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Synthesis of Some Novel Semicarbazone and Thiosemicarbazone Derivatives of Isatin as Possible Biologically Active Agents

Masoumeh Divar[1,2], Ali Khalafi-Nezhad[2], Kamiar Zomorodian[3], Razieh Sabet[4], Zahra Faghih[5], Maryam Jamali[4], Hasti Pournaghz[4] and Soghra Khabnadideh[1*]

[1]Faculty of Pharmacy, Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[2]Department of Chemistry, College of Sciences, Shiraz University, Shiraz 71454, Iran.
[3]Basic Sciences in Infectious Diseases Research Center, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.
[4]Faculty of Pharmacy, International Branch, Shiraz University of Medical Sciences, Shiraz, Iran.
[5]Cancer Immunology Group, Shiraz Institute for Cancer Research, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran.

Authors' contributions

This work was carried out in collaboration between all authors. Authors MD and AKN designed, synthesized and analyzed the data of the Isatin derivatives. Authors KZ, RS, ZF, MJ, HP and SK prepared the antimicrobial and cytotoxicity tests. Authors MD and SK wrote the paper. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The development of new beneficial agents is one of the crucial aims in medicinal chemistry. Isatin is considered as a vital class of bioactive compounds exhibiting different biological activities. A series of nine new Semicarbazone ***(12a-i)*** and six new Thiosemicarbazone ***(14a-c, 14f-h)*** derivatives of isatin were prepared.

**Corresponding author: E-mail: khabns@sums.ac.ir;*

Methods: Synthesis of the desired compounds was done in two steps. In the first step, we alkylated the isatin ring by using different alkyl halides in DMF and in the presence of Ca_2H. In the second step, the alkylated isatin was reacted with semicarbazide or thiosemicarbazide in ethanol and amount of acetic acid. Chemical structures of all the products were confirmed by IR[i], ^{1}H NMR[ii], ^{13}C NMR, and elemental analysis. The cytotoxic activities of the compounds were evaluated by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) method against MCF-7[iii] and MDA-231[iv] breast cancer cell lines. Antimicrobial activities of these compounds against different species of microorganisms including Gram positive and Gram negative bacteria as well as fungi were also determined by broth micro-dilution method as recommended by CLSI (Clinical and Laboratory Standard Institute).

Results: Some of our compounds showed moderate cytotoxic activity but showed notable antibacterial and antifungal effects.

Conclusion: Replacement of aminophenyl benzothiazole with isatin had reduced the antimicrobial activities of the compounds. Maybe a complex of our synthesized compounds with some metals could be used to improve their antimicrobial activities.

[i] Infrared.
[ii] Nuclear Magnetic Resonance.
[iii] Michigan Cancer Foundation-7.
[iv] Mammary Drive Adenocarcinoma.

Keywords: Synthesis; isatin; semicarbazone; thiosemicarbazone; MTT.

1. INTRODUCTION

Recently much interest has been shown in isatin and its derivatives. They possess a broad spectrum of biological activities including antimicrobial, anticonvulsant, antineoplastic, antiviral, antihypertensive, anti-inflammatory as well as enzymatic inhibition activities [1-19]. Isatin scaffold is an important pharmacophore responsible for biological activity, applicable lead structure for the synthesis of efficient chemotherapeutic agents [19].

Isatin is a synthetically flexible substrate, which can be used for synthesis of large varieties of schiff base transition metal complexe derivatives [20-23] and heterocyclic compounds. Also it is used as a starting material for various drug syntheses. It is obvious from literature that isatin derivatives are known to be linked with a broad spectrum of biological activities.

Among isatin derivatives, isatin semicarbazones and thiosemicarbazones (Fig. 1) have been found to show various chemotherapeutic properties such as anti-cancer, anti-microbial, anti-tuberculosis, anti-ulcer, anti-viral, anti-plasmodial, as well as enzymatic inhibition [1,2,6-8,11,13,17,18,24,25].

It has been reported that some isatin thiosemicarbazone analogs have anti-HIV activity which is comparable with non-nucleoside reverse-transcriptase inhibitors (NNRTIs). The desired structures have been designed based on the derived pharmacophoric model with the thiosemicarbazone moiety (=N–NH–CS–N-) constituting the –body- and the aryl ring of isatin and bulky diethyl moiety constituting the –wings- which was found to fulfill the specification of the pharmacophoric distance map by complying within the defined range [2]. The relationship between the structures of some anti-HIV drugs and isatin thiosemicarbazones are shown in Fig. 2.

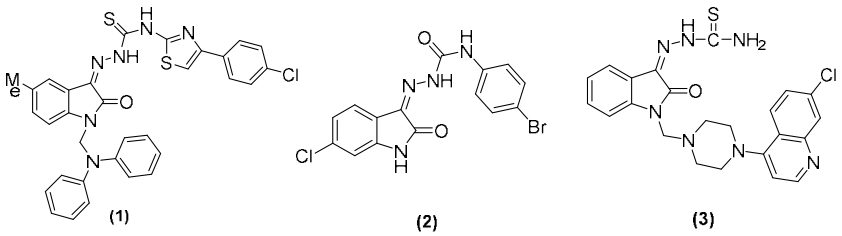


Fig. 1. Structures of some bioactive semicarbazone and thiosemicarbazone derivatives of isatin, anticonvulsant, antibacterial, antifungal *(1,2)*, and antiplasmodial agents *(3)*

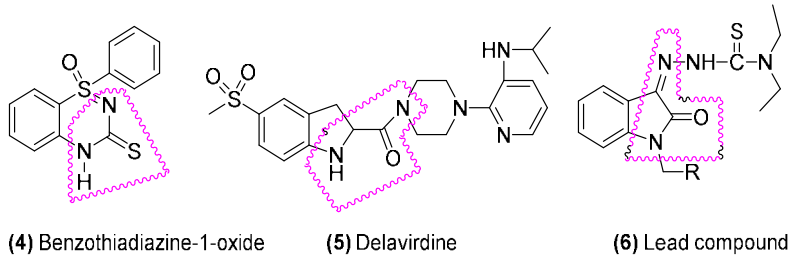


Fig. 2. Schematic representation of a butterfly-like conformation of the existing NNRTIs and the lead compound

On the other hand, *N*-Alkylated indoles are well known to exhibit anticancer activity [26-28]. However, little had been reported on the antineoplastic activity of *N*-alkyl and *N*-aryl isatins. Compound ***7*** was the first N-alkyl isatin described in 2003 to induce apoptosis in a panel of human cancer cell lines, but not normal cells, at micro molar concentrations [28]. Usually it was found that normal cell lines such as peripheral blood lymphocytes and human mammary epithelial cells were resistant to N-(3,4-dichlorobenzyl)-1*H*-indole-2,3-dione ***(7)*** induced apoptosis, while cancer cell lines of lymphoid source were the most responsive. Compound ***8*** also has showed a strong preference for UCH-L1(Ubiquitin C-terminal hydrolase) over UCH-L3 (An isoform of the enzyme) [27] (Fig. 3).

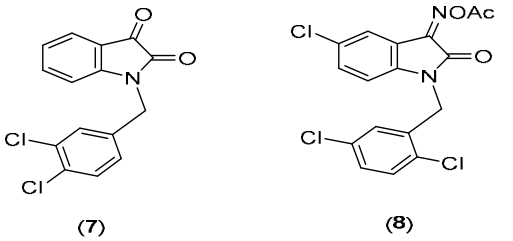


Fig. 3. Examples of the first synthetic cytotoxic N-substituted isatin based molecules

Encouraged by this and in continuation of our work on bioactive isatin derivatives [29] and according to the excellent biological activities of N-substituted isatins, a new category of these compounds have been synthesized. They were used to synthesize a new class of isatin semicarbazones and thiosemicarbazones which may exhibit better or different types of biological properties. The synthesized compounds were then screened for their *in vitro* cytotoxic effects on breast cancer cell lines, as well as their anti-fungal and antibacterial activities.

2. MATERIALS AND METHODS

All chemicals were obtained from *Merck* (Germany) or *Fluka* (Switzerland). Solvents were purified and dried according to reported methods [30] and stored over molecular sieves.

All yields refer to isolated products after chromatography and other indicated purification methods. The products were characterized by comparison of their physical data with those of known samples or by their full spectral data analysis. Infrared (IR) spectra were run on a *Shimadzu FTIR-8300* spectrophotometer, ν_{max} in cm^{-1}. The ^{1}H-NMR (250 MHz) and ^{13}C-NMR (62.5 MHz) were run on a *Bruker Advanced DPX-250*, *FT-NMR* spectrometer using tetramethylsilane (TMS) as internal standard in pure deuterated solvents ($CDCl_3$, DMSO-d_6 and D_2O). Chemical shifts are given in the δ scale in part per million (ppm) and *J* in Hz. The bonds are assigned as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (br), doublet of doublet (dd) and complex. Microanalyses were performed on a *Thermo Finigan, Flash EA,* and CHN Analyzer. Melting points were recorded on a *Büchi B 545* apparatus in open capillary tubes and were uncorrected.

The progress of reactions was followed with TLC using silica gel *SILG/UV 254* plates. Column chromatography was carried out on a silica gel 60 Merck (230-240 mesh) in glass columns (2 or 3 cm diameter) using 15-30 grams of silica gel per one gram of the crude Product.

2.1 Synthesis of N-substituted Isatin Intermediates

The synthesis of N-substituted isatin derivatives are considered, as prospective precursors in the present study. N-alkylated isatins ***(10a-i)*** were prepared by using of calcium hydride as a base in DMF and revealed that the method is suitable

for the preparation of all N-substituted isatins. Then in the next step, prerequisite N-substituted isatin intermediates were used to synthesize the desired semicarbazone and thiosemicarbazone analogs of isatin.

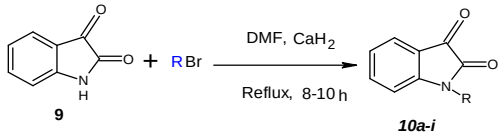


Fig. 4. Synthesis of intermediate *10a-i*

2.1.1 Synthesis of Isatin semicarbazone derivatives

N-substituted isatins ***(10a-i)*** were reacted with semicarbazide ***11*** in aqueous ethanol (50%) containing a catalytic amount of glacial acetic acid under reflux conditions to give the corresponding isatin semicarbazones ***(12a-i)*** in good to excellent yields (85–95%).

2.1.1.1 Spectroscopic data for semicarbones

Data for (E)-1-(1-cyclopentyl-2-oxindolin-3-yeliden) semicarbazide (12a):

In a double-neck round bottomed flask (100 ml,) a mixture of 1-cyclopentylindoline-2,3-dione (***10a***) (2.15 g, 0.01 mol), semicarbazide(1.12 g, 0.015 mol) and catalytic amount of acetic acid (2-3 drops) was dissolved in ethanol (15 ml). The solution was refluxed for 7 hours. After this time the reaction was completed as indicated by TLC. The reaction mixture was kept in a refrigerator overnight. Filtration of the reaction mixture followed by washing with cool ethanol (2×5 ml) and recrystalization from MeOH/H_2O gave a pure, yellow crystalline solid. (2.3 g, 85%), mp 215-216º C, R_f(EtOAc/*n*-hexane, 1:8) 0.53, ^{1}H-NMR (250 MHz, DMSO-d_6) δ 10.16 (1H, br. s), 7.30-8.10 (4H, m), 6.84 (2H, br. s), 4.04 (1H, m), 1.60-2.06 (8H, m), ^{13}C-NMR (62.5 MHz,DMSO-d_6) δ 163.14, 155.88, 154.84, 142.63, 131.32, 130.10, 121.69, 115.31, 109.86, 51.87, 27.43, 24.55, MS (EI, 70 eV) *m/z* (%): 272.33 $[M+H]^+$ (272.31 calcd for $C_{14}H_{16}N_4O_2$), *Anal. Calcd.* For $C_{14}H_{16}N_4O_2$: C, 58.31, H, 5.59, N, 19.43. *Found*: C, 58.70,H, 6.01, N, 19.20, IR (KBr) ν_{max}: 3455, 3238, 3165, 2957, 1666, 1638, 2985, 1203, 692.

Data for (Z)-2-(1-(2-methylbenzyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboxamide (12b):

Pure yellow crystals (2.6 g, 87%), mp 224-225º C, R_f(EtOAc/*n*-hexane, 1:3) 0.85, ^{1}H-NMR (250 MHz, DMSO- d_6) δ 11.65 (1H, bs), 6.84-8.12 (10H, m), 4.92 (2H, s), 2.35 (3H, s), ^{13}C-NMR (62.5 MHz,, DMSO- d_6) δ 163.5, 157.43, 147.43, 140.72, 134.82, 132.83, 129.43, 128.93, 126.93, 126.73, 125.65, 121.70, 117.87, 40.96, 17.85, MS (EI, 70 eV) *m/z* (%): 308.35 $[M+H]^+$(308.34 calcd for $C_{17}H_{16}N_4O_2$), *Anal. Calcd*.for $C_{17}H_{16}N_4O_2$: C, 66.22, H, 5.23, N, 18.17. *Found:* C, 66.12.0, H, 5.8, N, 18.09, IR (KBr)ν_{max}: 3475, 3289, 3240, 1687, 1642, 1443, 740.

Data for (Z)-ethyl 2-(3-(2-carbamoylhydrazono)-2-oxoindolin-1-yl) propanoate (12c):

Pure yellow crystals (2.8 g, 93%), mp 194-195º C, R_f(EtOAc/*n*-hexane, 1:3) 0.85, ^{1}H-NMR (250 MHz, DMSO-d_6) δ 7.03-7.6 (4H, m), 6.87 (1H, b.s), 3.67 (1H, q, *J*=7.5 Hz), 3.33 (2H, b.s) 1.49 (2H, q, *J*=7.5 Hz) 1.23 (3H, d, *J*=7.5Hz), ^{13}C-NMR (62.5 MHz, DMSO-d_6), δ 163.38, 160.70, 156.03, 154.84, 143.11, 130.25, 124.95, 121.85, 115.07, 109.05, 29.05, 19.42, 13.53, 13.49 , MS (EI, 70 eV) *m/z* (%): 304.32 $[M+H]^+$ (304.31calcd for $C_{14}H_{16}N_4O_4$),*Anal. Calcd*.for $C_{14}H_{16}N_4O_4$: C, 88.26, H, 5.30, N, 18.41. *Found*: C, 88.56, H, 5.50, N, 18.10, IR (KBr) ν_{max}: 3869, 3345, 3263, 3170, 2936, 1725, 1670, 1648, 1078, 683.

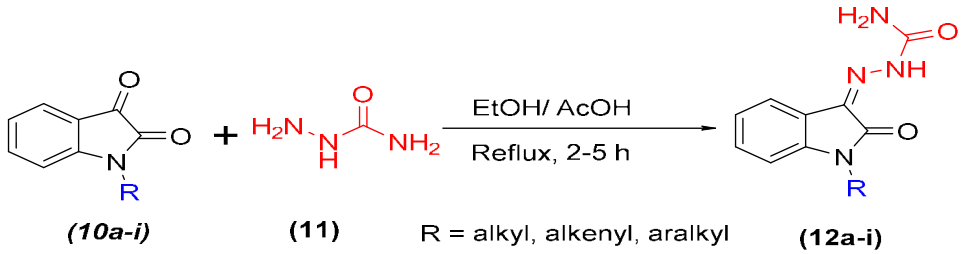


Fig. 5. Synthesis of compounds *12a-i*

Data for (Z)-1-(2-oxo-1(2-oxo-3-phenoxypropyl) indolin- ylidene) semicarbazide (12d):

Pure yellow crystals (3.0 g, 86%), mp 196-197º C, R_f(EtOAc/*n*-hexane, 3:1) 0.63, ^{1}H-NMR (250 MHz, DMSO-d_6): δ 10.29 (1H , b.s), 7.80 -8.11 (9H, m), 6.88 (2H, b.s) 5.21 (2H, s) 4.71 (2H, s), ^{13}C-NMR (62.5 MHz, DMSO-d_6) δ 167.81, 163.52, 155.67, 142.78, 135.50, 131.75, 131.38, 128.38, 128.13, 127.89, 124.96, 122.36, 115.06, 109.33, 66.47, 40.97, MS (EI, 70 eV) *m*/*z* (%): 352.37 $[M+H]^+$ (352.35 calcd for $C_{18}H_{16}N_4O_4$), *Anal. Calcd*.for $C_{18}H_{16}N_4O_4$: C, 61.36, H, 4.58, N, 15.90. *Found*: C, 61.30, H, 4.47, N, 15.72, IR (KBr) ν_{max}: 3751, 3438, 3291, 3180,2364,1752, 1693, 1504, 1468, 699.

Data for (Z)-1-(1-(but-3-en-2-yl)-2oxindolin-3 ylidene) semicarbazide (12e):

Pure yellow crystals (2.4 g, 95%), mp 202-203º C, R_f(EtOAc/*n*-hexane, 1:3) 0.50, ^{1}H-NMR (250 MHz, DMSO-d_6) δ 12.26 (1H, s), 7.50-8.51 (4H, m), 7.39 (2H, b.s) 5.75-5.88 (1H, m) 5.08-5.15(2H, dd, *J*=7.5-10 Hz) 4.33-4.35 (1H, m, *J*=5Hz) 3.15 (3H, d),^{13}C-NMR (62.5 MHz, DMSO-d_6) δ 178.64, 160.82, 142.78, 135.59, 132.98, 131.00, 130.86, 127.27, 125.87, 122.98, 120.75, 119.50, 110.38, 18.83, MS (EI, 70 eV) *m*/*z* (%): 258.29 $[M+H]^+$ (258.28 calcd for $C_{13}H_{14}N_4O_2$), *Anal. Calcd*.for $C_{13}H_{14}N_4O_2$: C, 60.45, H, 5.46, N, 21.69. *Found*: C, 60.61, H, 5.92, N, 21.85, IR (KBr) $\nu_{max.:}$3352, 3330, 3170, 1676, 1598, 1453, 630.

Data for (Z)-1-(1-sec-butyl-2-oxoindolin-3-ylidene) semicarbazide (12f):

Pure yellow crystals (2.3 g, 89%),mp 178-179º C, R_f(EtOAc/*n*-hexane, 1:3) 0.41, ^{1}H-NMR (250 MHz, DMSO-d_6) δ 10.24 (1H, b.s), 7.02-8.09 (4H, m), 6.86 (2H, s), 4.27-4.33 (1H, m), 1.93-1.99 (2H, m) 1.35 (3H, d, *J*=7.5 Hz), 0.75 (3H, t, *J*=5 Hz),^{13}C-NMR (62.5 MHz, DMSO-d_6). δ 163.27, 155.87, 142.91, 132.26, 131.37, 125.10, 121.60, 115.19, 110.03, MS (EI, 70 eV) *m*/*z* (%): 260.31 $[M+H]^+$ (260.30 calcd for $C_{13}H_{16}N_4O_2$), *Anal. Calcd*. for $C_{13}H_{16}N_4O_2$: C, 59.99, H, 6.20, N, 21.79. *Found*: C, 59.79, H, 6.27, N, 21.95, IR (KBr) $\nu_{max.}$: 3372, 3342, 1676, 1598, 1453, 650.

Data for (E)-1-(1-butyl-2-oxindolin-3ylidene) semicarbazide (12g):

Pure yellow crystals (2.5 g, 88%), mp 197-198º C, R_f(EtOAc/n-hexane, 1:3) 0.87, ^{1}H-NMR (250 MHz, DMSO-d_6):δ 11.57 (bs, 1H, NH), 7.02-8.13 (complex, 4H, isatin), 6.88 (bs, 2H, NH_2), 5.12 (m, 2H, N-CH_2), 4.05 (m, 2H, N-CH_2-CH_2) 1.48 (m, 2H, CH_2-CH_3) 1.07(t, 3H, terminal CH_3), ^{13}C-NMR (62.5MHz, DMSO-d_6):δ 13.9, 14.11, 48.54, 61.11, 109.35, 115.28, 122.18, 125.18, 131.38, 142.02, 155.77, 162.91,169.71. Anal.Calcd.for $C_9H_{12}N_4O_2$: C, 51.92, H, 5.81, N, 26.91. Found: C, 51.66, H, 5.20, N, 26.70, IR ν_{max}: 34921, 3380, 3167, 1660, 1548, 1472, 730.

Data for (E)-1-(1-allyl-2-oxoindolin-3-yeliden) semicarbazide (12h):

Pure yellow crystalline (2.2 g, 91%),m.p 218-220º C, R_f(EtOAc/n-hexane, 1:5) , ^{1}H-NMR (250 MHz, DMSO-d_6): δ 10.17,(broad singlet, 1H.=N-NH) 6.98-8.12,(complex,4H ,isatin), 6.87,(broad singlet, 2H, NH2) 5.75-5.88 (m, 1H, =CH) 5.08-5.15(dd, 2H, =CH2, *J*=7.5-10) 4.33-4.35(d, 2H, N-CH2, *J=5*),^{13}C-NMR(62.5MHz,DMSO-d_6):δ 64.89, 109.64, 114.30, 120.76, 121.99, 124.84, 129.43, 131.33, 143.37, 155.82, 157.98, 163.5,Anal.Calcd.for $C_{12}H_{12}N_4O_2$: C, 59.01, H, 4.95, N, 22.94,O,13.10. Found: C, 58.98, H, 4.93, N, 22.89, O, 13.7, IR (KBr) ν_{max}:3352, 3330, 3170, 1676, 1598, 1453, 630.

Data for (E)-1-(2-oxo-1-(2-phenoxy ethyl) indolin-3- ylidene) semicarbazide (12i):

Recrystalization from MeOH/H_2O gave(2.9, 89%) gave pure, yellow crystalline solid of corresponding semicarbzide, mp 212-213º C, R_f(EtOAc/n-hexane (0.55), 1:1) 0.72, ^{1}H-NMR (250 MHz, $CDCl_3$): δ 11.51 (bs, 1H, NH), (complex, 11H, aryl, isatin, NH_2), (m, 4H, CH_2-CH_2), ^{13}C-NMR (62.5 MHz, DMSO, d_6):δ 39.75, 64.89, 109.66, 114.30, 115.02, 120.76, 122.00, 124.85, 129.44, 131.34, 132.09, 143.37, 155.82, 157.97, 163.53, Anal.Calcd.for $C_{17}H_{16}N_4O_3$: C, 62.95, H, 4.97, N, 17.27, Found: C, 62.71, H, 5.04, N, 17.80, IR (KBr) ν_{max} : 3396, 3340, 3120, 1696, 1548, 1475, 590.

2.1.2 Synthesis of isatin thiosemicarbazone derivatives

Based on the structure of isatin, besides their medicinal importance of thiosemicarbazone compounds, new thiosemicarbazone derivatives were synthesized from N-substituted isatin derivatives. For this purpose, six analogs of the synthesized N-substituted isatins ***(10a-c, 10f-h)*** were reacted with thiosemicarbazide ***13*** in aqueous ethanol (50%) containing a catalytic amount of glacial acetic acid under reflux conditions to give the corresponding isatin thiosemicarbazones ***(14a-c, 14f-h)*** in excellent yields (88–92%).

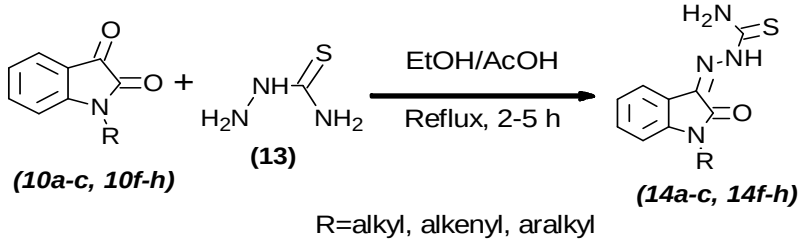


Fig. 6. Synthesis of compounds *14a-c, 14f-h*

2.1.2.1 Spectroscopic data for thiosemicarbones

Typical Procedure and Data for Synthesis of (Z)-1-(1-cyclopentyl-2-oxindolin-3-yeliden) thiosemicarbazide: (14a):

In a double-neck round bottomed flask (100 ml,) a mixture of 1-allylindoline- 2, 3-dione ***(10a)*** (2.15 g, 0.01 mol),thiosemicarbazide(1.36 g, 0.015 mol) and a catalytic amount of acetic acid (2-3 drops) was dissolved in ethanol (15 ml). The solution was refluxed for 7 hours. After this time the reaction was completed as indicated by TLC and then the reaction mixture was kept in a refrigerator overnight. Filtration of the reaction mixture followed by washing with cool ethanol (2×5 ml) and recrystalization from MeOH/H_2O gave pure yellow crystalline of corresponding semicarbazide (2.5 g, 88%) mp 233-234º C, R_f(EtOAc/*n*-hexane, 1:8) 0.57, ^{1}H-NMR (250 MHz, DMSO-d_6): δ 12.90 (broad singlet, 1H, NH), 6.88-7.52 (complex, 4H, isatin), 6.54 (broad singlet, 2H, NH_2), 4.64 (m, 1H, N- CH, cyclopentyl), 1.60-2.02 (complex, 8H, cyclopentyl), ^{13}C-NMR (62.5 MHz,DMSO-d_6): δ 25.10, 28.05, 52.51, 110.59, 120.96, 122.83, 131.27, 142.64, 161.03, 179.74, *Anal. Calcd.* For $C_{14}H_{16}N_4O_2$:C, 61.75, H, 5.92, N, 20.58. Found: C, 61.70,H, 6.01, N, 20.20,IR (KBr) v_{max}: 3452, 3228, 3155, 1676, 1498, 1203, 692.

Data for (Z)-2-(1-(2-methylbenzyl)-2-oxoindolin-3-ylidene) hydrazinecarbothioamide (14b):

Pure yellow crystals(2.9 g, 90%), mp 231-232º C, R_f(EtOAc/*n*-hexane, 1:3) 0.75, ^{1}H-NMR (250 MHz, DMSO- d_6) δ 12.38 (1H, br.s), 9.09(1H, s), 8.75 (1H, s) 7.01-7.74 (8H, m), 4.93 (2H, s), 2.35 (3H, s), ^{13}C-NMR (62.5 MHz, DMSO- d_6) δ 178.64, 160.82, 142.78, 135.59, 132.98, 131.00, 130.86, 127.27, 125.87, 122.98, 120.75, 119.50, 110.38, 18.38, MS (EI, 70 eV) *m*/*z* (%): 324.41 $[M+H]^+$ (324.40 calcd for $C_{17}H_{16}N_4OS$), *Anal. Calcd*.for $C_{17}H_{16}N_4OS$: C, 62.94, H, 4.97, N, 17.27. *Found:* C, 62.12, H, 5.01, N, 17.09, IR (KBr)v_{max}: 3452, 3348, 3167, 2995, 2754, 1656, 1468, 1058, 6793.

Data for (Z)-ethyl 2-(3-(2-carbamothioyl-hydrazono)-2-oxoindolin-1-yl)propanoate (14c):

Pure yellow crystals (2.9 g, 92%), mp 98-99º C, R_f(EtOAc/*n*-hexane, 1:3) 0.75, ^{1}H-NMR (250 MHz, DMSO-d_6) δ 12.46 (1H, br.s), 9.11 (1H, s), 8.76 (1H, s) 7.08-7.74 (4H, m), 5.16-5.25 (1H, q, *J*=7.5 Hz), 4.08-4.17 (2H, q, *J*=7.5 Hz), 1.53 (3H, d, *J*=7.5 Hz) 1.32 (3H, t, *J*=7.5),^{13}C-NMR (62.5 MHz, DMSO-d_6) δ 178.65, 169.26, 160.27, 141.73, 131.07, 130.28, 123.02, 120.90, 119.42, 110.25, 61.32, 48.73, 14.09, 13.94 , MS (EI, 70 eV) *m*/*z* (%): 320.38 $[M+H]^+$ (320.37 calcd for $C_{14}H_{16}N_4O_3S$), *Anal. Calcd*.for $C_{14}H_{16}N_4O_3S$: C, 52.49, H, 5.03, N, 17.49. *Found*: C, 52.56, H, 5.50, N, 18.10, IR(KBr)v_{max}:3889, 3374, 3253, 3166, 2596, 1728, 1696, 1492, 1102, 747.

Data for (Z)-1-(1-sec-butyl-oxoindolin-3-ylidene) thiosemicarbzide (14f):

Pure yellow crystals (2.5 g, 92%), mp 178-179º C, R_f(EtOAc/*n*-hexane, 1:3) 0.51, ^{1}H-NMR (250 MHz, DMSO-d_6) δ 12.69 (1H, br.s), 9.07 (1H, s), 8.88 (1H, s)7.09-8.03 (4H, m), 4.23-4.29 (1H, m), 1.72-1.97 (2H, qd, *J*=7.5, 46 Hz), 1.40 (3H, d, *J*=7.5 Hz), 0.75 (3H, t, *J*=7.5),^{13}C-NMR (62.5 MHz, DMSO-d_6) δ 163.27, 155.87, 142.91, 132.26, 131.37, 125.10, 121.60, 115.19, 110.03, 49.31, 25.56, 17.43, 10.98, MS (EI, 70 eV) *m*/*z* (%): 276.38 $[M+H]^+$ (276.36 calcd for $C_{13}H_{16}N_4OS$), *Anal.Calcd*.for $C_{13}H_{16}N_4OS$: C, 56.50, H, 5.84, N, 20.27. *Found*: C, 56.79, H, 5.27, N, 20.95, IR (KBr) v_{max}: 3482, 3320, 3165, 1676, 1498, 1223, 700.

Data for (Z)-1-(1-butyl-2-oxindolin-3ylidene) thiosemicarbazide (14g):

Pure yellow crystals (2.3 g, 85%), mp 220-222º C, R_f(EtOAc/*n*-hexane, 1:3) 0.77, ^{1}H-NMR (250

MHz, DMSO-d_6):δ 12.29 (b.s, 1H, NH), 9.14 (s, 1H, NH (HS-C=NH), 8.83 (s, 1H, SH (HS-C=NH) 7.04-7.76 (complex, 4H, isatin), 5.17 (t, 2H, N-CH_2, *J*=7.1 Hz), 4.09-4.17 (m, 2H, N-CH_2), 1.56-1.67 (m, 2H, N-CH_2-CH_2) 1.097 (t, 3H, terminal CH_3, *J*=7.5), ^{13}C-NMR (62.5MHz, DMSO-d_6):δ 13.88, 48.75, 61.33, 110.23, 119.42, 120.88, 123.00, 130.26, 131.04, 141.70, 160.27, 109.23, 178.68, *Anal. Calcd.* for $C_9H_{12}N_4OS$: C, 56.50, H, 5.84, N, 20.27. *Found*: C, 56.66, H, 5.46, N, 20.70, IR (KBr)v_{max}: 3452, 3228, 3155, 1664, 1428, 1250, 680.

Data forSynthesis of (Z)-1-(1-allyl-2-oxoindolin-3-yeliden) thiosemicarbazide (14h):

Pure yellow crystals (2.4 g, 93%),m.p 216-218º C R_f(EtOAc/n-hexane, 1:5), 0.79,^{1}H-NMR (250 MHz, DMSO-d_6):δ 12.77,(broad singlet, 1H, =N-NH) 6.83-7.76,(complex,4H ,isatin), 6.80 (broad singlet, 2H, NH2) 5.75-5.88 (m, 1H, =CH) 5.16-5.23(dd, 2H, =CH2,*J*=2.5-15 Hz) 4.31-4.33 (dd, 2H, N-CH2, *J*=2.5, 5Hz),^{13}C-NMR(62.5MHz,DMSO-d_6): δ 61.25, 110.24, 117.33, 119.32, 120.9, 122.85, 130.99, 131.39, 142.60, 160.42, 178.63, Anal. Calcd. for $C_{12}H_{12}N_4OS$: C, 55.37, H, 4.65, N, 21.52. Found: C, 55.98, H, 4.93, N, 21.89, IR (KBr) v_{max}: 3352, 3330, 3170, 1676, 1598, 1183, 630.

2.2 Cytotoxic Effect of Isatin Derivatives on MCF-7 and MDA-231 Breast Cancer Cell Lines

Two human breast cancer cell-lines, MCF-7 and MDA-231 were obtained from the national cell bank of Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in sterile T25 flasks in complete culture medium containing Roswell Park Memorial Institute (*RPMI*) 1640 medium (Biosera, UK), supplemented with 10% Fetal Bovine Serum (FBS) (Biosera, UK), 100 units/ml penicillin and 100 µg/ml streptomycin (Biosera, UK), were incubated at 37℃ in an incubator with humidified atmosphere and 5% CO_2. Both cell lines grow in monolayers, so in order to detach and disperse into single cells, after 70-80% confluence, the culture medium was removed and the cells were washed with 1× Phosphate Buffer Saline (PBS) to remove FBS and was treated with 1 ml of 0.25% trypsin-EDTA solution. The cells were then counted and their viability was determined using trypan blue exclusion test. The cells with more than 95% viability were diluted to the appropriate concentration and transferred into a sterile 96-microtiter plate.

2.2.1 MTT cell proliferation assay

The cell lines were treated with different concentrations of isatin semicarbazone and isatin thiosemicarbazone derivatives from 0.1 µg/ml to 200 µg/ml for 48 hours. Cytotoxic effects of these derivatives were determined through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell proliferation assay and their activity compared with Cisplatin as a positive control. This is a colorimetric assay, which works based on the reduction of tetrazolium salts. When a metabolic event or chemical induces apoptosis or necrosis, the yellow tetrazolium bromide is converted to purple formazans by the action of mitochondrial dehydrogenase enzyme in metabolically active viable cells. The formazans crystals could then be solubilized and quantified by spectrophotometry.

The powdered chemicals were dissolved in 100 µl DMSO as a stock (20 mg/ml). The chemicals were then diluted serially in a complete culture medium to obtain 200, 100, 50, 10, 1 and 0.1 µg/ml concentrations. The same concentrations were also made by adding DMSO to the culture medium as controls.

The cells were harvested and seeded in 10×10^3 concentration per well in 100µl complete medium. Three wells were considered for each chemical's concentration. Three untreated wells (cells without treatment) as well as three wells of cell culture medium alone were also included as negative controls. The cells were then incubated for 24 hours and treated with different concentrations of chemicals. Cisplatin in different concentrations were also added to separate wells as positive control. The plate was incubated for 48 hours. After incubation time, 10µl of MTT reagent (0.5 mg/ml) was added to each well including the controls. The plate was incubated again for 3 hours at 37° C until the purple intracellular crystals appeared. To dissolve formazan crystals, 100 µl DMSO was added to all wells and left at room temperature for 1 hour. The optical density of the test and control wells were read at 570 nm.

2.3 Antimicrobial Activities

2.3.1 Microorganisms

The antimicrobial activities of the synthetic compounds were determined standard species of *C. albicans* (ATCC 10261), *C. dubliniensis*

(CBS 8501), *C. tropicalis* (ATCC 750), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 90030),*C. parapsilosis* (ATCC 4344), *Cryptococcus neoformanse* (H99), *Aspergillus flavus* (ATCC 64025), *A. fumigates* (ATCC 14110), *A. clavatus* (CBS 51465), *Exophiala dermatitis* (ATCC14110), *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *E. fecalis* (ATCC51299).The reference antifungal compounds fluconazole (Sigma, St. Louis, MO, USA), for yeasts and *Aspergillus* species, and ciprofloxacin were used as standard drugs.

2.3.2 Determination of minimum inhibitory concentration

MICs were determined using broth micro dilution method as recommended by the CLSI with some modifications [26-28]. Briefly, for determination of antifungal activities against fungi, serial dilutions of the synthetic compounds (1.0 to 512.0 µg/ml) were prepared in 96-well microliter plates using RPMI-1640 media (Sigma, St. Louis, USA) buffered with MOPS (Sigma, St. Louis, USA). To determine the antibacterial activities, serial dilutions of the synthetic compounds (1.0 to 512.0 µg/ml) were prepared in Muller-Hinton Broth media (Merck, Darmstadt, Germany). Test fungi or bacteria strains were suspended in the media and the cell densities were adjusted to 0.5 McFarland standards at 530 nm wavelength using a spectrophotometric method (this yields stock suspension of $1\text{-}5\times10^6$ cells/ml for yeast and $1\text{-}1.5\times10^8$ cells/ml for bacteria). One hundred microliter of the working inoculums were added to the microliter plates which were incubated in a humid atmosphere at 30℃ for 24-48 h (fungi) or at 37℃ for 24 h (bacteria). Two hundred microliter of the inoculated medium was included as a sterility control (blank). In addition, growth controls (medium with inoculums but without drugs) were also included. The growth in each well was compared with that of the growth control well. MICs were visually determined and defined as the lowest concentration of the synthetic compounds or drugs which produced no visible growth. Each experiment was performed in triplicate.

3. RESULTS AND DISCUSSION

3.1 Synthesis

N-alkyl isatin intermediates prepared by alkylation reactions and were optimized to follow more efficiently using DMF that had not been previously dried and at the reflux condition. The use of CaH_2 is advantageous as a consequence of the ease with which it can be handled and exposed to the atmosphere, particularly in humid climates, in comparison to NaH [31]. The results for this step are summarized in Table 1.

In the next step semicarbazone or thiosemicarbazone were added to the N-alkylated isatin via a shiff base reaction. It should be emphasized that the purification of synthesized semicarbazones and thiosemicarbazones were carried out exclusively by recrystallization procedure. Purification by column chromatography was unsatisfactory using these type of chemicals because silica gel acts as a lewis acid that interacts with these compounds and decomposes them, leading to a decrease of yield as well as being a cumbersome isolation procedure (Table 2 and Table 3).

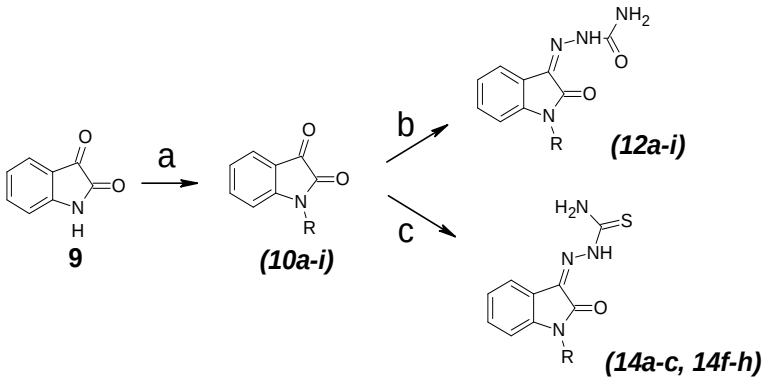


Fig. 7. General procedure for synthesis of isatin derivatives

a: CaH_2, DMF, b: Semicarbazide, ethanol, acetic acid, c: Thiosemicarbazide, ethanol, acetic acid

Table 1. Results for synthesis of N-substituted isatin intermediates

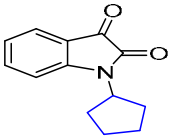
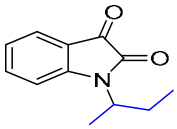
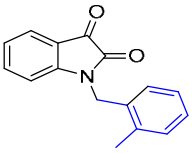
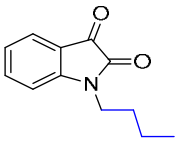
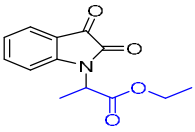
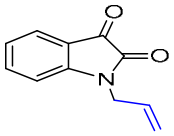
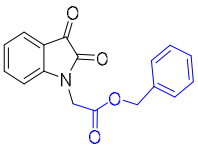
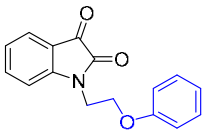
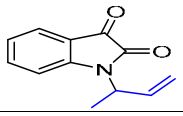
Compd.	Chemical structure	Time (h)	Yeild (%)	Compd.	Chemical structure	Time (h)	Yeild (%)
10a		8.5	76	***10f***		10	70
10b		9	65	***10g***		8	73
10c		8.5	69	***10h***		9.5	72
10d		10	73	***10i***		9	54
10e		8	72				

Table 2. Results for synthesis of isatin semicarbazone derivatives

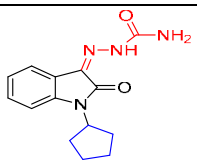
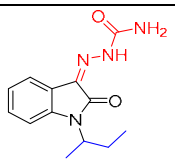
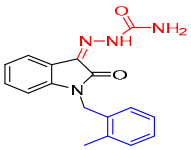
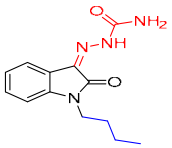
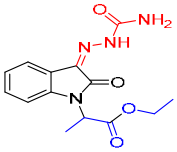
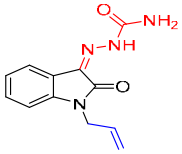
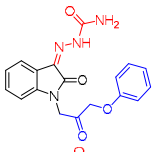
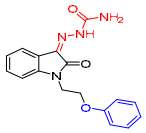
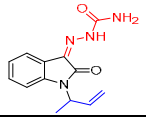
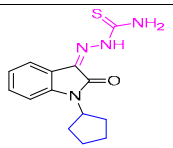
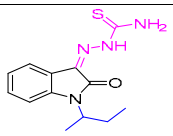
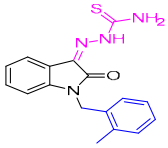
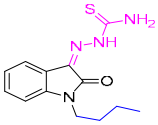
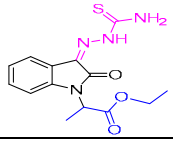
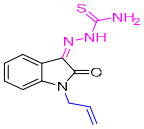
Compd.	Chemical structure	Time (h)	Yeild (%)	Compd.	Chemical structure	Time (h)	Yeild (%)
12a		4	85	***12f***		4	89
12b		3.5	87	***12g***		5	88
12c		4.5	93	***12h***		5	91
12d		3	86	***12i***		5	89
12e		5	95				

Table 3. Results for synthesis of isatin thiosemicarbazone derivatives

Compd.	Chemical structure	Time (h)	Yeild (%)	Compd.	Chemical structure	Time (h)	Yeild (%)
14a		5	88	***14f***		3	92
14b		5	90	***14g***		5	85
14c		3.5	92	***14h***		4	93

3.2 Cytotoxicity Test

Excel 2013 and Curve Expert 1.4 software was used for calculating the data. The values from triplicate wells were summed and the average of the values were determined. The average value of the blank wells was subtracted from the average of test wells. The inhibitory effect of each chemical was measured by the following formula: $100 - (\frac{\text{ODtest}-\text{ODblank}}{\text{ODnegative}} \times 100)$, (ODtest=Optical Density of the sample, ODblank=Optical Density of the blank, ODnegative=Optical Density of negative control).

A plot of the absorbance versus treatment values was depicted for each chemical, using Curve Expert 1.4 software. The concentration, which inhibits proliferation of 50% or 30% of the cells was considered as IC_{50}or IC_{30} value, respectively.

IC_{50} of Cisplatin was 10.4 and 24.21µg/ml for MCF-7 and MDA-MB-231 cell lines, respectively. Our compounds did not show any noticeable cytotoxic activity on MCF-7 cell line but they showed their cytotoxic activity on MDA-MB-231 cell line with an IC_{50} value equal to 120-130µg/ml. Of the tested compounds, ***14c*** was the most active with an IC_{50}=122.74 µg/ml. There was no recognizable difference between semicarbazone and thiosemicarbazone derivatives (Table 1).

Some of the synthesized isatin thiosemicarbazone derivatives, in particular compound ***14c***, have partial cytotoxic activity against the breast cancer cell lines. It was previously reported that isatin thiosemicarbazone derivatives could exhibit their antitumor activities through forming complexes with metal ions which in turn leads to inhibition of ribonuclease reductase enzyme [32]. Also binding to DNA were proposed as a probable mechanism of their cytotoxicity [33,34].

Table 4. Cytotoxic effect (IC_{50}, µg/ml) of Isatin derivatives on breast cancer cell lines

Isatin semicarbazonell carbazone	MDA-231	MCF-7
12a	223.92	218.84
12c	237.88	204.53
12d	215.28	256.91
12g	262.09	>1000
Isatin thiosemicarbazone		
14h	2116.41	>1000
14c	122.74	ND*
14g	259.68	>1000

**ND= Not Detected*

3.3 Antimicrobial Susceptibility Test

As it has been previously shown that some isatin derivatives have antibacterial and antifungal activities [35,36], we decided to test our compounds against some selected species of bacteria and fungi. We used broth microdilution method to examine the synthesized compounds against some Gram-positive and Gram-negative bacteria and also fungi.

We found that semicarbazone and thiosemicarbazone derivatives of isatin do not possess any antibacterial and antifungal activities at concentrations up to 512 µg/ml.

4. CONCLUSION

Singh et al. have reported that semicarbazone and thiosemicarbazone moieties which were linked to aminophenylbenzothiazole, had antimicrobial activities against Gram-positive and Gram-negative bacteria [37]. Our study showed that replacement of aminophenylbenzothiazole with isatin had reduced the antimicrobial activities of the compounds. In another study, Konstantinovic *et al*. showed that complexes of isatin thiocarbazone with metals such as Pd(II), Zn(II) and Hg(II) had enhanced their antimicrobial activities in comparison to their respective ligands [38]. Hence, we conclude that if we could make a complex of our synthesized compounds using a range of metals, then this may potentially improve their antimicrobial activities.

To conclude, this study showed the synthesis and *in vitro* determination of the cytotoxic and antimicrobial activities of nine new isatin semicarbazones ***(12a-i)*** and six new reported thiosemicarbazones ***(14a-c, 14f-h)***.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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