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Evaluation of Antioxidant, Antimicrobial and Anticancer activity of Thiazole Tagged Isatin Hydrazones

[1]Venkateshwarlu Eggadi, [1]Umasankar Kulandaivelu, [3]Sharvana Bhava Bandaru Sheshagiri, [2]Venkateshwar Rao Jupalli

[1]Vaagdevi College of Pharmacy, Warangal-506001, Telungana, India
[2]Department of Pharmaceutical Chemistry, Talla Padmavathi College of Pharmacy, Warangal-506009, Telungana, India

Abstract: Isatin and its derivatives is versatile lead molecule for potential bioactive agents and shows wide spectrum of activities. In this study, we evaluated antioxidant, antimicrobial and cytotoxic activity of isatin-3-[N^2-(2-benzalaminothiazol-4-yl)] hydrazone derivatives using well defined models. Antioxidant activity of the isatin derivatives (**Va-Vj**) was evaluated by using the 1, 1-diphenyl-2-picryl hydrazine radicals scavenging assay. The antimicrobial activity is evaluated by cup plate method and anticancer activity is evaluated by MTT assay against HBL-100 & HeLa cell lines. Compound **Vh** (R = 5-Cl, R^1 = OH & R^2 = OCH_3) showed good antioxidant activity with the IC_{50} of 8.09 μM. In addition **Ve** and **Vi** have showned most active antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* with a Zone of Inhibition (mm) 20, 16, 18 and 14, 12, 15 on respective organism at 100 μg/disc. The compound **Vi** have produced a good antifungal activity against *Aspergillus niger* and *Clostridium vericulata* with the zone of inhibition values of 9 and 8 mm respectively. These isatin derivatives also among the test compounds, compound **Vd** (R = 5-Cl, R^1 = OH & R^2 = OCH_3) and compound **Vh** (R = 5-Cl, R^1 = OH & R^2 = OCH_3) have shown nearly equal cytotoxic activity with IC_{50} values of 246.53 μM and 247.29 μM against HBL-100 cell lines and HeLa cell lines respectively. From the results, isatin derivatives showed powerful antioxidant activity, antimicrobial and anticancer activity may be due to the halogens substituted at 5^{th} position of isatin. The standard drugs used were ampicillin, clotrimazole, cisplatin and ascorbic acid for antibacterial, antifungal, anticancer and antioxidant respectively.

Keywords: Isatin derivatives**;** zone of inhibition; cytotoxic activity; DPPH method.

1. Introduction

Oxidative stress has been implicated as a major role in the onset and progression of a vast variety of clinical abnormalities including neurodegenerative disorders. Free radicals play an important role in many physiological and pathological conditions.[1] In general, the generation and scavenging of oxygen free radicals is balanced and any imbalance or excessive amounts of active radicals may contribute to disease development. It has been found that, free radical reactions can produce deleterious modifications in membranes, proteins, enzymes, DNA and increasing the risk of diseases.[2] Therefore, it is important to find effective scavengers of free radicals for prevention and treatment of such disorders.

Infections caused by multi-drug resistant bacteria are of major health concern worldwide. Particularly important are infections caused by the Gram-positive bacteria *Staphylococcus aureus* and species of the genus *Enterococcus*, due to increasing incidence of infections caused by these microorganisms in hospitals and communities, and their ability of developing antibiotic resistance to multiple antibiotics. Due to some serious side effects in newly introduced antibacterial agents such as semi-synthetic streptogramins quinupristin/ dalfopristin, daptomycin, the development of a diversified series of antimicrobials still remains a necessity.[3] Indole and its analogous are good pharmacophore for designing several chemotherapeutic reagents which exhibit wide spectrum of antimicrobial activities.[4]

The development of new anticancer therapeutic agents is one of the fundamental goals in medicinal chemistry. Cytotoxicity and genotoxicity of anticancer drugs to the normal cells are major problems in cancer therapy and engender the risk of inducing secondary malignancy.[5] A dose of anticancer drug sufficient to kill tumor cells is often toxic to the normal tissue and leads to many side effects, which in turn, limits its treatment efficacy. In recent years, there has been a concerned search for the discovery and development of novel selective anticancer agents, devoid of many of the unpleasant side effects of conventional anticancer agents. The synthesis of a newer class of anticancer agents is in need of time.

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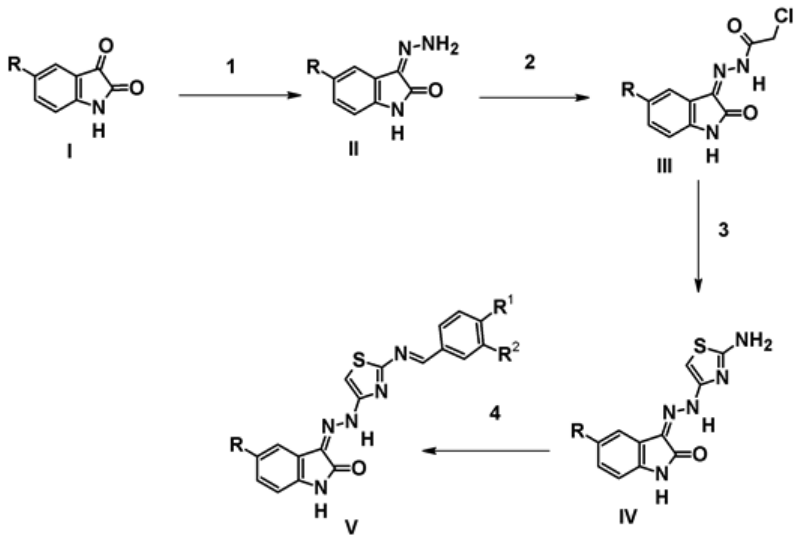
*corresponding author: VE Tel: +919848835092 ;
E-mail: eggadivenkey@gmail.com



Scheme 1: NH_2NH_2. H_2O (80 %), EtOH, Conc. H_2SO_4, reflux; 2. Chloroacetyl chloride, EtOH, reflux; 3. Thiourea, EtOH, reflux; 4. Aryl aldehyde EtOH, Conc. H_2SO_4

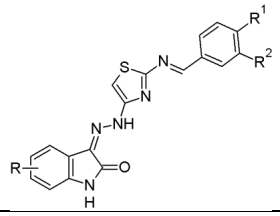
Isatin is a naturally occurring indole derivative (indole-2, 3-dione) that is found in the brain, body fluids and other tissues.[6] Isatin readily crosses the blood-brain barrier, suggesting its possible action on the CNS, and it possesses a wide range of activities such as selective inhibitor of monoamine oxidase-B[7], antipsychotic[8], anticonvulsant[9], anxiolytic[10], sedative-hypnotic[11], apoptosis[12], antimicrobial[13], anticancer and cytotoxic[14], analgesics and anti-inflammatory[15], antidiabetic[16] and diuretic activities[17]. The present investigation was to screen our isatin derivatives on their pharmacological profiles by using well-defined preclinical models. Thus, in the present study, the antimicrobial, antioxidant and cytotoxicity of the isatin derivatives were evaluated.

2. Results and Discussion

2.1. Chemistry

The final compounds **Va-Vj** were synthesized following the synthetic route outlined in Scheme 1. The respective isatin (**I**) were reacts with 99% hydrazine hydrate offered the isatin hydrazones (**II**). The isatin-3-[N^2-(chloroacetyl)] hydrazones (**III**) were prepared by a reaction of respective isatin hydrazones with chloroacetyl chloride. The condensation of chloroacetyl derivatives of isatin hydrazones with thiourea in absolute ethanol gives isatin-3-[N^2-(2-aminothiazol-4-yl)] hydrazone (**IV**). The intermediate isatin-3-[N^2-(2-aminothiazol-4-yl)] hydrazone was characterized by their spectral data. ^{1}H NMR spectrum (DMSO-d_6, δ, ppm) of the compound exhibited characteristic absorption peaks at 13.48 (s, 1H, lactam), 11.31 (s, 1H, NHCO), 6.86 to 7.91 (m, 4H, Ar-H), 6.84 (s, 1H, thiazole-H), 4.85 (s, 2H, -NH_2). IR spectrum (KBr, cm^{-1}) showed absorption frequencies at 3238 (NH_2), 3158 (NH), 1688 (C=O, lactam), 1591 (C=C, aromatic), 1551 (C=N). Mass spectrum: m/z 259 (72%), 245 (100%) and 160 (4%). Finally the isatin-3-[N^2-(2-benzalaminothiazol-4-yl)] hydrazones (**Va-Vj**) were prepared by respective isatin aminothiazolyl hydrazone were condensed with different aromatic aldehydes (Table 1). All the final isatin derivatives were freely soluble in DMSO, methanol and ethanol. However, intermediates and final compounds have been further purified by recrystallization from appropriate solvents(s) and characterized by their physical and spectral data. The representative compound in the series **Va** [isatin-3-[N^2-(2-benzalaminothiazol-4-yl)] hydrazones] was characterized by their spectral data. ^{1}H NMR spectrum (DMSO-d_6, δ, ppm) at 10.94 (s, 1H, lactam), 8.67 (s, 1H, NNH), 7.91–8.07 (m, 9H, Ar-H), 7.47 (s, 1H, N=CH), 6.97 (s, 1H, thiazole-H). Its IR spectrum (KBr, cm^{-1}) showed absorption frequencies at 3432 (NH), 3180 (NH), 1728 (C=O, lactam), 1618 (C=C, aromatic), 1545 (C=N). Mass spectrum, m/z: 347.7 (7%), 272.7 (100%) and 244.7 (25%). On the basis of the above, the other derivatives were characterized and conformed.

Table 1. Physical data of Isatin-3-[N^2-(2-benzalaminothiazol-4-yl)]hydrazones



Code	R	R^1	R^2	MF	MW
Va	H	H	H	$C_{18}H_{13}N_5OS$	347
Vb	H	Cl	H	$C_{18}H_{12}ClN_5OS$	381
Vc	H	$N(CH_3)_2$	H	$C_{20}H_{18}N_6OS$	390
Vd	H	OH	OCH_3	$C_{19}H_{15}N_5O_3S$	393
Ve	5-CH_3	Cl	H	$C_{19}H_{14}ClN_5OS$	395
Vf	5-CH_3	OH	OCH_3	$C_{20}H_{17}N_5O_3S$	407
Vg	5-CH_3	H	H	$C_{19}H_{15}N_5OS$	361
Vh	5-Cl	OH	OCH_3	$C_{19}H_{14}ClN_5O_3S$	427
Vi	5-Cl	Cl	H	$C_{18}H_{11}Cl_2N_5OS$	416
Vj	5-NO_2	OH	OCH_3	$C_{19}H_{14}N_6O_5S$	438

2.2. Antimicrobial Activity

Antibacterial Activity: The compounds **Va-Vj** and ampicillin were assessed for their *In vitro* antibacterial activity against two gram positive (*B. subtilis* and *S. aureus*) and two gram negative (*E. coli* and *P. vulgaris*). Results of antibacterial activity were plate method indicated that, the compound **Ve** and **Vi** showed most antibacterial activity against two gram positive bacteria

Table 2. Antimicrobial bacterial activity of isatin (**Va-Vj**) derivatives and *Zone of Inhibition* (in mm) at 100 µg/mL.

Code	Antibacterial Activity				Antifungal Activity			
	B. subtilis	***S. aureus***	***E. coli***	***P. vulgaris***	***A. niger***	***C. verticulata***	***F. oxysporum***	***A. flavus***
Va	10	--	--	--	8	7	--	--
Vb	12	10	18	12	8	6	--	--
Vc	11	9	--	--	5	5	--	--
Vd	10	8	--	--	5	4	--	--
Ve	20	16	18	--	9	7	--	--
Vf	12	--	--	--	5	--	--	--
Vg	12	10	--	--	5	--	--	--
Vh	12	--	--	--	5	5	--	--
Vi	14	12	15	--	9	8	--	--
Vj	12	10	9	--	5	4	--	--
***Ampicillin**	22	20	18	17	---	---	---	---
***Clotrimazole**	---	---	---	---	22	19	20	22

* 10 µg/mL concentration

and one gram negative bacteria and **Vj** also active but less effective than the above compounds. The moderate activity produced by **Vb** against two gram positive and two gram negative bacteria and mild activity was produced by **Vc**, **Vd** and **Vg** against two gram positive bacteria only. The **Va**, **Vf** and **Vh** shown antibacterial activity against *B. subtilis* only.

Antifungal Activity: The antifungal activity of isatin derivatives compared with standard clotrimazole was assessed against four fungal organisms. The synthesized isatin (**Va-Vj**) derivatives showed variation in the level of activity against the four human pathogenic fungal strains tested (Table 2). The compound **Vi** have produced a maximum zone of inhibition and **Vj** show minimum zone of inhibition (in mm) against *A. niger* and *C. vericulata* at 100 µg/mL, concentration. The compounds **Va, Vb, Vc, Vd, Ve, Vf, Vg** and **Vh** shown mild to moderate antifungal activity against *A. niger* and *C. vericulata* organisms. No compound showed antifungal activity against *F. oxysporum, A. flavus*.

The previous literature revealed that, the introduction of electron withdrawing groups at positions 5, 6 and 7 greatly increased activity of isatin with substitution at the 5th position being most favorable.[22] This is not surprising, as C5 substitution has previously been associated with increased biological activity for a range of indole-based compounds[23] and the presence of the substituted aromatic ring at 3rd position has been reported to be associated with antimicrobial properties [24, 25]. The various substituent at 3rd position of the isatin which were reported, were various substituted phenyl ring moieties,[26,27] heterocyclic rings[28-30] and aliphatic system[31]. A comparison of antimicrobial action in a series of halogenated compounds revealed that, substitution at the 5th position of isatin with chlorine, bromine or fluorine produced more active compounds.[32, 33] Electron withdrawing substituent and the presence of nitro group at 5th position may also modulate efficacy as an antimicrobial agent.

From *in vitro* antibacterial and antifungal data, it was confirmed that, the compounds containing strong electron withdrawing (chlorine group: **Vi**) and electron donating (methyl: **Ve**) groups at 5th position and chlorine substitution on the aromatic ring (polar group) exhibited significant activity. But overall, all the compounds have displayed antibacterial and antifungal activity. The presence of electron withdrawing substituents on the phenyl ring at 3rd position and also nitro group at 5th position would be expected to increase the lipophilic character of the molecule, facilitating transport across the microorganism cell membrane and increasing antimicrobial activity.[22] Compound **Vj** also expected to increase the lipophilic character of the molecule, facilitating transport across the microorganism cell membrane and increasing antimicrobial activity. Our finding is supported by substitution[34,35] at 5th and 3rd position of isatin derivatives showed antibacterial and antifungal activity. The antimicrobial activity our isatin derivatives are also showing more potent antimicrobial activity than the new isatin containing thiazole derivatives[36].

2.3. Cytotoxic Activity

The results of the evaluation have been viewed by taking cisplatin (IC_{50} 25 µM) as the standard one. Table 3 presenting the data on cytotoxicity of ten isatin-3-[N^2-(2-benzalaminothiazol-4-yl)] hydrazones reveals that, they have exhibited moderate activity against HBL-100 & HeLa cell lines. Among the test compounds, compound **Vd** (R = 5-Cl, R^1 = OH & R^2 = OCH_3) and compound **Vh** (R = 5-Cl, R^1 = OH & R^2 = OCH_3) have shown cytotoxic activity with IC_{50} values of 246.53 µM and 247.29 µM against HBL-100 cell lines and HeLa cell lines respectively but compared with cisplatin exhibited less cytotoxic activity. Other compounds have not shown any activity against both the cell lines employed.

Among the synthesized 2-indolinones, compounds with halogen atom (electron withdrawing groups) at C5 position showed the most potent activity than other synthesized compounds. This is not surprising, as C5 substitution has previously been associated with increased biological activity for a range of indole-based compounds[37,38]. Previous studies have shown that, strong electronegative atom substitution such as chloro/bromo at the C5 position of the aromatic ring increases the liphophilicity of molecules and is responsible for enhanced cytotoxicity in MTT model.[39]

2.4. *In vitro* Antioxidant Activity by DPPH

The antioxidant activity of isatin derivatives was shown in Table 3. It could be observed from the table that, the test compounds have shown potent/mild/moderate antioxidant activity with IC_{50} values in the range of 8.09 to 19.61 µM. Among the compounds tested, compound Vh (R = 5-Cl, R^1 = OH & R^2 = OCH_3) has shown potent free radical scavenging activity. Compounds **Vj** and **Vd** with IC_{50} values of 8.12 and 9.42 µM has also found to be potent antioxidant activity where, as the rest of the compounds have shown moderate (**Vf**; IC_{50} = 10.12) to

mild (**Va-Vc**, **Ve-Vg** & **Vi**) free radical scavenging activity compared to standard reference ascorbic acid (IC_{50} value is 5.87μM). The structure activity relationship study showed that, the antioxidant activity of these isatin derivatives could be attributed to electron donating nature of the substituents like ⁻OH, ⁻CH_3 and Cl^-, reduce free radical DPPH and prevented the damage of cells[18]. The 3H-Spiro [1, 3-benzothiazole-2, 3'-indol]-2' (1'H) – one[19] and novel bis-isatin carbohydrazone derivatives showed stronger antioxidant activity.[20] This antioxidant activity is mainly due to one or more the groups like ⁻OH, ⁻CH_3 and Cl^-, since they are known to be good hydrogen donors.[21] Our compounds showed better antioxidant activity than other derivatives may be the same hydrogen donors.

Table 3. Cytotoxic and Antioxidant activities of compounds **Va-Vj**

Code	IC_{50} (mM)		
	HBL-100	HeLa	DPPH
Va	NA	NA	19.61
Vb	297.36	265.43	19.51
Vc	NA	NA	18.12
Vd	246.53	NA	9.42
Ve	324.36	297.62	18.58
Vf	316.98	NA	10.12
Vg	NA	NA	18.75
Vh	281.76	247.29	8.09
Vi	331.20	276.79	14.69
Vj	307.29	NA	8.12
Cisplatin	25.00	25.00	---
Ascorbic acid	---	---	5.87

3. Experimental

***Materials and Methods*:** All the reagents and chemicals were obtained commercially from Merck, Spectrochem or CDH, India. Purified solvents of reagent grade were purified, dried by standard procedure. Monitoring of reactions and homogeneity of the intermediate and final compounds were carried out by thin layer chromatography on silica gel plates using n-hexane-ethyl acetate as mobile phase and visualized in either iodine or UV chambers. Melting points were determined using Thermonik Melting Point Apparatus (Campbell electronics, India) by open capillary method and are uncorrected. Infrared (IR) spectra were taken on a FT (IR Spectrophotometer IR (Prestige 21 (Shimatzu Corporation, Japan) from 400 cm^{-1} using KBr discs. ^1H (NMR spectra were recorded at 400 MHz in DMSO (d6 using a Bruker Avance 400 instrument (Bruker Instruments Inc., USA). *Test organisms: Bacillus subtilis* (NCIM 2921) and *Staphylococcus aureus* (NCIM 2079) and two gram negative bacteria viz., *Escherichia coli* (NCIM 2068) and *Proteus vulgaris* (NCIM 2027), *Aspergillus niger* (NCIM 596), *Aspergillus flavus* (NCIM 555), *Fusarium oxysporum* (NCIM 1072) and *Cunninghamella verticulata* (NCIM 1185).

3.1. Chemistry

Synthesis of isatin derivatives (**Va–Vj**).

3.1.1. General procedure for synthesis of isatin hydrazones.[40]

3.1.2. General procedure for synthesis of isatin-3-[N^2-(chloroacetyl)] hydrazones.[41]

3.1.3. General procedure for synthesis of isatin-3-[N^2-(2-aminothiazol-4-yl)] hydrazones.[42.]

3.1.4. Synthesis of isatin-3-[N^2-(2-benzalaminothiazol-4-yl)] hydrazones.[9,16,17]

3.2. *In vitro* Antioxidant Activity by DPPH

DPPH (1, 1-diphenyl-2-picryl hydrazine) free radical scavenging capabilities of isatin (**Va–Vj**) were evaluated by the method of Blois.[43] Briefly, to the 0.1 ml of different concentrations of test compounds, 2.5 ml of methanol and 0.5 ml of 0.2 mM DPPH solutions were added and mixed thoroughly and the absorbance was read at 517 nm against blank samples. Ascorbic acid was used as a reference standard. The IC_{50} values were calculated and it is the concentration of sample required to scavenge 50% of DPPH free radicals.

3.3. Antimicrobial testing

The isatins were tested for their antimicrobial activity using disc diffusion method. Bacterial species were sub-cultured on nutrient agar medium and fungal species on potato dextrose agar medium, which were then incubated at 37°C for 24 h and 27°C for 48 h respectively. Test substances dilution was made with dimethylformamide itself to obtain a solution of 100 μg/mL concentration were impregnated on sterile discs. Ampicillin and Clotrimazole were used as positive controls. The disc impregnated with ethyl acetate was used as negative control. The discs were placed on the surface of the nutrient agar for bacteria and incubated at 37 °C for 24 h, and on the surface of the potato dextrose agar for fungi and incubated at 27 °C for 48 h. Inhibition zones were calculated as the difference between disc diameter (6 mm) and the diameters of inhibition [44].

3.4. Cytotoxic Activity

Anticancer activity of the sample was measured using 3-(4, 5–dimethyl thiazol–2yl)–2, 5–diphenyl tetrazolium bromide (MTT) assay.[45] The monolayer culture of Hep2 cells at a concentration of 10 cells/mL/well was seeded in 24 well titre plates. The cells were permitted to adhere for 24 h, and the growth medium (MEM) removed using micropipette and the monolayer of cells washed twice with MEM without FBS to remove dead cells and excess FBS 1 mL of medium (without FBS) containing different dilution of drugs were added in respective wells; 200 μL of MTT (5 mg/mL in PBS) were added to each well, and the cells incubated for a further 6–7 h in 5% CO_2 incubator. After removal of the medium, 1 mL of DMSO was added to each well. Absorbance was recorded at the wavelength of 570 nm.

Conclusion: From the results, it can be concluded that isatin derivatives have antioxidant, antimicrobial and cytotoxic activities.

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