (III) reduction. In the assessment of antioxidant capacity, both synthetically and biologically relevant free radicals were used. $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals are not biologically relevant, but are commonly used as indicator compounds in testing hydrogen transfer capacity that is related to antioxidant activity. a-Alfa sembolü Tocopherol was used as reference antioxidant. For comparison, Table 1 presents the results of the antioxidant activities, expressed as EC_{50}, TEAC and FRAP values.

All of the tested compounds demonstrated the ability to inhibit LPO. Compound **1a** was the most efficient inhibitor with the lowest EC_{50} value of 0.066±0.002 mM. Its inhibitory effect on Fe^{+3}/ascorbic acid-induced lipid peroxidation was nearly 2 times higher than that of reference compound, α-tocopherol. Compounds **1b**-**e** and **2a** showed almost similar inhibitory effects on TBARS formation, which were not significantly different than that of α-tocopherol.

Compounds **1a**-**e** showed similar degrees of efficacy in their scavenging activities against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals. The EC_{50} values for these compounds (0.27-0.29 mM) were comparable to that of a-tocopherol (0.28 mM).

In $ABTS^{\bullet+}$ scavenging method, the antioxidant activities of the tested compounds were expressed also as the Trolox Equivalent Antioxidant Capacity (TEAC). The TEAC reflects the ability of hydrogen or electron-donating antioxidants to scavenge the ABTS radical cation compared with that of Trolox. There was no significant difference between the TEAC values of **1a**–**e** which were comparable to that of α-tocopherol at the concentration of 2.5 mM, whereas the TEAC values of **2a**–**e** were significantly lower.

FRAP value reflects the electron donation capacity of the compounds. At a concentration of 2.5 mM, **1a**–**e** showed the highest ferric reducing antioxidant powers, which

Table 1. Antioxidant activities of **1a**-**e** and **2a**-**e** expressed as EC_{50}, TEAC and FRAP values

Compound	EC_{50} (mM)[a]			TEAC[b]	FRAP[c]
	Anti-LPO	DPPH	ABTS	mM/L	mM/L
1a	0.066 ± 0.002[1]	0.27 ± 0.038[1]	0.88 ± 0.10[1,2]	2.18 ± 0.02[1]	2.57 ± 0.13[1,2,3]
1b	0.127 ± 0.004[2]	0.28 ± 0.002[1]	1.06 ± 0.03[2,3]	2.17 ± 0.01[1]	2.50 ± 0.05[1]
1c	0.122 ± 0.002[2]	0.28 ± 0.005[1]	1.01 ± 0.45[1,2,3]	2.16 ± 0.01[1]	2.31 ± 0.05[2]
1d	0.124 ± 0.001[2]	0.29 ± 0.025[1]	0.96 ± 0.01[1]	2.16 ± 0.02[1]	2.33 ± 0.05[2]
1e	0.121 ± 0.006[2]	0.28 ± 0.009[1]	0.88± 0.06[1]	2.16 ± 0.01[1]	2.66 ± 0.06[3]
2a	0.122 ± 0.008[2]	0.80 ± 0.076[2,3]	1.30 ± 0.13[3]	1.80 ± 0.05[2]	1.91 ± 0.06[4]
2b	0.237 ± 0.003[3]	0.88 ± 0.030[3]	1.66 ± 0.05[4]	1.75 ± 0.08[2]	1.77 ± 0.01[4]
2c	0.250± 0.010[3,4]	0.97 ± 0.018[2]	1.63 ± 0.08[4]	1.83 ± 0.05[2]	1.34 ± 0.04[5]
2d	0.248 ± 0.002[4]	1.19 ± 0.510[4]	1.61 ± 0.04[4]	1.88± 0.02[2]	1.35± 0.06[5]
2e	0.239 ± 0.006[3,4]	1.59 ± 0.055[5]	1.59 ± 0.05[4]	1.86 ± 0.01[2]	1.69 ± 0.03[4]
α-tocopherol	0.128 ± 0.006[2]	0.28 ± 0.001[1]	1.02 ± 0.05[1,2,3]	2.16 ± 0.04[1]	2.75 ± 0.05[3]

[a]EC_{50} value: The effective concentration at which the antioxidant activity was 50 %; $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals were scavenged by 50%, respectively.
[b]Expressed as mmol Trolox equivalents at a concentration of 2.5 mM.
[c]Expressed as mmol ferrous ions eqivalents at a concentration of 2.5 mM.
Values were the means of three replicates ± standard deviation. Values with different superscript numbers in the same column were significantly ($p < 0.05$) different.

were almost similar to that of the reference at the same concentration.

When these data were examined, it was observed that –Cl substitution at the 5- position of the benzothiazole system had a negative effect on antioxidant activity, since compounds **2a**-**e** were found to be less active in all antioxidant tests carried out. Amongst the compounds **1a**-**e**, R_1-ethyl substituted **1a** was a significantly better inhibitor of lipid peroxidation.

2.2.2. Cytotoxic activity

Compounds **1a**-**e** and **2a**-**e** were further examined for cytotoxicity using the mouse fibroblast (NIH/3T3) and human prostate adenocarcinoma (PC-3) cell lines. The cytotoxicity results presented in Table 2 were expressed as the concentrations inhibiting 50% cell growth (IC_{50}). For six of the test compounds, IC_{50} values could not be calculated due to solubility problems. However, maximum deaths in the

Table 2. Cytotoxic activities of **1a**-**e** and **2a**-**e** against mouse fibroblast (NIH/3T3) and human prostat adenocarcinoma (PC-3) cell lines

Compound	IC_{50} (μg/mL)[a]	
	NIH/3T3	PC-3
1a	>145 (>0.62 mM)[b]	>145
1b	>132.5 (>0.54 mM)	>132.5
1c	>140 (>0.54 mM)	>140
1d	66.76 (0.24 mM)	98.41 (0.35 mM)
1e	21.47 (0.072 mM)	53.42 (0.18 mM)
2a	>137.5 (>0.51 mM)	>137.5
2b	77.52 (0.28 mM)	98.74 (0.35 mM)
2c	>135 (>0.46 mM)	>135
2d	>137.5 (>0.44 mM)	>137.5
2e	47.48 (0.14 mM)	50.44 (>0.15 mM)

[a]IC_{50}, the concentration inhibiting 50% cell growth.
[b]IC_{50} values converted to mM were presented in brackets.

highest concentrations were determined. In the NIH/3T3 cell line, the percentage cell death induced by compounds **1a**, **1b**, **1c**, **2a**, **2c** and **2d** were 21.4%, 25.8%, 21.5%, 24.3%, 38.8% and 36.8%, and IC_{50} values of **1d**, **1e**, **2b** and **2e** were 66.76, 21.47, 77.52, 47.48 µg/mL, respectively. In the PC-3 cell line, the percentage cell death induced by compounds **1a**, **1b**, **1c**, **2a**, **2c** and **2d** were 43.5%, 37.6%, 33.5%, 50.5%, 52.3% and 44.6%, and IC_{50} values of **1d**, **1e**, **2b** and **2e** were 98.41, 53.42, 98.74 and 50.44 µg/mL, respectively.

Both in mouse normal fibroblast and human adenocarcinoma cells, 50% growth inhibition was not achieved at levels up to 132.5-145 µg/mL for **1a**, **1b**, **1c**, **2a**, **2c** and **2d**. Compounds **1d**, **1e**, **2b** and **2e** were found to be slightly cytotoxic to both normal and cancer cells. Compound **1a** which displayed promising antioxidant effects, showed a differential between active and cytotoxic doses. **1a** demonstrated anti-LPO (EC_{50}=0.066 mM) and $DPPH^{\bullet}$ scavenging (EC_{50}=0.27 mM) activities at concentrations lower than those cytotoxic for the NIH/3T3 mammalian cells (IC_{50}= >0.62 mM). Based on the percentage cell death rates, it can be concluded that **1a** is slightly more selective for cancer cells (43.5%) than normal mammalian cells (21.4%).

3. Conclusion

In the search for effective antioxidant agents, we achieved the synthesis of novel spirobenzothiazoline derivatives. The structures were confirmed by microanalysis, spectrometry and X-ray crystallography. In general, 5-nonsubstituted spirobenzothiazolines (**1a**-**e**) demonstrated notable inhibitory capacity on lipid peroxidation, reducing power and scavinging effects on $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals, that were similar to that of α-tocopherol in all antioxidant tests carried out. Compound **1a** incorporating an ethyl group on the cyclohexane ring was found to be the most potent antioxidant agent. The compound exhibited strong inhibitory effect on the Fe^{3+}/ascorbic acid-induced lipid peroxidation, which was nearly 2-fold higher than that of the reference compound, α-tocopherol. *In vitro* cytotoxicity of compounds **1a**-**e** and **2a**-**e** against mouse normal fibroblast (NIH/3T3) and human prostat adenocarcinoma (PC-3) cells were further studied. 50% inhibition of cell growth against normal and cancer cells was not achieved for **1a**, **1b**, **1c**, **2a**, **2c** and **2d** derivatives at highest concentrations tested. Compound **1a** exhibited anti-LPO and $DPPH^{\bullet}$ scavenging activities at concentrations lower than those cytotoxic for normal fibroblast cells. Compound **1a** was also found to be slightly more selective for cancer cells than normal mammalian cells.

4. Materials and Methods

4.1.Chemistry

Melting points were determined in open capillary tubes with a Buchi B-540 melting point apparatus and are uncorrected. Microanalyses were performed on a Thermo Finnigan Flash EA 1112 elemental analyzer. IR spectra were recorded in KBr discs (υ_{max} in cm^{-1}) on a Perkin-Elmer 1600 FTIR and Shimadzu IRAffinity-1 FTIR spectrophotometer. ^{1}H-NMR (DMSO-d_6), ^{13}C-NMR (APT) (DMSO-d_6) and heteronuclearcorrelation ^{1}H-^{13}C (HSQC, HMBC) (DMSO-d_6) spectra were run on Varian^{UNITY}INOVA (500 MHz) instrument. Chemical shifts are reported as d (ppm) relative to TMS as internal standard and coupling constants (*J*) are given in hertz (Hz). MS (ESI+/-) were determined on a Finnigan LCQ Advantage Max mass spectrometer. (cyc.: cyclohexane, ar.:aromatic, *:broad/distorted)

4.1.1.General procedure for the synthesis of 5-chloro/nonsubstituted-4'-substituted-3H-spiro[1,3-benzothiazole-2,1'-cyclohexanes] (**1a**-**e**, **2a**-**e**)

A mixture of 2-aminothiophenol/2-amino-4-chlorothiophenol (0.01 mol) and an appropriate cyclohexanone (0.01 mol) in absolute ethanol (50 mL) was refluxed on a water bath for 8 h. The solvent was evaporated in a crystallizing dish at room temperature and the residue was recrystallized from ethanol/water.

*4'-Ethyl-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane](**1a**)*

Beige crystals (56.7%); mp 87.5-88.5 °C; IR(KBr): υ_{max} =3341 (N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 0.85 and 0.86 (3H, 2t, J=7.3 Hz, 4-CH_2CH_3-cyc.), 1.03-1.38 (5H, m, 4-CH_2CH_3-cyc., CH/CH_2-cyc.), 1.56-1.74 (4H, m, CH/CH_2-cyc.), 2.09-2.13 (2H, m, CH/CH_2-cyc.), 6.37 and 6.55 (1H, 2s, NH), 6.45-6.54 (2H, m, Ar.-H), 6.79 (1H, td*, J=7.8; 1.5 Hz, Ar.-H), 6.90-6.93 (1H, m, Ar.-H); ^{13}C-NMR (APT) (DMSO-d_6/125MHz): δ 12.13, 12.27 (4-CH_2CH_3-cyc.), 28.98, 29.50, 30.54 (CH_2-cyc./4-CH_2CH_3-cyc.), 38.23 (CH-cyc.), 40.94 (CH_2-cyc.), 77.00, 81.68 (C-cyc.), 109.23, 109.79 (Ar.-CH), 118.54, 118.81 (Ar.-CH), 121.93, 121.95 (Ar.-CH), 124.91, 125.63 (Ar.-C), 125.54, 125.68 (Ar.-CH), 147.56, 147.72 (Ar.-C); MS (ESI+) m/z (%):234.1 ($M+H^+$, 100). Anal. Calcd for $C_{14}H_{19}NS$ (233.37): C, 72.05; H, 8.21; N, 6.00. Found: C, 71.84; H, 7.76; N, 5.95.

*4'-Propyl-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane](**1b**)*

Brown needles (67.2%); mp 72-74 °C; IR(KBr): υ_{max} =3337 (N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 0.85 and 0.86

(3H, 2t, J=7.3 Hz, 4-$CH_2CH_2CH_3$-cyc.), 1.05-1.38 (7H, m, 4-$CH_2CH_2CH_3$-cyc., CH/CH_2-cyc.), 1.55-1.74 (4H, m, CH/CH_2-cyc.), 2.07-2.12 (2H, m, CH/CH_2-cyc.), 6.36 and 6.55 (1H, 2s, NH), 6.45-6.53 (2H, m, Ar.-H), 6.79 (1H, t*, J=7.8 Hz, Ar.-H), 6.91 (1H,dd*, J=7.3;2.1 Hz, Ar.-H); ^{13}C-NMR (HSQC) (DMSO-d_6/125MHz): δ 14.92, 14.95 (4-$CH_2CH_2CH_3$-cyc.), 20.27, 20.34 (4-$CH_2CH_2CH_3$-cyc.), 29.31, 30.92 (CH_2-cyc.), 36.20 (CH-cyc.), 39.17 (4-$CH_2CH_2CH_3$-cyc.), 40.96 (CH_2-cyc.), 77.20, 81.67 (C-cyc.), 109.22, 109.77 (Ar.-CH), 118.54, 118.80 (Ar.-CH), 121.94 (Ar.-CH), 124.91, 125.61 (Ar.-C), 125.54, 125.68 (Ar.-CH), 147.55, 147.72 (Ar.-C); MS (ESI+) m/z (%):248.1 (M+H$^+$, 100). Anal. Calcd for $C_{15}H_{21}NS$ (247.40): C, 72.82; H, 8.56; N, 5.66. Found: C, 72.98; H, 8.75; N, 5.31.

4'-tert-Butyl-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane] ***(1c)***

Beige crystals (55.6%); mp 115-117 °C; IR(KBr): υ_{max} =3318 (N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 0.80-0.88 (9H, m, 4-$C(CH_3)_3$-cyc.), 1.00-1.42 (3H, m, CH/CH_2-cyc.), 1.56-1.72 (4H, m, CH/CH_2-cyc.), 2.15-2.23 (2H, m, CH/CH_2-cyc.), 6.37 and 6.60 (1H, 2s, NH), 6.45-6.57 (2H, m, Ar.-H), 6.77-6.80 (1H, m, Ar.-H), 6.91 (1H, d, J=7.8 Hz, Ar.-H); ^{13}C-NMR (APT) (DMSO-d_6/125MHz): δ 24.28, 25.32 (CH_2-cyc.), 28.06, 28.26 (4-$C(CH_3)_3$-cyc.), 32.70, 32.84 (4-$C(CH_3)_3$-cyc.), 41.36, 41.49 (CH_2-cyc.), 46.88, 47.21 (CH-cyc.), 77.73, 81.62 (C-cyc.), 109.21, 110.10 (Ar.-CH), 118.51, 118.93 (Ar.-CH), 121.83, 121.93 (Ar.-CH), 124.86, 125.78 (Ar.-C), 125.46, 125.66 (Ar.-CH), 147.64, 147.78 (Ar.-C); MS (ESI+) m/z (%):262.3 (M+H$^+$, 100). Anal. Calcd for $C_{16}H_{23}NS$ (261.43): C, 73.51; H, 8.87; N, 5.36. Found: C, 73.24; H, 8.77; N, 5.21.

*4'-Phenyl-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane]**(1d)*** [22]

White crystals (69.0%); mp 109-110 °C; IR(KBr): υ_{max} =3320 (N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 1.58-1.69 (2H, m, CH_2-cyc.), 1.82-1.96 (4H, m, CH_2-cyc.), 2.22-2.27 (2H, m, CH_2-cyc.), 2.52-2.58 (1H, m, CH-cyc.), 6.47 and 6.58 (1H, 2s, NH), 6.49-6.57 (2H, m, Ar.-H), 6.80-6.85 (1H, m, Ar.-H), 6.94-6.97 (1H, m, Ar.-H), 7.16-7.33 (5H, m, 4-Ar.(H)-cyc.); ^{13}C-NMR (HSQC) (DMSO-d_6/125MHz): δ 30.73, 32.19, 40.89, 41.21 (CH_2-cyc.), 42.65, 42.75 (CH-cyc.), 77.46, 81.13 (C-cyc.), 109.37, 109.62 (Ar.-CH), 118.67, 118.80 (Ar.-CH), 122.00, 122.03 (Ar.-CH), 124.86, 125.65 (Ar.-C), 125.42, 125.80 (Ar.-CH), 126.68, 126.77 (4-Ar.(CH)-cyc.), 127.29, 127.59 (4-Ar.(CH)-cyc.), 128.93, 129.09 (4-Ar.(CH)-cyc.), 146.82, 147.17 (4-Ar.(C)-cyc.), 147.54, 147.73 (Ar.-C); MS (ESI+) m/z (%):282.1 (M+H$^+$, 100). Anal. Calcd for $C_{18}H_{19}NS$ (281.42): C, 76.82; H, 6.81; N, 4.98. Found: C, 76.85; H, 6.42; N, 4.96.

*4'-(4-Hydroxyphenyl)-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane]**(1e)***

Beige powder (58.6%); mp 150-152 °C; IR(KBr): υ_{max} =3536, 3343, 3320 (O-H, N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 1.51-1.66 (2H, m, CH_2-cyc.), 1.77-1.85 (4H, m, CH_2-cyc.), 2.20 (2H, d*, J=12.7 Hz, CH_2-cyc.), 2.41-2.46 (1H, m, CH-cyc.), 6.44 and 6.57 (1H, 2s, NH-disappeared on D_2O exchange), 6.48-6.56 (2H, m, Ar.-H), 6.67 (1H, d, J=8.3 Hz, 4-Ar.(H)-cyc.), 6.68 (1H, d, J=8.5 Hz, 4-Ar.(H)-cyc.), 6.79-6.83 (1H, m, Ar.-H), 6.95 (1H, dd, J=7.3; 3.2 Hz, Ar.-H), 7.00 (1H, d, J=8.3 Hz, 4-Ar.(H)-cyc.), 7.09 (1H, d, J=8.3 Hz, 4-Ar.(H)-cyc.), 9.08 and 9.11 (1H, 2s, OH-disappeared on D_2O exchange); ^{13}C-NMR (APT) (DMSO-d_6/125MHz): δ 31.01, 32.48, 40.97, 41.29 (CH_2-cyc.), 41.76, 41.93 (CH-cyc.), 77.56, 81.19 (C-cyc.), 109.34, 109.62 (Ar.-CH), 115.62, 115.78 (4-Ar.(CH)-cyc.), 118.64, 118.77 (Ar.-CH), 121.98, 122.01 (Ar.-CH), 125.45, 125.88 (Ar.-C), 125.62, 125.77 (Ar.-CH), 128.04, 128.33 (4-Ar.(CH)-cyc.), 137.03, 137.37 (4-Ar.(C)-cyc.), 147.55, 147.63 (Ar.-C), 156.15, 156.22 (4-Ar.(C-OH)-cyc.); MS (ESI-) m/z (%):296.2 (M-H$^-$, 100). Anal. Calcd for $C_{18}H_{19}NOS$ (297.41): C, 72.69; H, 6.44; N, 4.71. Found: C, 72.62; H, 6.46; N, 4.30.

*5-Chloro-4'-ethyl-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane]**(2a)*** [23]

Beige crystals (45.5%); mp 108-110 °C; IR(KBr): υ_{max} =3310 (N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 0.85-0.87 (3H, m, 4-CH_2CH_3-cyc.), 1.17-1.34 (5H, m, 4-CH_2CH_3-cyc., CH/CH_2-cyc.), 1.57-1.73 (4H, m, CH/CH_2-cyc.), 2.07-2.13 (2H, m, CH/CH_2-cyc.), 6.41 (1H, d, J=2.0 Hz, Ar.-H4), 6.47-6.50 (1H, m, Ar.-H6), 6.74 and 6.95 (1H, 2s, NH-disappeared on D_2O exchange), 6.90 and 6.91 (1H, 2d, J=8.3 Hz, Ar.-H7); ^{13}C-NMR (HMBC) (DMSO-d_6/125MHz): δ 12.10, 12.25 (4-CH_2CH_3-cyc.), 28.94 (4-CH_2CH_3-cyc.), 29.43, 30.42 (CH_2-cyc.) 37.22, 38.08 (CH-cyc.), 40.99 (CH_2-cyc.), 79.48, 82.55 (C-cyc.), 108.18, 108.59 (Ar.-CH), 117.53, 117.69 (Ar.-CH), 122.78 (Ar.-CH), 123.80, 124.29 (Ar.-C), 129.92, 130.05 (Ar.(C-Cl)), 148.97, 149.13 (Ar.-C); MS (ESI+) m/z (%):268.4 (M+H$^+$, 100). Anal. Calcd for $C_{14}H_{18}ClNS$ (267.82): C, 62.79; H, 6.77; N, 5.23. Found: C, 62.63; H, 6.79; N, 5.24.

*5-Chloro-4'-propyl-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane](**2b**)*

Brown needles (61.4%); mp 80-82 °C; IR(KBr): υ_{max} =3379, 3310 (N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 0.83-0.87 (3H, m, 4-$CH_2CH_2CH_3$-cyc.), 1.02-1.31 (7H, m, 4-$CH_2CH_2CH_3$-cyc., CH/CH_2-cyc.), 1.56-1.74 (4H, m, CH/CH_2-cyc.), 2.09-2.12 (2H, m, CH/CH_2-cyc.), 6.40 (1H, d, J=2.0 Hz, Ar.-H4), 6.46-6.50 (1H, m, Ar.-H6), 6.75 and 6.96 (1H, 2s, NH), 6.89 (1H, d*, J=8.3 Hz, Ar.-H7); ^{13}C-NMR (APT) (DMSO-d_6/125MHz): δ 14.91, 14.93 (4-$CH_2CH_2CH_3$-cyc.), 20.26, 20.33 (4-$CH_2CH_2CH_3$-cyc.), 29.27, 30.81 (CH_2-cyc.), 36.06 (CH-cyc.), 39.10 (4-$CH_2CH_2CH_3$-cyc.), 41.01 (CH_2-cyc.), 79.47, 82.53 (C-cyc.), 108.19, 108.59 (Ar.-CH), 117.52, 117.68 (Ar.-CH), 122.75 (Ar.-CH), 123.81, 124.28 (Ar.-C), 129.92, 130.05 (Ar.(C-Cl)), 148.96, 149.13 (Ar.-C); MS (ESI+) m/z (%):282.4 (M+H$^+$, 100). Anal. Calcd for $C_{15}H_{20}$ClNS (281.84): C, 63.92; H, 7.15; N, 4.97. Found: C, 63.89; H, 7.00; N, 4.90.

*4'-tert-Butyl-5-chloro-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane](**2c**)* [24]

White crystals (44.3%); mp 180-182 °C; IR(KBr): υ_{max} =3370 (N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 0.83-0.86 (9H, m, 4-$C(CH_3)_3$-cyc.), 0.95-1.36 (3H, m, CH/CH_2-cyc.), 1.58-1.72 (4H, m, CH/CH_2-cyc.), 2.15-2.22 (2H, m, CH/CH_2-cyc.), 6.41 (1H, d, J=2.4 Hz, Ar.-H4), 6.47-6.51 (1H, m, Ar.-H6), 6.73 and 6.97 (1H, 2s, NH), 6.90 (1H, d, J=7.8 Hz, Ar.-H7); ^{13}C-NMR (APT) (DMSO-d_6/125MHz): δ 24.24, 25.23 (CH_2-cyc.), 28.03, 28.22 (4-$C(CH_3)_3$-cyc.), 32.69, 32.83 (4-$C(CH_3)_3$-cyc.), 41.39, 41.53 (CH_2-cyc.), 46.70, 47.03 (CH-cyc.), 78.58, 82.51 (C-cyc.), 108.20, 108.85 (Ar.-CH), 117.53, 117.75 (Ar.-CH), 122.69, 122.77 (Ar.-CH), 123.73, 124.50 (Ar.-C), 129.86, 130.05 (Ar.(C-Cl)), 149.03, 149.17 (Ar.-C); MS (ESI+) m/z (%):296.4 (M+H$^+$, 100). Anal. Calcd for $C_{16}H_{22}$ClNS (295.87): C, 64.95; H, 7.49; N, 4.73. Found: C, 64.91; H, 7.47; N, 4.64.

*5-Chloro-4'-phenyl-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane](**2d**)*

White powder (42.0%); mp 155-157 °C; IR(KBr): υ_{max} =3366 (N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 1.56-1.70 (2H, m, CH_2-cyc.), 1.83-1.89 (4H, m, CH_2-cyc.), 2.22-2.25 (2H, m, CH_2-cyc.), 2.53-2.59 (1H, m, CH-cyc.), 6.45 (1H, d, J=2.0 Hz, Ar.-H4), 6.50-6.52 (1H, m, Ar.-H6), 6.84 (1H, s, NH), 6.92 and 6.93 (1H, 2d, J=8.1 Hz, Ar.-H7), 7.16-7.31 (5H, m, 4-Ar.(H)-cyc. and NH); ^{13}C-NMR (APT) (DMSO-d_6/125MHz): δ 30.64, 32.06, 40.92, 41.26 (CH_2-cyc.), 42.39, 42.56 (CH-cyc.), 78.34, 82.01 (C-cyc.), 108.33, 108.51 (Ar.-CH), 117.67, 117.75 (Ar.-CH), 122.88 (Ar.-CH), 123.73, 124.21 (Ar.-C), 126.73, 126.80 (4-Ar.(CH)-cyc.), 127.28, 127.55 (4-Ar.(CH)-cyc.), 128.96, 129.10 (4-Ar.(CH)-cyc.), 129.99, 130.15 (Ar.(C-Cl)), 146.70, 146.99 (4-Ar.(C)-cyc.), 148.93, 149.14 (Ar.-C); MS (ESI+) m/z (%):316.4 (M+H$^+$, 100). Anal. Calcd for $C_{18}H_{18}$ClNS (315.86): C, 68.45; H, 5.74; N, 4.43. Found: C, 68.81; H, 5.47; N, 4.24.

*5-chloro-4'-(4-hydroxyphenyl)-3H-spiro[1,3-benzothiazole-2,1'-cyclohexane] (**2e**)*

Beige needles (45.0%); mp 166-168 °C; IR(KBr): υ_{max} =3566, 3422, 3389 (O-H, N-H); ^{1}H-NMR (DMSO-d_6/500MHz): δ 1.48-1.66 (2H, m, CH_2-cyc.), 1.77-1.86 (4H, m, CH_2-cyc.), 2.21 (2H, d*, J=11.7 Hz, CH_2-cyc.), 2.41-2.46 (1H, m, CH-cyc.), 6.44 (1H, d, J=2.0 Hz, Ar.-H4), 6.49-6.52 (1H, m, Ar.-H6), 6.66-6.69 (2H, m, 4-Ar.(H)-cyc.), 6.81 and 7.17 (1H, 2s, NH), 6.92 and 6.93 (1H, 2d, J=7.8 Hz, Ar.-H7), 7.00 (1H, d, J=8.3 Hz, 4-Ar.(H)-cyc.), 7.08 (1H, d, J=8.3 Hz, 4-Ar.(H)-cyc.), 9.10 and 9.11 (1H, 2s, OH); ^{13}C-NMR (APT) (DMSO-d_6/125MHz): δ 30.92, 32.36, 41.00, 41.34 (CH_2-cyc.), 41.49, 41.76 (CH-cyc.), 78.43, 82.07 (C-cyc.), 108.30, 108.51 (Ar.-CH), 115.64, 115.78 (4-Ar.(CH)-cyc.), 117.64, 117.72 (Ar.-CH), 122.86 (Ar.-CH), 123.76, 124.23 (Ar.-C), 128.03, 128.30 (4-Ar.(CH)-cyc.), 129.96, 130.20 (Ar.(C-Cl)), 136.90, 137.18 (4-Ar.(C)-cyc.), 148.95, 149.15 (Ar.-C), 156.18, 156.24 (4-Ar.(C-OH)-cyc.); MS (ESI+) m/z (%):332.4 (M+H$^+$, 100). Anal. Calcd for $C_{18}H_{18}$ClNOS (331.86): C, 65.15; H, 5.47; N, 4.22. Found: C, 64.78; H, 5.47; N, 3.93.

4.2. Biological activity assays

4.2.1. Antioxidant activity assays

Antioxidative effect on lipid peroxidation (LPO)

LPO assay was assessed by the method described by Duh *et al.* [25]. The formation of LPO products was assayed by the measurement of thiobarbituric acid reactive substances (TBARS) according to Buege and Aust [26]. The percentage inhibition of LPO was calculated using the following equation:

Inhibition effect (%) = (1 - Absorbance of sample/Absorbance of control) x100.

DPPH radical (DPPH$^•$) scavenging activity

The DPPH$^•$ scavenging activity of the extract was measured

according to the procedure described by Brand-Williams et al. [27] and calculated by the following equation:

$DPPH^{\bullet}$ scavenging activity (%) = (1 - Absorbance of sample at 517 nm/Absorbance of control at 517 nm) x 100.

Total radical-trapping antioxidant potential (TRAP) assay

The total radical-trapping antioxidant potential of the extract was measured using the trolox equivalent antioxidant capacity (TEAC) assay as described by Re *et al.* [28]. The ability to scavenge ABTS radical cation ($ABTS^{\bullet +}$) was calculated by the following equation:

$ABTS^{\bullet +}$ scavenging activity (%) = (1 - Absorbance of sample at 734 nm/Absorbance of control at 734 nm) x 100.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure of Benzie and Strain [29]. The standard curve was constructed using iron sulfate heptahydrate solution (0.125 – 2 mM), and the results were expressed as mM Fe^{2+} equivalents.

Statistical analysis

All measurements were made in triplicate. The results were statistically analyzed with Student's *t*-test by using the computer software Statistical Package for Social Sciences for windows (SPSS version 21; SPSS, Inc. Chicago IL, USA). Results were considered significant at $p < 0.05$.

4.2.2. MTT proliferation assay

Cytotoxic potentials of newly synthesized compounds were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in mouse fibroblast (NIH/3T3, CRL-1658) and human prostate adenocarcinoma (PC-3, CRL-1435) cell lines which were obtained from American Type Culture Collection (ATCC, USA). The MTT assay is based on colorimetric changes related to the number of viable cells via mitochondrial activity. In viable cells the yellow tetrazolium salt MTT is reduced into blue/purple colored formazan crystals. The optical density (OD) of the formazan is then read at 590 nm [30].

Cells were grown in Dulbecco's modified Eagle's medium/ Ham's F-12 (DMEM-F12) supplemented with with 10% fetal bovine serum and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) in a humidified incubator with 5% CO_2 at 37 °C. When 80-85% confluency in a flask was provided, cells were trypsinized and seeded at 10^5 cells into each well of 96-well plates. After 24h incubation for cell attachment, cells were treated with various concentrations of the test compounds. The test chemicals were dissolved in dimethyl sulfoxide (DMSO) and diluted in DMEM-F12 that given final concentration range of 0-145 µg/mL. After 24h treatment, 20µL MTT (5 mg/mL in phosphate buffered saline) was added to each well and incubated for 3h at 37 °C. All medium was discarded and formazan crytals were dissolved in 100µL DMSO for 5 min on an orbital shaker to form a homogeneus blue/purple color. ODs were determined at 590 nm, against 670 nm reference wavelength, using a microplate spectrophotometer system (Epoch, Germany). DMEM-F12, 1% DMSO and 1% sodium dodecyl sulfate (SDS) were used as growth, solvent and positive controls in each plate, respectively. Each compound was tested in duplicates in a plate and each plate was repeated in triplicates. All ODs were corrected by substracting the ODs of blank wells and mean OD values of each test chemical were calculated.

The percentage of inhibitory effects of test compounds were calculated as follows:

Inhibition% = 100 – ($_{mean}$cOD of test compound / $_{mean}$cOD of 1%DMSO)x100

cOD: corrected OD

The authors declared no conflict of interest.

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