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Short communication

Novel *R*-(+)-limonene-based thiosemicarbazones and their antitumor activity against human tumor cell lines

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ABSTRACT

In an attempt to develop potent and selective antitumor agents, a series of novel thiosemicarbazones derived from a natural monoterpene *R*-(+)-limonene was synthesized and their antitumor activity was evaluated. Overall, the majority of tested compounds exhibited considerable inhibitory effects on the growth of a wide range of cancer cell lines. Almost all of tested thiosemicarbazones were especially sensitive to prostate cells (PC-3). Derivatives **5**, **6**, **8**, **9**, **10**, **11** and **13** presented the most potent antitumor activity against PC-3 cells. These compounds showed lower value of GI_{50} (0.04–0.05 μM) than the reference drug paclitaxel, besides a high selectivity for the same cell line. The 4-fluorobenzaldehyde derivative **10** was the most selective compound for prostate cells, while 2-hydroxybenzaldehyde derivative **8** was the most active compound, with potent antitumor activity against all tested cell lines.

1. Introduction

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths in 2008 with the highest incidences to lung, stomach, liver, colon and breast cancer [1]. Only in Brazil, the Brazilian national cancer institute (INCA) estimated that over 500.000 new cases of cancer were reported in 2012. Melanoma, prostate, lung and stomach cancers were found to be the most common types among men, while women were more affected by breast and uterus types [2].

Thiosemicarbazones are molecules that have demonstrated high antitumor potential [3–6]. This property is attributed to the ability to inhibit the enzyme ribonucleoside diphosphate reductase (RDR), which is involved in the biosynthesis of deoxyribonucleic acid (DNA) [7]. A well-known representative compound is Triapine, 3-aminopyridine-2-carboxaldehyde-thiosemicarbazone.This promising anticancer agent is under clinical trials due its significant cytotoxic activity against various tumor cell lines, such as prostate, pancreas, kidney, ovary and lung [8]. Similar to Triapine, most of the active thiosemicarbazones have aryl or alkyl groups on the carbon at the imine moiety. Nevertheless, more substituted thiosemicarbazones might present improved activities, considering that the presence of bulky groups at *N*-4 position is believed to enhance their biological activities [9].

Terpenes are also known to possess antitumor property among a broad spectrum of biological activities [10,11]. Monoterpene moieties, such as citronellal and citral, attached on the imine group of thiosemicarbazones showed interesting antitumor activity against leukemia cells. This class of compounds is widely employed as starting material in the synthesis of a large number of biomolecules, since they are inexpensive reactants and readily available in pure enantiomeric forms [12].

As previously reported [13,14], our research group pioneered the preparation of imine carbon-substituted thiosemicarbazones containing a terpenic unit linked at *N*-4 position, with the aim of associating biological active compounds. The new thiosemicarbazones presented an enhanced pharmacological effect when evaluated *in vitro*, as antitumor and antiprotozoal agents, corroborating our expectations. In a previous work, thiosemicarbazones derived from

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(−)-α-bisabolol were found to drastically inhibit the growth of eight cancer cell lines. The compounds were especially active against myeloid leukemia, highlighting the methyl-phenyl-ketone thiosemicarbazone as the most active for this type of tumor cells [13].

In continuing our search for new compounds that display antitumor activity derived from natural terpenes abundant in Brazilian flora, we selected *R*-(+)-limonene **1**. Besides being a very suitable chiral starting material towards the synthesis of thiosemicarbazones due its exocyclic double bond [15], numerous reports of antitumor activity are found in the literature for this monoterpene. Chemopreventive and chemotherapeutic action was described for *R*-(+)-limonene itself against several types of carcinomas, such as melanoma, prostate and stomach [11,16]. Good results were also achieved for patients either with breast cancer or in treatment for colon cancer [17]. Additionally, pharmacokinetic studies revealed that this monoterpene shows low toxicity in patients with advanced stages of cancer, supporting its use as a chemotherapeutic agent [18].

Based on our previous promising results and the studies presented above, in this paper we report the synthesis of a series of novel thiosemicarbazones containing *R*-(+)-limonene moiety at *N*-4 position and their antitumor activity against several human tumor cell lines.

2. Results and discussion

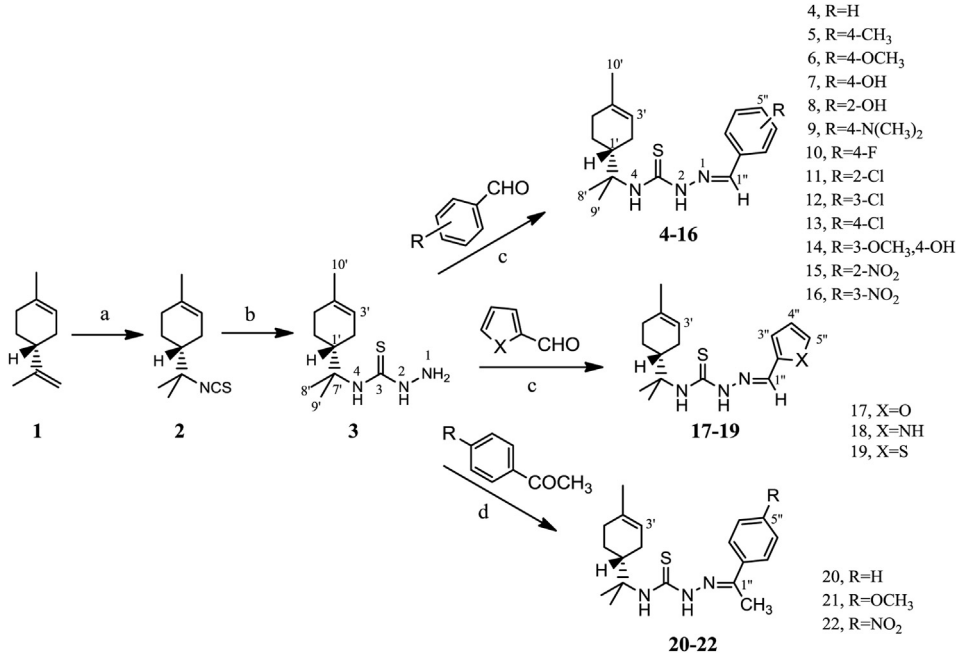
2.1. Chemistry

Using the same synthetic protocol from the previous reports [13–15,19], the synthesis of the target compounds was accomplished according to the reaction sequence in Scheme 1. The preparation of synthetic precursor isothiocyanoterpene **2** and thiosemicarbazide **3** was described previously by our research group [15,19]. The synthesis of thiosemicarbazones **4**–**22** was initiated by chemo and regioselective addition of HSCN to exocyclic double bond of *R*-(+)-limonene **1**, in order to obtain the isothiocyanoterpene **2** with retaining of chiral integrity from the original natural monoterpene [15]. Compound **2** was, then, reacted with hydrazine providing thiosemicarbazide **3** [19]. *R*-(+)-limonene thiosemicarbazones **4**–**22** were obtained by condensation of equimolar amounts of thiosemicarbazide **3** with different oxo compounds (aldehydes and ketones) in ethanol, using hydrochloric acid 10% as catalyst. The reaction was conducted until complete consumption of **3** occurred, leading to thiosemicarbazones **4**–**22** in good yields (68–95%) after recrystallization from ethanol. All compounds were characterized by their spectroscopic data (IR, ^{1}H and ^{13}C NMR, MS data).Table 1

2.2. Antitumor activity

An *in vitro* bioassay was carried out to investigate the antitumor potential effect of *R*-(+)-limonene and synthesized compounds. Hence, the antitumor activity was evaluated against ten different human tumor cell lines: glioma (U251), melanoma (UACC-62), breast (MCF-7), ovarian with phenotype of multiple drug resistance (NCI-ADR/RES), kidney (786-0), lung, non-small cell type (NCI-H460), prostate (PC-3), ovarian (OVCAR-3), colon (HT-29) and leukemia (K-562). Non-tumor cell line (VERO, renal, green monkey) was used as parameter for evaluation of the cytotoxicity of the compounds. Chemotherapeutic agent paclitaxel was used as standard positive control. The antitumor activity (μM) of tested compounds is given by three parameters for each cell line: GI_{50} (molar concentration that inhibits 50% cell growth), TGI (molar concentration to total inhibition of the cell growth) and LC_{50} (molar concentration that leads to 50% cell death). For any parameter, compounds with values greater than 100 μM were considered inactive against the specific cell line. The average activity was calculated by a mean graph midpoint (MG-MID – antilogarithm of mean of log GI_{50} or antilogarithm of mean of log TGI) for all synthesized compounds.

All of tested compounds inhibited the growth of the cell lines in a dose-dependent manner. The greatest decrease on the dose–response curve was displayed by 2-hydroxy derivative **8** (GI_{50} average activity of 2.2 μM and TGI average activity of 11.5 μM),



Scheme 1. Reagents and conditions: (a) $KHSO_4$, KSCN, $CHCl_3$, rt, 24 h; (b) $NH_2NH_2{\cdot}2HCl/NaHCO_3/H_2O$, EtOH, reflux, 3 h; (c) EtOH, HCl 10%, r.t. 1–2 h.; (d) EtOH, HCl 10%, reflux 2–12 h.

Table 1
GI_{50} values (in μM) for synthesized compounds.

Compd	Cancer cell lines											
	Glioma U251	Melanoma UACC-62	Breast MCF7	Ovarian resistant NCI-ADR/RES	Lung NCI-H460	Kidney 746-0	Prostate PC-3	Ovarian OVCAR-03	Colon HT-29	Leukemia K-562	MG-MID[a]	VERO
1	>100	NT	>100	>100	>100	>100	>100	>100	>100	23.9	84.9	>100
2	29.1	NT	57.6	>100	>100	>100	>100	>100	>100	4.0	57.3	>100
3	0.2	NT	0.9	39.3	42.0	>100	44.5	41.9	60.1	>100	18.3	49.5
4	26.3	31.6	8.4	>100	13.0	9.9	1.3	8.7	8.8	6.3	12.0	20.5
5	89.2	75.9	15.2	78.5	75.7	87.2	<0.04[b]	67.2	20.2	1.6	18.6	81.4
6	11.4	13.3	31.2	43.8	22.5	8.9	<0.04[b]	7.9	8.4	1.5	6.6	12.2
7	>100	>100	>100	82.1	>100	>100	0.6	>100	>100	>100	58.9	>100
8	6.4	2.8	2.2	3.7	1.9	4.6	<0.04[b]	5.5	10.9	0.8	2.2	3.1
9	57.7	45.1	26.8	>100	53.4	87.0	<0.04[b]	>100	46.5	22.6	25.7	75.6
10	>100	>100	>100	>100	>100	>100	<0.04[b]	>100	>100	>100	45.7	>100
11	80.6	19.3	11.0	75.0	64.5	85.5	0.05	15.1	71.3	5.6	17.0	74.6
12	7.9	9.0	8.0	67.5	1.4	9.2	0.6	13.6	7.9	5.4	6.6	14.8
13	69.5	11.2	18.3	65.2	7.8	74.9	0.05	8.6	13.6	0.3	7.9	70.0
14	11.7	14.7	14.8	23.9	>100	12.9	0.3	53.5	<0.04[b]	1.9	6.3	19.7
15	20.2	10.6	1.2	19.2	5.0	68.5	1.7	8.5	7.9	0.3	6.0	21.6
16	>100	>100	>100	>100	>100	>100	9.5	>100	0.6	45.6	43.6	>100
17	9.7	10.7	9.3	9.8	13.5	11.7	13.5	10.8	7.2	0.1	6.6	5.1
18	0.08	14.8	3.9	78.0	18.0	20.8	2.8	33.5	27.5	23.4	9.8	20.5
19	13.5	2.6	4.8	13.6	10.8	11.5	17.8	13.1	17.8	17.6	10.7	10.1
20	>100	>100	20.7	>100	>100	>100	>100	>100	29.5	>100	75.9	>100
21	43.4	95.3	5.0	>100	90.6	>100	83.6	74.9	18.7	54.0	51.3	>100
22	7.0	7.2	8.0	9.9	50.8	21.7	62.6	7.7	29.9	83.4	18.6	67.1
PCT	0.003	0.01	0.005	0.04	0.1	0.02	0.1	0.2	0.001	0.1	0.022	0.03

NT = not tested.
PCT = paclitaxel (reference drug).
[a] Average activity, MG-MID.
[b] The lowest concentration tested was 0.25 μg mL^{-1}. Data are means of representative experiment done in triplicate.

followed by compounds **6**, **12**–**15**, **17** and **18**, whose GI_{50} average activity values were lower than 10 μM. *R*-(+)-limonene showed moderate activity against K-562 cells (GI_{50} 23.9 μM), confirming the anticancer potential assigned to this monoterpene in the literature [20]. The incorporation of the –NCS group into the terpene resulted in an increase in activity of isothiocyanoterpene **2**, especially for K-562, MCF-7 and U251 cells at concentration of 2- to 6-fold lower than its precursor. For thiosemicarbazide **3**, obtained by the incorporation of hydrazine into isothiocyanate structure, the results exhibited a drastic decrease on the growth of all tumor cells evaluated, especially glioma U251 (GI_{50} 0.2 μM) and breast MCF-7 (GI_{50} 0.9 μM) cells. Compound **3** showed to be highly selective for U251 cells. Benzaldehyde-thiosemicarbazones (**4**–**16**) presented the best inhibitory activity for prostate PC-3 cells, with GI_{50} ranging from 0.04 to 9.5 μM. Being highly selective for this cell line, derivatives **5**, **6**, **8**–**11** and **13** showed a more prominent inhibitory effect (GI_{50} within 0.04–0.05 μM) than the reference drug Paclitaxel (GI_{50} 0.1 μM) and cytostatic inhibitory concentrations lower than 2.0 μM. 4-Fluorobenzaldehyde derivative **10**, exclusively active for PC-3 cells, is being the subject of further antitumor studies due to its outstanding result. Potent antitumor activity was also found for benzaldehyde derivatives **5**, **6**, **8**, **13**–**15** against myeloid leukemia with GI_{50} lower than 1.9 μM. Considering the most sensitive cell line for benzaldehyde- thiosemicarbazones, PC-3 cells, it was noticed that the nature of the substituent on the phenyl ring influences the activity. In general, the derivatives with electron-donating substituents **5**, **6**, **8**, **9** and with halo-electron-withdrawing substituents **10**, **11**, **13**, exhibited cytostatic effects on PC-3 cells (TGI 0.7–1.7 μM) at concentration of 4- to 50-fold lower than the others (Table 2).

Broadening the possibilities of structural variations in order to find structure–activity relationships, heteroaromatic aldehyde-derived **17**–**19** and ketone-derived thiosemicarbazones **20**–**22** were evaluated against the same tumor cell lines. Differently from what was observed for the benzaldehyde-thiosemicarbazone series, the heteroaromatic derivatives did not display a significant selectivity against prostate cell line, although other very attractive values of antitumor activity were achieved for them. Markedly, furan derivative **17** showed high activity against K562 cells (GI_{50} 0.10 μM), whereas pyrrole derivative **18** exhibited a potent cytostatic activity (3.9 μM) against U251 cells, in addition to a highly selective inhibitory activity (GI_{50} 0.08 μM) for the same cell line. Thiophene derivative **19** inhibited more significantly the growth of melanoma cell line (UACC-62) with GI_{50} value of 2.6 μM. The replacement of hydrogen for a methyl group at the imine moiety for acetophenone-thiosemicarbazones **20**–**22** promoted a detrimental effect on the antitumor activity. Only moderate antitumor activity was observed for compound **22** containing an electron-withdrawing group at the phenyl ring, while the derivatives **20** and **21** showed small role in the growth inhibitory activity.

Taking the LC_{50} values into account, thiosemicarbazones **4**, **6**, **8**, **9** and **15** were more lethal to PC-3 cells than to other cell lines, as well as the most potent compound. 2-hydroxybenzaldehyde thiosemicarbazone **8** was the most active derivative against the majority of cell lines evaluated, including PC-3, with LC_{50} value of 12.3 μM.

In addition to the great activity and selectivity of some compounds against prostate PC-3 cells, an outstanding result was the citotoxicity of thiosemicarbazones. Derivatives **7**, **10**, **16**, **20** and **21** presented a GI_{50} value for VERO cell line (non-tumor cells) higher than 100 μM, which might suggest these compounds have a low toxicity to normal cells. Even considering the most active thiosemicarbazone (2-hydroxy derivative **8**), a 100-fold decrease in toxicity was found in comparison with the reference drug Paclitaxel.

3. Conclusion

We have synthesized a series of novel thiosemicarbazones derived from bulky group *R*-(+)-limonene. The vast majority of

Table 2
TGI values (in μM) for synthesized compounds.

Compd	Cancer cell lines											
	Glioma U251	Melanoma UACC-62	Breast MCF-7	Ovarian resistant NCI-ADR/RES	Lung NCI-H460	Kidney 746-0	Prostate PC-3	Ovarian OVCAR-03	Colon HT-29	Leukemia K-562	MG-MID[a]	VERO
1	>100	NT	>100	>100	>100	>100	>100	>100	>100	>100	100	>100
2	>100	NT	>100	>100	>100	>100	>100	>100	>100	>100	100	>100
3	10.1	NT	46.7	>100	>100	>100	>100	>100	>100	>100	71.1	>100
4	71.4	>100	84.4	>100	53.9	67.0	5.7	20.6	22.2	47.6	44.0	>100
5	>100	>100	93.6	>100	>100	>100	0.9	>100	94.5	56.8	58.3	>100
6	71.9	70.4	37.7	50.4	47.8	29.6	1.2	21.2	33.6	30.7	28.5	70.1
7	>100	>100	>100	>100	>100	>100	53.8	>100	>100	>100	93.9	>100
8	10.6	10.8	17.5	10.9	8.4	12.1	0.9	11.8	16.6	>100	11.5	11.2
9	>100	>100	>100	>100	>100	>100	<0.7	>100	>100	>100	60.9	>100
10	>100	>100	>100	>100	>100	>100	<0.9	>100	>100	>100	61.3	>100
11	>100	98.0	98.3	>100	81.0	>100	0.7	83.6	>100	66.2	56.0	>100
12	35.8	39.8	61.0	>100	21.4	26.9	6.6	80.5	53.0	>100	41.2	>100
13	>100	>100	>100	97.4	21.1	>100	1.7	26.4	70.5	16.9	40.2	>100
14	32.6	42.3	63.9	>100	>100	35.4	8.3	>100	<0.7	85.2	33.0	91.8
15	>100	92.2	94.7	>100	32.7	>100	6.4	25.8	70.8	>100	56.3	>100
16	>100	>100	>100	>100	>100	>100	88.0	>100	21.9	>100	84.1	>100
17	81.5	14.1	53.7	14.1	10.8	9.8	27.2	13.8	26.9	>100	24.9	14.7
18	3.9	49.9	11.5	>100	57.5	>100	>100	>100	>100	>100	51.4	>100
19	27.4	19.0	11.5	41.0	20.8	26.7	62.8	35.1	57.8	>100	33.4	13.4
20	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	100	>100
21	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	100	>100
22	29.3	46.8	77.5	>100	83.7	>100	>100	29.3	>100	>100	69.4	>100
PCT	0.1	0.5	0.4	0.4	0.08	0.8	<0.03	<0.03	<0.03	0.1	0.13	NT

NT = not tested.
PCT = paclitaxel (reference drug).
[a] TGI (in μM) mean-graph midpoint (MG-MID) = average sensitivity of all cell lines toward the tested compounds.

compounds presented excellent growth inhibitory activity against most of the cancer cell lines tested. The precursor thiosemicarbazide **3** was able to induce growth inhibition of U251 cells. Among the examined series, the highest anticancer activity and selectivity against PC-3 cells were displayed by thiosemicarbazones **5**, **6**, **8**, **9**, **10**, **11** and **13**, of which 4-fluoro-derivative **10** was the most selective. All compounds were found to be at least 100-fold less toxic than the reference drug, and 2-hydroxy-derivative **8** was found to be the most active one against the majority of cell lines tested. Based on the results of this investigation, we shall carry out further studies to investigate the mechanisms of action of these compounds.

4. Materials and methods

4.1. General experimental procedure

Melting points were determined in a Micro-Quimica MQAPF-301 apparatus. The optical rotations were determined in a Perkin Welmer polarimeter 343 model at 20 °C, using chloroform as solvent. IR spectra were recorded on a BOMEM model MB-100 spectrometer. Low resolution mass spectra were recorded by means of a SHIMADZU-GC/MS model QP 2000A spectrometer at 70 eV with a probe for solids. Direct Infusion ESI-(+)-FTMS, performed in an LTQ-XL-Orbitrap Discovery (Thermo Scientific, Bremen, Germany), operating in 5.2 kV of capillary voltage, capillary temperature of 285 °C, sheath gas (nitrogen) flow rate at 10 arbitrary units and sample solution flow rate of 10 μL/min. Data acquisition was performed in the positive ion mode within the *m/z* range of 300–400. Raw data were treated on Xcalibur 2.1 (Thermo Scientific, San Jose, California, USA) and mass accuracy was the utilized parameter for compound characterization. ^{1}H- and ^{13}C NMR spectra were recorded in a Varian model Mercury plus 300 spectrometer at 300 MHz and 75.45 MHz, respectively, with $CDCl_3$ and CD_3OD-d_4 as solvent and TMS as the internal standard. The chemical shifts (δ) are given in ppm relative to TMS. Chromatography column was performed on silica gel Merck 230–400 mesh ASTM. All reagents were purchased from commercial suppliers.

4.2. General procedure for synthesis of target compounds

4.2.1. Isothiocyanoterpene *2*

Isothiocyanoterpene **2**, *R*-4-(2-isothiocyanatopropan-2-yl)-1-methylcyclohex-1-ene was prepared as described in literature [15].

4.2.2. Thiosemicarbazide *3*

Thiosemicarbazide **3**, (+)-*N*(4)-{*R*-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]}-thiosemicarbazide, was prepared as described in literature [19].

4.2.3. Thiosemicarbazones *4–22*

2.86 mmol (0.29 mL) of benzaldehyde and drops of solution 10% hydrochloridric acid were added to a solution of 2.86 mmol (0.65 g) of -(+)-*N*(4)-{*R*-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]}-thiosemicarbazide **3** in ethanol. The mixture was stirred under at room temperature and the product was recrystallized from ethanol. White crystals; yield 77% for (+)-(*R*)-*N*(1)-(phenyl-methylene)-*N*(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide **4**; mp. 150–152 °C; $[\alpha]_D$ +30; (KBr/cm^{-1}) (NH) 3342, (NH) 3131, 1600 (C=N). 1291 (C=S). EI-MS *m/z* 315 ($M^{+\bullet}$). ^{1}H NMR (300 MHz $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.39 (3H, m, H-4″/H-5″/H-6″), 7.60 (3H, m, NH, H-4/H-3″/H-7″), 7.81 (1H, s, C=N, H-1″), 9.37 (1H, s, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 120.6 (C-3′), 127.4 (C-3″/C-7″), 129.1 (C-4″/C-6″), 130.5 (C-5″), 133.6 (C-4′), 134.3 (C-2″), 141.3 (C=N, C-1″) and 175.6 (C=S, C-3). HRMS (ESI) calcd for $C_{18}H_{25}N_3S$ 316.1842 $[M + H]^+$ found 316.1841.

4.2.4. (+)-(R)-N(1)-[(4-methylphenyl)-methylene]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide ***5***

White crystals; yield 95%; mp. 168–170 °C; $[\alpha]_D$ +30; IR (KBr/cm^{-1}): (NH) 3356 and 3323, (C=N) 1606, (C=S) 1259; EI-MS *m/z* 329 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.37 (3H, s, H-8″), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.19 (2H, d, *J* = 7.6, H-4″/H-6″), 7.31 (1H, s, C=N, H-1″), 7.48 (2H, d, *J* = 7.8, H-3″/H-7″), 7.59 (1H, sl, H-4), 10.07 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 21.6 (C-8″) 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 120.6 (C-3′), 127.1 (C-4″/C-6″), 131.1 (C-2″), 134.1 (C-3″/C-7″), 133.6 (C-4′), 138.2 (C-5″), 141.1 (C=N, C-1″) and 175.4 (C=S, C-3). HRMS (ESI) calcd for $C_{19}H_{27}N_3S$ 330.1998 $[M + H]^+$ found 330.1995.

4.2.5. (+)-(R)-N(1)-[(4-methoxyphenyl)-methylene]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide ***6***

Light yellow crystals; yield 90%; mp. 153–156 °C; $[\alpha]_D$ +32; IR (KBr/cm^{-1}): (NH) 3289 and 3404, (C = N) 1611, (C=S) 1225; EI-MS *m/z* 345 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 3.85 (3H, s, H-8″), 5.36 (1H, m, H-3′), 6.92 (2H, d, *J* = 7.6, H-4″/H-6″), 7.55 (2H, m, H-4/H-3″/H-7″), 7.80 (1H, s, C=N, H-1″), 9.49 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 55.9 (C-8″), 59.0 (C-7′), 120.6 (C-3′), 114.4 (C-4″/C-6″), 126.3 (C-2″), 128.9 (C-3″/C-7″), 133.6 (C-4′), 141.1 (C=N, C-1″), 161.5 (C-5″) and 175.3 (C=S, C-3). HRMS (ESI) calcd for $C_{19}H_{27}N_3OS$ 346.1948 $[M + H]^+$ found 346.1952.

4.2.6. (+)-(R)-N(1)-[(4-hydroxyphenyl)-methylene]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide ***7***

Light yellow crystals; yield 73%; mp. 175–179 °C; $[\alpha]_D$ +31; IR (KBr/cm^{-1}): (NH) 3469 and 3358, (C=N) 1598, (C=S) 1230; EI-MS *m/z* 331 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 6.85 (2H, d, *J* = 8.4, H-4″/H-6″), 7.47 (2H, d, *J* = 8.7, H-3″/H-7″), 7.79 (2H, s, C=N, H-1″/OH), 9.49 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 115.8 (C-4″/C-6″), 120.6 (C-3′), 125.1 (C-2″), 128.9 (C-3″/C-7″), 133.6 (C-4′), 142.6 (C=N, C-1″), 159.3 (C-5″) and 174.8 (C=S, C-3). HRMS (ESI) calcd for $C_{18}H_{25}N_3OS$ 332.1791 $[M + H]^+$ found 332.1794.

4.2.7. (+)-(R)-N(1)-[(2-hydroxyphenyl)-methylene]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide ***8***

Light yellow crystals; yield 75%; mp. 160–163 °C; $[\alpha]_D$ +33; IR (KBr/cm^{-1}): (NH) 3272 and 3376, (C=N) 1595, (C=S) 1285; EI-MS *m/z* 331 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 6.73 (1H, s, NH, H-4), 6.95 (1H,m, H-6″), 6.99 (1H, m, H-4″), 7.25 (1H, m, H-5″), 7.33 (1H, m, H-7″), 8.08 (1H, s, C=N, H-1″), 9.49 (1H, s, OH), 9.87 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 117.0 (C-4″), 117.4 (C-2″), 120.4 (C-6″), 120.6 (C-3′), 131.8 (C-5″), 132.3 (C-7″), 133.6 (C-4′), 146.2 (C=N, C-1″), 157.5 (C-3″), and 174.9 (C=S, C-3). HRMS (ESI) calcd for $C_{18}H_{25}N_3OS$ 332.1791 $[M + H]^+$ found 332.1794.

4.2.8. (+)-(R)-N(1)-{[4-(dimethylamino)-phenyl]-methylene}-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide ***9***

Yellow crystals; yield 89%; mp. 177–182 °C; $[\alpha]_D$ +29; IR (KBr/cm^{-1}): (NH) 3415 and 3378, (C=N) 1592, (C=S) 1250; EI-MS *m/z* 358 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 3.09 (6H, $N(CH_3)_2$), 5.36 (1H, m, H-3′), 6.69 (2H, d, *J* = 8.7, H-4″/H-6″), 7.47 (2H, d, *J* = 8.5, H-3″/H-7″), 7.69 (1H, s, C=N, H-1″), 7.77 (1H, sl, H-4), 8.40 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 40.3 (C-8″), 41.0 (C-1′), 59.0 (C-7′), 112.0 (C-4″/C-6″), 120.6 (C-3′), 121.2 (C-2″), 128.8 (C-3″/C-7″), 133.6 (C-4′), 142.4 (C=N, C-1″), 151.9 (C-5″) and 175.6 (C=S, C-3). HRMS (ESI) calcd for $C_{20}H_{30}N_4S$ 359.2264 $[M + H]^+$ found 359.2260.

4.2.9. (+)-(R)-N(1)-[(4-fluorophenyl)-methylene]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide ***10***

White crystals; yield: 80%; mp 178–180 °C; $[\alpha]_D$: +40°; IR (KBr/cm^{-1}): (NH) 3334 and 3136, (C=N) 1541, (C=S) 1311; EI-MS: *m/z* 333 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, sl, H-3′), 7.10 (2H, t, *J* = 8.7, H-4″/H-6″), 7.53 (1H, sl, NH, H-4), 7.59 (2H, m, H-3″/H-7″), 7.82 (1H, sl, C=N, H-1″), 9.52 (1H, sl, NH, H-2). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 116.3 (C-4″/C-6″), 120.6 (C-3′), 129.2 (C-3″/C-7″), 129.9 (C-2″), 133.6 (C-4′), 140.2 (C=N, C-1″), 164.1 (C-5″) and 175.6 (C=S, C-3). HRMS (ESI) calcd for $C_{18}H_{24}FN_3S$ 334.1748 $[M + H]^+$ found 334.1752.

4.2.10. (+)-(R)-N(1)-[(2-chlorophenyl)-methylene]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide ***11***

Withe crystals; yield 94%; mp. 161–164 °C; $[\alpha]_D$ +30; IR (KBr/cm^{-1}): (NH) 3322 and 3149, (C=N) 1595, (C=S) 1263; EI-MS *m/z* 349 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.30 (2H,m, H-4″/H-5″), 7.38 (1H, m, H-7″), 7.54 (1H, s, NH, H-4), 7.80 (1H, m, H-6″), 8.27 (1H, s, C=N, H-1″), 10.07 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 120.6 (C-3′), 127.0 (C-6″), 127.2, (C-4″), 130.4 (C-7″), 131.1 (C-2″), 131.2 (C-5″), 133.6 (C-4′), 134.6 (C-3″), 138.0 (C=N, C-1″) and 174.8 (C=S, C-3). HRMS (ESI) calcd for $C_{18}H_{24}ClN_3S$ 350.1452 $[M + H]^+$ found 350.1453.

4.2.11. (+)-(R)-N(1)-[(3-chlorophenyl)-methylene]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide ***12***

White crystals; yield 88%; mp. 165–167 °C; $[\alpha]_D$ +31; IR (KBr/cm^{-1}):(NH) 3633 and 3231, (C=N) 1603, (C=S) 1272; EI-MS *m/z* 349 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.33 (2H, m, H-6″/H-5″), 7.45 (1H, m, H-3″), 7.58 (1H, s, NH, H-4), 7.60 (1H, m, H-7″), 7.97 (1H, s, C=N, H-1″), 10.66 (1H, s, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 120.5 (C-3′), 125.7 (C-6″), 126.7 (C-7″), 130.1 (C-3″/C-5″), 133.6 (C-4′), 134.0 (C-1′), 135.0 (C-4″), 135.7 (C-2″), 140.1 (C=N, C-1″) and 175.6 (C=S, C-3). HRMS (ESI) calcd for $C_{18}H_{24}ClN_3S$ 350.1452 $[M + H]^+$ found 350.1455.

4.2.12. (+)-(R)-N(1)-[(4-chlorophenyl)-methylene]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide ***13***

White crystals; yield 91%; mp. 170–173 °C; $[\alpha]_D$ +30, IR (KBr/cm^{-1}): (NH) 3390 and 3155, (C=N) 1595, (C=S) 1300; EI-MS *m/z* 349 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.36 (2H, d, *J* = 8.4, H-4″/H-6″), 7.53 (2H, d, *J* = 8.7, H-3″/H-7″), 7.94 (2H, s, C=N, H-1″/NH, H-4), 10.35 (1H, s, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$):

δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 120.6 (C-3′), 128.4 (C-4″/C-6″), 129.2 (C-3″/C-7″), 132.3 (C-2″), 133.6 (C-4′), 136.1 (C-5″), 140.4 (C=N, C-1″) and 175.4 (C=S, C-3). HRMS (ESI) calcd for $C_{18}H_{24}ClN_3S$ 350.1452 $[M + H]^+$ found 350.1458.

4.2.13. (+)-(R)-N(1)-[(4-hydroxy-3-methoxyphenyl)-methylene]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide **14**

White crystals; yield 80%; mp. 139–142 °C; $[\alpha]_D$ +38; IR (KBr/cm^{-1}): (NH) 3334 and 3136, (C=N)1541, (C=S) 1311, (C–O–CH_3) 1265, (C–OH 1200); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 3.92 (3H, s, OCH_3), 5.36 (1H, m, H-3′), 6.94 (1H, d, J = 8.4, H-6″), 7.06 (1H, dd, J = 8.1, 1.8, H-7″), 7.16 (1H, d, J = 1.5, H-3″), 7.61 (1H, s, C=N, H-1″), 7.65 (1H, sl, NH, H-4), 8.80 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 56.0 (OCH_3), 59.0 (C-7′), 107.8 (C-3″), 114.8 (C-6″), 120.6 (C-3′), 122.8 (C-7″), 125.9 (C-2″), 133.6 (C-4′), 141.6 (C=N, C-1″), 147.1 (C-5″), 148.3 (C-4″) and 175.7 (C=S, C-3). HRMS (ESI) calcd for $C_{19}H_{27}N_3O_2S$ 362.1897 $[M + H]^+$ found 362.1903.

4.2.14. (+)-(R)-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-N(1)-[(2-nitrophenyl)-methylene]-thiosemicarbazide **15**

Yellow crystals; yield 89%; mp. 172–174 °C; $[\alpha]_D$ +43; IR (KBr/cm^{-1}): (NH) 3424 and 3241, (C=N) 1591, (C=S) 1244; EI-MS m/z 360 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.51 (1H, m, H-6″), 7.52 (1H, s, H-4), 7.61 (1H, m, H-5″), 7.65 (2H, m, H-4″/H-7″), 8.38 (1H, s, C=N, H-1″), 9.43 (1H, s, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 120.6 (C-3′), 124.9 (C-6″), 128.4 (C-2″) 128.9 (C-7″), 130.4 (C-5″), 133.2 (C-4″), 133.6 (C-4′), 136.6 (C=N, C-1″), 148.3 (C-3″) and 174.8 (C=S, C-3). HRMS (ESI) calcd for $C_{18}H_{24}N_4O_2S$ 361.1693 $[M + H]^+$ found 361.1698.

4.2.15. (+)-(R)-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-N(1)-[(3-nitrophenyl)-methylene]-thiosemicarbazide **16**

Light yellow crystals; yield 94%; mp. 158–160 °C; $[\alpha]_D$ +33°; IR (KBr/cm^{-1}): (NH) 3491 and 3364, (C=N) 1600, (C=S) 1232; EI-MS m/z 360 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.52 (1H, s, NH, H-4), 7.60 (1H, m, H-7″), 7.64 (1H, m, H-6″), 7.93 (1H, m, H-3″), 8.16 (1H, s, C=N, H-1″), 8.23 (1H, m, H-5″), 9.43 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 115.3 (C-7″), 115.8 (C-6″), 120.5 (C-3′), 128.9 (C-3″/C-5″), 133.6 (C-4′), 135.7 (C-2″), 142.6 (C=N, C-1″), 159.3 (C-4″) and 175.2 (C=S, C-3). HRMS (ESI) calcd for $C_{18}H_{24}N_4O_2S$ 361.1693 $[M + H]^+$ found 361.1699.

4.2.16. (+)-(R)-N(1)-(furan-2-yl-methylene)]-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide **17**

White crystals; yield 80%; mp. 138–139 °C; $[\alpha]_D$ +44°; IR (KBr/cm^{-1}): (NH) 3343 and 3310, (C=N) 1311, (C=S) 1302; EI-MS m/z 305 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 6.50 (1H, m, H-4″), 6.69 (1H, m, H-3″), 7.52 (1H, s, NH, H-4), 7.52 (1H, m, H-5″), 7.64 (1H, s, C=N, H-1″), 9.13 (1H, s, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 112.2 (C-4″), 113.6 (C-3″), 120.5 (C-3′), 130.6 (C=N, C-1″), 133.6 (C-4′), 144.8 (C-5″), 149.1 (C-2″) and 175.4 (C=S, C-3). HRMS (ESI) calcd for $C_{16}H_{23}N_3OS$ 306.1635 $[M + H]^+$ found 306.1629.

4.2.17. (+)-(R)-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-N(1)-[(1H-pyrrol-2-yl)-methylene]-thiosemicarbazide **18**

Light yellow crystals; yield 68%; mp. 116–118 °C; $[\alpha]_D$ +60°; IR (KBr/cm^{-1}): (NH) 3363 and 3290, (C=N) 1321, (C=S) 1295; EI-MS m/z 304 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 6.21 (1H, m, H-4″), 6.44 (1H, m, 5″), 6.87 (1H, m, 3″), 7.32 (1H, sl, NH, H-4), 7.55 (1H, s, C=N, H-1″), 8.77 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 110.8 (C-4″), 115.2 (C-3″), 120.5 (C-3′),122.2 (C-5″), 126.6 (C-2″), 133.6 (C-4′), 134.0 (C=N, C-1″) and 175.1 (C=S, C-3). HRMS (ESI) calcd for $C_{16}H_{24}N_4S$ 305.1794 $[M + H]^+$ found 305.1801.

4.2.18. (+)-(R)-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-N(1)-[(thiophen-2-yl)-methylene]-thiosemicarbazide **19**

White crystals; yield 83%; mp. 187–189 °C; $[\alpha]_D$ +30°; IR (KBr/cm^{-1}): (NH) 3336 and 3315, (C=N) 1301, (C=S) 1290; EI-MS m/z 321 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.50 (3H, s, H-9′), 1.64 (3H, s, H-10′), 1.79 (2H, m, H-6′), 1.98 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.56 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.05 (1H, dd, J = 5.1 and 3.6, H-3″), 7.24 (1H, dd, J = 3.6 and 0.9, H-4″), 7.36 (1H, m, H-5″), 7.54 (1H, sl, NH, H-4″), 8.08 (1H, s, C=N, H-1″), 10.06 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.0 (C-1′), 59.0 (C-7′), 120.5 (C-3′), 127.9 (C-4″), 128.2 (C-5″), 130.5 (C-3″), 133.6 (C-4′), 136.1 (C=N, C-1″), 138.8 (C-2″) and 175.1 (C=S, C-3). HRMS (ESI) calcd for $C_{16}H_{23}N_3S_2$ 322.1406 $[M + H]^+$ found 322.1410.

4.2.19. (+)-(R)-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-N(1)-(1-phenyl-ethylidene)-thiosemicarbazide **20**

White crystals; Yield: 75%; mp. 136–137 °C; $[\alpha]_D$: +39°; IR (KBr/cm^{-1}): (NH) 3343 and 3293, (C=N) 1541, (C=S) 1298; EI-MS: m/z 329 ($M^{+\bullet}$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.51 (3H, s, H-9′), 1.65 (3H, s, H-10′), 1.82 (2H, m, H-6′), 1.99 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.26 (3H, s, H-8″), 2.66 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.41 (3H, m, H-4″/H-5″/H-6″), 7.67 (2H, m, H-3″/H-7″), 7.84 (1H, s, NH, H-4), 8.41 (1H, s, NH, H-2). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 13.5 (C-8″), 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.2 (C-1′), 59.0 (C-7′), 120.6 (C-3′), 126.3 (C-4″/C-6″), 128.8 (C-3″/C-7″), 129.8 (C-5″), 134.6 (C-4′), 137.7 (C-2″), 144.3 (C=N, C-1″) and 176.1 (C=S, C-3). HRMS (ESI) calcd for $C_{19}H_{27}N_3S$ 330.1998 $[M + H]^+$ found 330.2001.

4.2.20. (+)-(R)-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-N(1)-[1-(4-methoxyphenyl)-ethylidene]-thiosemicarbazide **21**

White crystals; Yield: 88%; mp. 146–149 °C; $[\alpha]_D$: +36°; IR (KBr/cm^{-1}): (NH) 3340 and 3289, (C=N) 1380, (C=S) 1250; EI-MS m/z 359 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.51 (3H, s, H-9′), 1.65 (3H, s, H-10′), 1.82 (2H, m, H-6′), 1.99 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.22 (3H, s, H-8″), 2.66 (1H, m, H-1′), 3.84 (3H, s, OCH_3), 5.36 (1H, m, H-3′), 6.91 (2H, dd, J = 8.1, 2.1, H4″/H6″), 7.62 (2H, dd, J = 8.1, 2.1, H-3″/H-7″), 7.80 (1H, sl, NH, H-4), 8.39 (1H, sl, NH, H-2). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 13.4 (C-8″), 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.2 (C-1′), 55.5 (OCH_3), 59.0 (C-7′), 114.1 (C-4″/C-6″), 120.6 (C-3′), 127.7 (C-3″/C-7″), 134.2 (C-2″), 134.6 (C-4′), 145.2 (C=N, C-1″), 160.9 (C-5″) and 176.0 (C=S, C-3).

4.2.21. (+)-(R)-N(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-N(1)-[1-(4-nitrophenyl)-ethylidene]-thiosemicarbazide ***22***

Yellow crystals; Yield: 94%; mp. 196–198 °C; $[\alpha]_D$: +33°; IR (KBr/cm^{-1}): (NH) 3340 and 3289, (C=N) 1370, (C=S) 1220; EI-MS *m/z* 374 ($M^{+\bullet}$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8′), 1.51 (3H, s, H-9′), 1.65 (3H, s, H-10′), 1.82 (2H, m, H-6′), 1.99 (2H, m, H-2′), 2.05 (2H, m, H-5′), 2.31 (3H, s, H-8″), 2.66 (1H, m, H-1′), 5.36 (1H, m, H-3′), 7.75 (1H, sl, NH, H-4), 7.82 (2H, dd, J = 8.6, 2.1, H4″/H6″), 8.26 (2H, dd, J = 8.6, 2.1, H-3″/H-7″), 8.50 (1H, sl, NH, H-2). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 13.5 (C-8″), 23.5 (C-10′), 24.2 (C-9′), 24.4 (C-6′), 24.5 (C-8′), 26.7 (C-2′), 31.3 (C-5′), 41.2 (C-1′), 59.0 (C-7′), 120.6 (C-3′), 124.1 (C-4″/C-6″), 126.9 (C-3″/C-7″), 134.6 (C-4′), 142.5 (C-2″), 143.7 (C-5″), 148.3 (C=N, C-1″) and 176.1 (C=S, C-3). HRMS (ESI) calcd for $C_{19}H_{26}N_4O_2S$ 375.1849 $[M + H]^+$ found 375.1855.

4.3. Evaluation of antitumor activity

The synthesized compounds were evaluated *in vitro* against ten human cancer cell lines consisting of glioma (U251), melanoma (UACC-62), breast (MCF-7), ovarian with phenotype of multidrug resistance (NCI-ADR/RES), lung, non-small cell type (NCI-H460), kidney (746-0), prostate (PC-3), ovarian (OVCAR-03), colon (HT-29) and leukemia (K-562). Cell lines were obtained from National Cancer Institute (Frederick, MD, USA). Normal cell line (VERO, renal, green monkey), from the Rio de Janeiro Cell Bank, was also used. Stock cell cultures were grown in medium containing RPMI 1640, supplemented with 5% of fetal bovine serum. Experimental cultures were supplemented also with penicillin:streptomycin (10 μg/mL : 10 UI/mL). The tests were performed by the colorimetric method with sulforhodamine B, according to NCI standard protocol and paclitaxel was used as positive control. Assays were performed in 96-well plate using four concentrations at 10-fold dilutions (0.25 μg mL^{-1} to 250 μg mL^{-1}) for each compound tested. Three measurements were obtained at the beginning of incubation (time zero, T_0) and 48 h post-incubation for compound-free (C) and tested (T) cells. Cell proliferation was determined according to the equation $100 \times [(T - T_0)/C - T_0]$, for $T_0 < T \leq C$, and $100 \times [(T - T_0)/T_0]$, for $T \leq T_0$ and a concentration–response curve for each cell line was plotted using software ORIGIN 8.0 (OriginLab Corporation) [21]. The antitumor activity was deduced from dose–response curves and three dose–response parameters (GI_{50}, TGI and LC_{50}) were calculated by sigmoidal regression using software ORIGIN 8.0® (OriginLab Corporation) [22]. The average activity (MG-MID – antilogarithm of mean of log GI_{50} or antilogarithm of mean of log TGI) of synthesized compounds was also determined using MS Excel software. Compounds with GI_{50} values <100 μM were considered active.

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