Arabian Journal of Chemistry (2015) xxx, xxx–xxx

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Synthesis, characterization and cytotoxic investigations of novel bis(indole) analogues besides antimicrobial study

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Received 27 March 2015; accepted 23 May 2015

KEYWORDS

Indole-3-acylhydrazides;
Indole-3-carboxaldehyde;
N-prenylated indole-3-carboxaldehyde;
Bis(indole) analogues;
Antimicrobial activity;
Anticancer activity

Abstract Two series of novel bis(indole) analogues *viz*., *N*′-((5-substituted-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)alkanehydrazides **(7a–f)** and *N*′-((5-substituted-1-(3-methylbut-2-enyl)-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)acetohydrazide **(8a–f)** were synthesized and characterized by spectral analysis. The target molecules were screened for their antimicrobial, anticancer activities and structure and activity relationship (SAR) was investigated. Compounds **7a**, **7c** and **8a** were found to be active in antimicrobial screening. Anticancer screening reveals that Compound **7c** was active against HeLa cell line with an IC_{50} of 43.1 μM and compound **7d** was found to be interesting candidate with an IC_{50} of 26.0 and 30.2 μM against Colo-205 and Hep G2 cell lines respectively.

1. Introduction

The indole skeleton is the interesting class of heterocyclic compounds which has been stimulated by both their unique chemical structure and the wide range of biological properties (Barraja et al., 2011). In particular, *C*-3-substituted indoles are important building blocks for the synthesis of many biologically active compounds which possess antimalarial (Agarwal et al., 2005), inhibitors of HIV-1 (Meanwell et al., 2009), antimicrobial (Lakshmi et al., 2010; Reddy et al., 2011), antioxidant (Lakshmi et al., 2010), anticancer (Lakshmi et al., 2010), cytotoxic (Gu et al., 1999), inhibitors

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Peer review under responsibility of King Saud University.

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http://dx.doi.org/10.1016/j.arabjc.2015.05.015

of hepatitis C virus (Jin et al., 2014), anti-diabetic (Rajesh et al., 2007), and neuro protective (Mohareb et al., 2011) activities. On the other hand, *N*-1 and *C*-3-substituted indole derivatives have been found to play an important role in many biologically active compounds especially with anti-inflammatory (Hall et al., 2008; Singh et al., 2008), anticancer (Singh et al., 2008; Madadi et al., 2014), anti-nociceptive (Adam et al., 2010) and antipsychotic (Madadi et al., 2013) activities. Marine indole alkaloids have emerged as an important structural class exhibiting antiviral, antimicrobial and antitumor activity (Dembitsky et al., 2005; Bao et al., 2005; Oh et al., 2005, 2006). Among these biologically active scaffolds, bis(indole) alkaloids with a broad spectrum of biological activities are being discovered from marine invertebrates such as sponges, bryozoans, coelenterates and tunicates (Yang and Cordell, 1997; Tsuda et al., 2005; Shin et al., 1999; Ryan and Drennan, 2009; Diana et al., 2010). Dragmacidin D and topsentins and their analogues exhibited activities *in vitro* and *in vivo* against P388 murine and human tumor cells (Wright et al., 1992; Tsujii and Rinehart, 1988). Coscinamides A–C were isolated from *Coscinoderma* sps a marine sponge and exhibit cycloprotection against HIV in the NCI assay (Bokesch et al., 2000; Bacher et al., 2001). Asterriquinone B1 was isolated from *Aspergillus terreus* which is a bis(indole) derivative with 2,5-dimethoxy benzoquinone as a spacer and is effective in inhibiting the growth of several transplantable animal tumors *in vivo* (Liu et al., 1999). Nortopsentins A–C were isolated from a marine sponge *Spongosorites ruetzleri* having a 2,4-bis(3-indolyl)imidazole skeleton and exhibited *in vitro* cytotoxicity against P388 cell line (Alvarez and Salas, 1991; Sakem and Sun, 1991; Kawasaki et al., 1996). The structures of the naturally occurring bis(indole) molecules are presented in Fig. 1.

A great limitation in the use of the reservoir of marine organisms for therapy is that only very small amounts of the biologically active substances are isolated from the natural material. Due to the interesting biological activities, different analogues of the marine nortopsentins have been synthesized in which the imidazole moiety of the nortopsentin was replaced by five- or six-membered heterocyclic rings such as thiazole (Parrino et al., 2015; Carbone et al., 2015, 2013; Carbone et al., 2014), pyrazinone (Jiang and Gu, 2000), pyrimidines, pyrazines (Jiang et al., 2001), thiophenes (Diana et al., 2007a), pyridines (Xiong et al., 2001), pyrazoles (Diana et al., 2007b) and 1,2,4-thiadiazoles (Kumar et al., 2011) which were reported for their cytotoxicity against several cancer cell lines.

The acylhydrazones [(hydrazide–hydrazone (—CO—NH—N=CH—)] are classified as important functional group which exhibit significant role in anti-inflammatory (Todeschini et al., 1998; Radhwan et al., 2007; Gaston et al., 1996; Almasirad et al., 2005; Murineddu et al., 2001), antimalarial (Gemma et al., 2006), antimicrobial (Masunari and Tavares, 2007; Kuçukguzel et al., 2002), anticonvulsant (Dimmock et al., 2000; Ragavendran et al., 2007), antitumor (Bernhardt et al., 2009; Whitnall et al., 2006; Vicini et al., 2006; Terzioglu and Gursoy, 2003; Abadi et al., 2003), analgesic (Lima et al., 2000), antiplatelet (Silva et al., 2004) and antitubercular (Vavrikova et al., 2011a; Manvar et al., 2008; Vavrikova et al., 2011b) activities. In recent years, researchers have turned their interest by replacing the central heterocyclic spacer of the bis(indole) alkaloids with biologically active long chain linkers such as glyoxylamide derivatives (Gupta et al., 2007), and acylhydrazones and their cytotoxic activity was evaluated against human cancer cell lines (Kumar et al., 2012). Keeping the literature survey in view, we plan to synthesize the novel bis(indole) analogues by replacing the central spacer (heterocyclic and long chain) with alkane hydrazone spacer. All the synthesized compounds were screened for antimicrobial and anticancer activities.

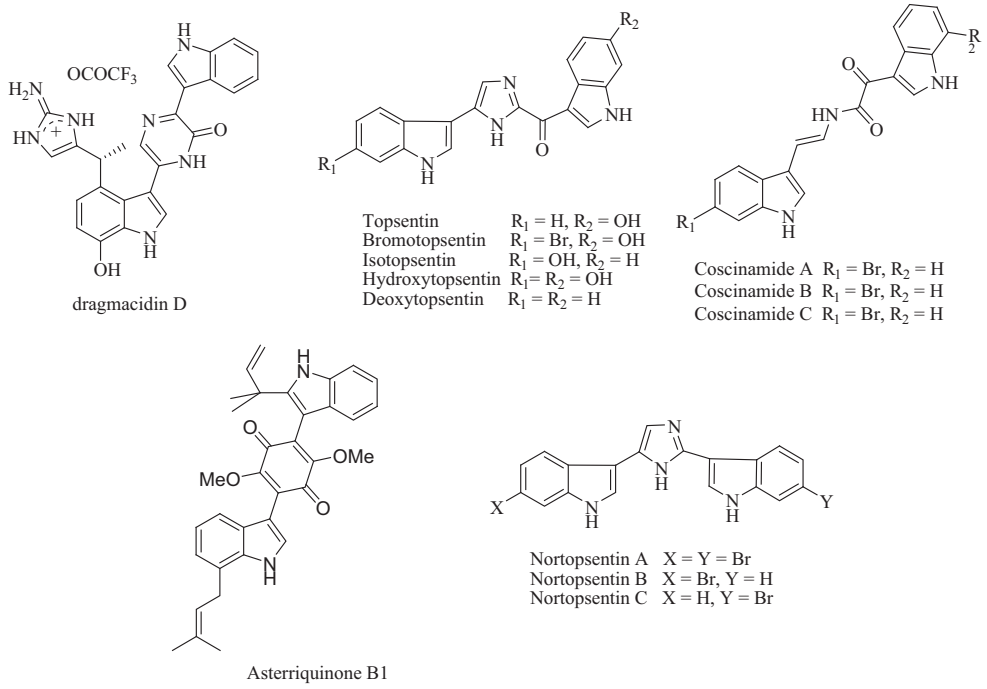


Figure 1 Naturally occurring bis(indole) molecules.

2. Results and discussion

2.1. Chemistry

Synthesis of two series of novel bis(indole) analogues *viz*., *N*′-((5-substituted-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl) alkanehydrazides **(7a–f)** and *N*′-((5-substituted-1-(3-methyl but-2-enyl)-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)aceto-hydrazide **(8a–f)** has been accomplished by adopting known synthetic routes. Indole-3-carboxylic acids namely, indole-3-acetic **(1a)**, indole-3-propionic **(1b)** and indole-3-butyric acid **(1c)** were selected as precursors, which on reflux with 4 N HCl in the presence of MeOH for 2–3 h to yield Indole-3-carboxylic methyl esters **(2a–c)**, and these methyl esters on reaction with hydrazine hydrate in the presence of MeOH under refluxing conditions for 2–3 h yield three different acyl-hydrazides chain linkers **(3a–c)** (Scheme 1).

Indoles **(4)** were formylated by Vilsmeier-Haack reaction to yield 3-Formyl Indoles **(5)**, that were prenylated by the reaction with Prenyl bromide in the presence of NaH, DMF at 0 °C for 15 min to yield *N*-prenylated 3-formyl indoles **(6)** (Scheme 2).

The series of *N*′-((5-substituted-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)alkanehydrazides **(7a–f)** were achieved by the condensation of indole-3-substituted acylhydrazides **(3a–c)** with 5-substituted-1*H*-indole-3-carbaldehyde **(5a,b)** in the presence of EtOH and catalytic amount of Glac. acetic acid under refluxing conditions for 20–45 min. Similarly the condensation of indole-3-substituted acylhydrazides **(3a–c)** with 5-substituted-1-(3-methylbut-2-enyl)-1*H*-indole-3-carbaldehyde **(6a,b)** in the presence of EtOH and 1–2 drops of Glac. acetic acid under refluxing conditions for 20–45 min yields novel *N*′-((5-substituted-1-(3-methylbut-2-enyl)-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)acetohydrazide **(8a–f)** (Scheme 3).

All the synthesized compounds were well characterized by advanced spectroscopic techniques (^{1}H, ^{13}C, Mass, IR and elemental analysis).

2.2. Bio evaluation

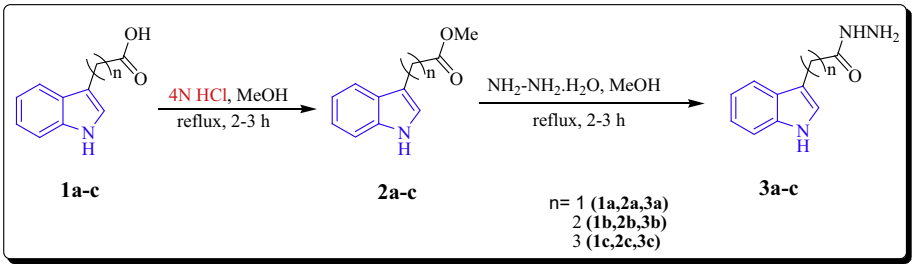
2.2.1. Antimicrobial activity

The newly synthesized and well characterized compounds **(7a–f)** and **(8a–f)** were screened for *in vitro* antibacterial activity against Gram positive bacterium (*Bacillus subtilis*), Gram negative bacteria (*Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*) and antifungal activity against *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans* using agar well diffusion assay and zones of inhibition of the test compounds were expressed in mm.

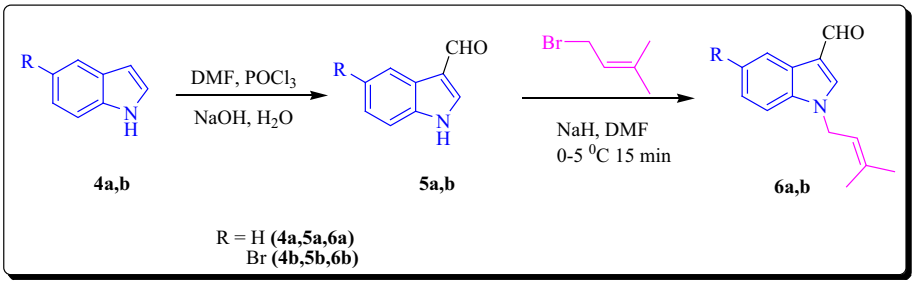
2.2.1.1. Antibacterial activity. The antibacterial activity of the test compounds **(7a–f)** and **(8a–f)** was compared with standard "ciprofloxacin" and the results are presented in Table 1.

As indicated in Table 1, most of the synthesized compounds generally showed potent antibacterial activity against all the tested bacterial strains. Compound **7a** shows good antibacterial activity against *B. subtilis*, *K. pneumonia* and *P. aeruginosa* with zones of inhibition of 22, 21 and 24 mm respectively. In case of *E. coli*, compound **7c** is more active with a zone of inhibition of 21 mm. On bacterial strains, compound **7a** is potent against *P. auregenosa* with a zone of 24 mm. Considering the activity of compounds **8**, with the exception of compound **8e**, showed a decreased bactericidal activity suggesting that the presence of the hydrophobic prenyl group does not improve the biological activity.

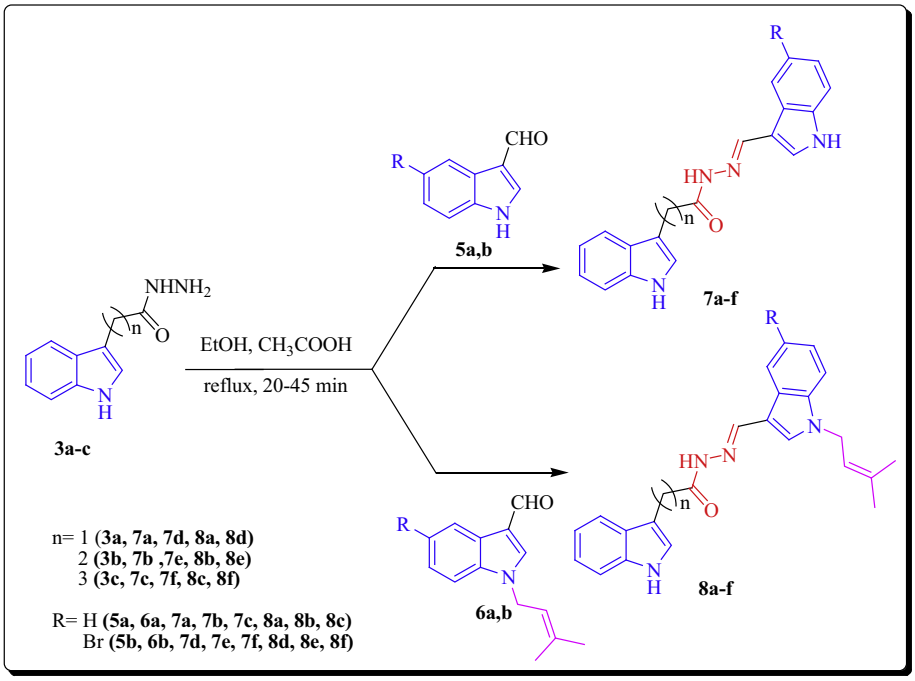
2.2.1.2. Antifungal activity. The antifungal activity of tested compounds **(7a–f)** and **(8a–f)** was compared with ketoconazole (standard) and the results are tabulated in Table 2.



Scheme 1 Synthesis of indole 3-substituted acylhydrazides **(3a-c)**.



Scheme 2 Synthesis of 5-substituted-1*H*-indole-3-carbaldehyde **(5a,b)** and 5-substituted-1-(3-methylbut-2-enyl)-1*H*-indole-3-carbaldehyde **(6a,b)**.



Scheme 3 Synthesis of *N*′-((5-substituted-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)alkanehydrazides **(7a–f)** and *N*′-((5-substituted-1-(3-methylbut-2-enyl)-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)acetohydrazide **(8a–f)**.

Table 1 Zones of inhibition (mm)[a] of compounds **7a–f** and **8a–f** against tested bacterial strains.

Entry	Compound	Bacterial strains			
		B. subtilis	*E. coli*	*K. pneumonia*	*P. auregenosa*
1	**7a**	22	19	21	24
2	**7b**	19	20	19	16
3	**7c**	18	21	20	20
4	**7d**	20	20	18	20
5	**7e**	19	14	15	23
6	**7f**	21	14	19	16
7	**8a**	18	16	20	19
8	**8b**	18	16	19	21
9	**8c**	16	14	14	18
10	**8d**	19	20	19	18
11	**8e**	20	17	20	20
12	**8f**	20	14	20	16
13	Standard[a]	24	27	22	26

Ciprofloxacin was used as standard.
[a] 100 μg/mL of compound in each well.

Table 2 Zones of inhibition (mm)[a] of compounds **7a–f** and **8a–f** against tested fungal strains.

Entry	Compound	*A. flavus*	*A. niger*	*C. albicans*
1	**7a**	16	18	14
2	**7b**	13	14	12
3	**7c**	12	16	13
4	**7d**	12	14	13
5	**7e**	15	12	14
6	**7f**	15	16	15
7	**8a**	14	13	16
8	**8b**	10	12	15
9	**8c**	12	10	9
10	**8d**	10	11	14
11	**8e**	9	10	12
12	**8f**	10	12	9
13	Standard[a]	21	23	19

Ketoconazole was used as standard.
[a] 100 μg/mL of compound in each well.

The results tabulated in Table 2 infer that compound **7a** was found to be interesting molecule with good antifungal activity against *A. flavus* and *A. niger* with zone of inhibition of 16 and 18 mm respectively. Compound **8a** is potent against *C. albicans* with a zone of 16 mm. Compounds **7a** and **8a** are acetohydrazide analogues, in which compound **(7a)** belongs to unprenylated series, whereas compound **8a** comes under prenylated series.

2.2.2. In vitro anti cancer activity

The anti-proliferative/cytotoxic activities of the newly synthesized bis(indole) analogues **(7a–f)** and **(8a–f)** were evaluated against three different types of human cancer cell lines, *viz*., human colorectal cancer **(Colo-205)**, human hepatocellular liver carcinoma **(Hep G2)**, and human cervical cancer **(HeLa)** using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay, according to the method of Mossman (Mosmann, 1983). The cytotoxic potency of the compounds varied between the cell lines suggesting a structural property of these compounds as possible determinants of their biological activity. The cytotoxic investigations were evaluated at IICT, Hyderabad, in association with Dr. J.V. Rao, Scientist, Biology division.

It is evident from the results that all the target compounds have shown significant cytotoxic activity against all the tested cell lines (Table 3). However, **8e** and **8f** are found to be inactive

against Colo-205 and Hep G2 cell lines at 300 μM concentration. Etoposide (a standard drug molecule) was used as a positive control in these assays and the IC_{50} values were recorded for Colo-205, Hep G2, and HeLa as 0.45, 4.75 and 23.3 μM respectively. Among the derivatives, about 45–55% of the test compounds have shown effective inhibition of growth in all the cell lines at less than 100 μM concentration.

Compound **7c** exhibited an excellent anti-proliferative activity against all the cell lines (IC_{50} values are less than 50 μM conc.) followed by **7d**, **8a**, **7e** and **7f**. Furthermore, results indicated based on the IC_{50} values, the Hep G2 cell lines appear to be more sensitive than Colo-205 and HeLa cell lines. Nevertheless, the order of relative sensitivity among the cell lines differs, when compared to the activity of etoposide. All the bis(indole) analogues **(7a–f)** and **(8a–f)** were found to be effective anti-proliferative agents against HeLa cells, with 2 to 10-fold less active than the IC_{50} value of etoposide. Similarly, the analogues **7c**, **7d**, **7e**, **7f** and **8a**, have exhibited potent anti-proliferative activity against Hep G2 cell lines. Likewise, these analogues (excluding **7f)** also showed moderate cytotoxicity against Colo-205 cell lines.

2.2.3. Structure Activity Relationship (SAR)

The compounds, 5-bromo substituted aceto and propanehydrazides **(7d** and **7e)** were found to be active against all the three cell lines than the corresponding unsubstituted aceto and propanehydrazides **(7a** and **7b)** which indicates that hydrophobic bromine increases the activity. But there is an exception from the above hypothesis that the presence of bromine on position 5 in butanehydrazide **(7f)** slightly decreases the cytotoxicity than its corresponding unsubstituted butanehydrazide **(7c)**.

Introduction of prenyl group increases the activity of **8a** against Colo-205, **8a** and **8b** against Hep-G2 and **8a**, **8b** and **8f** against HeLa cell lines. In prenylated series unsubstituted aceto and propanehydrazides (**8a** and **8b**) exhibited potent cytotoxicity than their corresponding 5-bromo substituted aceto and propanehydrazides **(8d** and **8e)** which indicates that hydrophobic bromine decreases the activity. In contrast, bromine on 5th position in butanehydrazide **(8f)** increases the cytotoxicity than its corresponding 5-bromo substituted aceto and propanehydrazides **(8d** and **8e)**. The relative cytotoxicity of prenylated series **(8a–f)** against the three cell lines is in the order of HeLa > Hep-G2 > Colo-205.

In unprenylated series **(7a–f)**, 5-bromo substituted aceto and propanehydrazides **(7d** and **7e)** and unsubstituted butanehydrazide **(7c)** exhibited potent cytotoxicity. In contrast the prenyl moiety alters the activity that unsubstituted aceto and propanehydrazides **(8d** and **8e)** and 5-bromo substituted butanehydrazide **(8f)** were the interesting candidates in prenylated series **(8a–f)**.

Table 3 *In vitro* cytotoxicity of bis (indole) derivatives **(7a–f)** and **(8a–f)** against Colo-205, Hep G2 and HeLa human cancer cells by MTT assay expressed in IC_{50} (μM).

Entry	Compound	[a]IC_{50} (μM)		
		Colo-205	Hep G2	HeLa
1	**7a**	58.1	247	232
2	**7b**	136	274	248
3	**7c**	35.6	34.7	43.1
4	**7d**	26.0	30.2	61.4
5	**7e**	34.9	37.5	101
6	**7f**	88.7	68.1	65.2
7	**8a**	42.1	34.3	64.3
8	**8b**	216	153	229
9	**8c**	177	103	157
10	**8d**	142	154	121
11	**8e**	NA	NA	187
12	**8f**	NA	NA	62.7
13	Standard[A]	0.45	4.75	23.3

NA indicates that the compound is inactive at 300 μM.

[a] IC_{50} is defined as the concentration, which results in a 50% decrease in cell number as compared with that of the control cultures in the absence of an inhibitor and was calculated using the respective regression analysis. The values represent the mean of three individual observations.

[A] Etoposide was used as positive control.

3. Conclusion

Among the novel series *N*′-((5-substituted-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)alkanehydrazides **(7a–f)** and *N*′-((5-substituted-1-(3-methylbut-2-enyl)-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)acetohydrazide **(8a–f)** compounds **7a** (*B. subtilis*, *K. pneumonia* and *P. aeruginosa*) and **7c** (*E. coli*) are potent for antibacterial activity and compounds **7a** (*Aspergillus sps*) and **8a** (*C. albicans*) exhibit good antifungal activity.

Anticancer screening reveals that compound **(7c)** was active against HeLa cell line with an IC_{50} of 43.1 μM and compound **(7d)** was found to be interesting candidate with an IC_{50} of 26.0 and 30.2 μM against Colo-205 and Hep G2 cell lines.

4. Experimental

4.1. General

All chemical reagents were obtained from Sigma Aldrich and were used without further purification. Melting points were determined in open capillaries and are uncorrected. Infrared (IR) spectra were recorded using FT-IR Bruker Alpha spectrometer, ESI-Mass spectra were recorded on Finnigan Matt Mass spectrometer and NMR (^1H and ^{13}C) spectra were recorded with a Bruker Ascend-400 and 200 MHz and Bruker Ascend-400 and Jeol JNM EX-90 spectrometer.

4.2. General procedure for preparation of indole-3-carboxylates *(2a–c)*

The indole-3-carboxylic acids **(1a–c)** (10 mmol) were esterified in a classical manner with methanol and 4 N HCl under reflux for 2–3 h. After completion, the reaction mixture was cooled and methanol was evaporated under reduced pressure. Then the residue was poured onto crushed ice and treated with 10% aq. $NaHCO_3$ solution dropwise till the reaction mixture became slightly basic in nature. The mixture was extracted with EtOAc and the organic layer was dried over anhydrous Na_2SO_4 and filtered and the solvent was evaporated under reduced pressure to get crude indole-3-carboxylates **(2a–c)**. Recrystallization of the crude products was carried in ethanol.

4.2.1. Methyl 2-(1H-indol-3-yl)acetate ***(2a)***

Yield: 92%., Brown viscous liquid., ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.75 (s, 3H), 3.84 (s, 2H), 7.08 (s, 1H), 7.10 (t, J = 7.2 Hz, 1H), 7.18 (t, J = 7.6 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.59 (d, J = 7.6 Hz, 1H), 8.22 (s, 1H)., ^{13}C NMR ($CDCl_3$, 22.4 MHz): δ 31.0, 51.9, 108.1, 111.1, 118.6, 119.5, 122.0, 123.2, 127.1, 136.1, 172.6. ESI-MS: m/z 212.06 $[M+Na]^+$, IR (KBr, cm^{-1}): 1221, 1732, 3335., Anal. Calcd. for $C_{11}H_{11}NO_2$: C, 69.83, H, 5.86, N, 7.40%, Found: C, 69.82, H, 5.90, N, 7.39%.

4.2.2. Methyl 3-(1H-indol-3-yl)propanoate ***(2b)***

Yield: 90%, Pale yellow solid, Mp: 50 °C, ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.42 (t, J = 7.6 Hz, 2H), 3.89 (t, J = 7.6 Hz, 2H), 3.69 (s, 3H), 7.01 (s, 1H), 7.14 (t, J = 7.2 Hz, 1H), 7.21 (t, J = 7.6 Hz, 1H), 7.27 (d, J = 8 Hz, 1H), 7.65 (d, J = 8 Hz, 1H), 7.99 (s, 1H). ^{13}C NMR ($CDCl_3$, 22.4 MHz): δ 20.1, 34.3, 50.9, 111.0, 113.2, 117.8, 118.1, 121.4, 121.5, 126.6, 136.1, 173.0. ESI-MS: m/z 204 $[M+H]^+$. IR (KBr, cm^{-1}): 1240, 1724, 3329. Anal. Calcd. for $C_{12}H_{13}NO_2$: C, 70.92, H, 6.45, N, 6.89%, Found: C, 70.90, H, 6.47, N, 6.86%.

4.2.3. Methyl 4-(1H-indol-3-yl)butanoate ***(2c)***

Yield: 93%., Pale cream fluffy flakes, Mp: 68 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.01–2.08 (m, 2H), 2.39 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 7.6 Hz, 2H), 3.65 (s, 3H), 6.98 (s, 1H), 7.10 (t, J = 7.2 Hz, 1H), 7.18 (t, J = 7.6 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.59 (d, J = 7.6 Hz, 1H), 7.94 (s, 1H)., ^{13}C NMR ($CDCl_3$, 22.4 MHz): δ 24.4, 25.2, 33.6, 51.4, 111.0, 115.4, 118.7, 119.1, 121.4, 121.8, 127.3, 136.3, 174.1., ESI-MS: m/z 240.09 $[M+Na]^+$., IR (KBr, cm^{-1}): 1248, 1714, 3336., Anal. Calcd. for $C_{13}H_{15}NO_2$: C, 71.87, H, 6.96, N, 6.45%, Found: C, 71.86, H, 6.99, N, 6.44%.

4.3. General procedure for preparation of indole-3-carboxylic acid hydrazides ***(3a–c)***: (Rapolu et al., 2013)

4.4. General procedure for the synthesis of 5-substituted 1H-indole-3-carbaldehyde ***(5a,b)***: *(James and Snyder, 1959; Choppara et al., 2015)*

To a solution of substituted indoles **(4a,b)** (42.6 mmol) in dry DMF (187.4 mmol) in an ice-salt bath, POCl3 (47.1 mmol) is subsequently added with stirring over a period of 30 min. After completion of addition, raise the temperature to 40 °C and stir the syrup for 1.5 h at that temperature. At the end of the reaction (as indicated by TLC) 25 g crushed ice was added to the reaction mixture. The obtained solution is transferred into 250 mL RB flask and added with NaOH (470 mmol) dissolved in 50 mL water with constant stirring and the resultant suspension is heated rapidly to the boiling point and allowed to cool to room temperature, after which it is placed in refrigerator overnight. The precipitate was filtered off and washed thrice with 100 mL water, yielding 5-substituted 1*H*-indole-3-carbaldehyde **(5a,b)**.

4.4.1. 1H-indole-3-carbaldehyde ***(5a)***: *(James and Snyder, 1959; Choppara et al., 2015)*

Yield: 92%., Brownish yellow solid., Mp: 196–198 °C., ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.14 (t, J = 7.2 Hz, 1H), 7.22 (t, J = 7.2 Hz, 1H), 7.34 (d, J = 8 Hz, 1H), 7.52 (s, 1H), 7.62 (d, J = 8 Hz, 1H), 8.12 (s, 1H), 9.52 (s, 1H). ^{13}C NMR (DMSO-d_6, 22.4 MHz): δ 111.4, 118.0, 119.4, 120.5, 122.4, 127.7, 131.82, 137.2, 182.7. ESI-MS: m/z 146.20 $[M+H]^+$. IR (KBr, cm^{-1}): 1229, 1632, 3442. Anal. Calcd. for C_9H_7NO: C, 74.47, H, 4.86, N, 9.65%, Found: C, 74.44, H, 4.91, N, 9.64%.

4.4.2. 5-bromo-1H-indole-3-carbaldehyde ***(5b)***: *(Choppara et al., 2015)*

Yield: 90%., Cream coloured solid., Mp: 192 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.34 (d, J = 8.8 Hz, 1H), 7.43 (d, J = 8.4 Hz, 1H), 7.75 (s, 1H), 8.25 (s, 1H), 8.32 (s, 1H), 9.94 (s, 1H). ^{13}C NMR (DMSO-d_6, 22.4 MHz): δ 113.0, 114.8, 117.3, 123.1, 125.6, 135.2, 136.7, 144.4, 183.9. ESI-MS: m/z 245.95 $[M+Na]^+$. IR (KBr, cm^{-1}): 1229, 1643, 3312. Anal. Calcd. for C_9H_6BrNO: C, 48.25, H, 2.70, N, 6.25%, Found: C, 48.22, H, 2.76, N, 6.24%.

4.5. General procedure for the synthesis of substituted 1-(3-methylbut-2-enyl)- 1H-indole-3-carbaldehyde ***(6a,b)***

To a solution of substituted 1*H*-indole-3-carbaldehydes **(5a,b)** (2.20 mmol) in dry DMF (5 mL) was added NaH (2.64 mmol, 60% oil dispersion) and the resulting mixture was stirred for 10 min in an ice bath. 3,3-dimethylallyl bromide (2.20 mmol) was added and the resulting mixture stirred for 15 min at 0 °C. The mixture was diluted with EtOAc (20 mL) and washed five times with distilled water (50 mL). The organic layer was dried over anhydrous Na_2SO_4 and filtered and the solvent was removed under reduced pressure to get crude residue which was purified by column chromatography (silica gel 100–200 mesh) using 8:2 (Hexane: EtOAc) as eluents affording **6a,b**.

4.5.1. 1-(3-methylbut-2-enyl)-1H-indole-3-carbaldehyde ***(6a)***

Yield: 87%., Brownish yellow crystals., Mp: 79–81 °C., ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.85 (s, 3H), 1.86 (s, 3H), 4.74 (d, J = 7.2 Hz, 2H), 5.44 (t, J = 7.2 Hz, 1H), 7.28–7.41 (m, 4H) 7.76 (s, 1H), 9.99 (s, 1H)., ^{13}C NMR ($CDCl_3$, 22.4 MHz): δ 17.9, 25.4, 44.6, 110.0, 117.7, 117.9, 121.8, 122.7, 123.6, 125.5, 137.3, 137.5, 138.7, 184.3., ESI-MS: m/z 214.20 $[M+H]^+$., IR (KBr, cm^{-1}): 1224, 1640., Anal. Calcd. for $C_{14}H_{15}NO$: C, 78.84, H, 7.09, N, 6.57%, Found: C, 78.80, H, 7.16, N, 6.55%.

4.5.2. 5-bromo-1-(3-methylbut-2-enyl)-1H-indole-3-carbaldehyde ***(6b)***

Yield: 89%., Pale pink solid., Mp: 95–98 °C., ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.85 (s, 3H), 1.86 (s, 3H), 4.71 (d, J = 7.2 Hz, 2H), 5.42 (t, J = 7.2 Hz, 1H), 7.25 (d, J = 9.6 Hz, 1H), 7.43 (dd, J = 2.0, 8.8 Hz, 1H), 7.73 (s, 1H), 8.47 (d, J = 2.0 Hz, 1H), 9.96 (s, 1H)., ^{13}C NMR ($CDCl_3$, 22.4 MHz): δ 12.7, 20.1, 39.4, 106.0, 110.8, 111.7, 119.0, 121.1, 121.4, 130.3, 132.6, 132.8, 133.8, 178.6., ESI-MS: m/z 314.01 $[M+Na]^+$., IR (KBr, cm^{-1}): 1162, 1654., Anal. Calcd. for $C_{14}H_{14}BrNO$: C, 57.55, H, 4.83, N, 4.79%, Found: C, 57.53, H, 4.90, N, 4.77%.

4.6. General procedure for preparation of N′-((5-substituted-1H-indol-3-yl)methylene)-n-(1H-indol-3-yl)alkanehydrazides ***(7a–f)***

A mixture of indole-3-carboxylic acid hydrazides **(3)** (1 mmol) and 5-substituted-1*H*-indole-3-carbaldehyde **(5)** in ethanol and glacial acetic acid (2 drops) was refluxed for 20–30 min. As the reaction progresses, *N′*-((5-substituted-1*H*-indol-3-yl)methylene)-n-(1*H*-indol-3-yl)alkanehydrazides **(7a–f)** separate out as a solid in the reaction mixture. After completion of the reaction, the solid product was collected by simple filtration. Then the solid was dried and purified by column chromatography (silica gel 100–200 mesh) using 6:4 (Hexane: EtOAc) as eluents affording pure compounds **(7a–f)**.

4.6.1. N′-((1H-indol-3-yl)methylene)-2-(1H-indol-3-yl)acetohydrazide ***(7a)***

Yield: 87%., Brown solid., Mp: 152 °C., ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.87 (s, 2H), 7.02–7.83 (m, 10), 8.12 (s, 1H), 8.52 (s, 1H), 10.21 (s, 1H), 10.89 (s, 1H)., ^{13}C NMR (DMSO-d_6, 22.4 MHz): δ 30.1, 108.1, 111.5, 114.3, 116.6, 116.9, 118.6, 119.7, 122.3, 123.1, 123.5, 127.5, 132.2, 132.5, 136.5, 137.5, 137.9, 140.6, 173.1. ESI-MS: *m/z* 317 $[M+H]^+$., IR (KBr, cm^{-1}): 1222, 1608, 1649, 3354, 3397, 3541., Anal. Calcd. for $C_{19}H_{16}N_4O$: C, 72.13, H, 5.10, N, 17.71%, Found: C, 72.11, H, 5.14, N, 17.69%.

4.6.2. N′-((1H-indol-3-yl)methylene)-3-(1H-indol-3-yl)propanehydrazide ***(7b)***

Yield: 84%., Pale yellow solid., Mp: 300 °C., ^{1}H NMR (DMSO-d_6, 200 MHz): δ 2.64 (t, J = 7.6 Hz, 2H), 2.87 (t, J = 7.6 Hz, 2H), 6.98–7.62 (m, 10H), 8.07 (s, 1H), 8.90 (s, 1H), 10.12 (s, 1H), 10.31 (s, 1H)., ^{13}C NMR (DMSO-d_6, 22.4 MHz): δ 26.3, 39.5, 111.7, 113.2, 116.8, 118.1, 119.0, 119.5, 120.2, 122.4, 123.2, 123.8, 126.1, 127.8, 128.7, 131.8, 136.0, 137.2, 140.1, 178.5., ESI-MS: *m/z* 331 $[M+H]^+$., IR (KBr, cm^{-1}): 1241, 1610, 1650, 3392, 3410, 3572., Anal. Calcd. for $C_{20}H_{18}N_4O$: C, 72.71, H, 5.49, N, 16.96%, Found: C, 72.70, H, 5.51, N, 16.95%.

4.6.3. N′-((1H-indol-3-yl)methylene)-4-(1H-indol-3-yl)butanehydrazide ***(7c)***

Yield: 92%., Yellow solid., Mp: 178 °C., ^{1}H NMR (DMSO-d_6, 200 MHz): δ 2.01–2.08 (m, 2H), 2.25 (t, J = 7.6 Hz, 2H), 2.80 (t, J = 7.6 Hz, 2H), 6.93–7.73 (m, 10H), 8.12 (s, 1H), 8.28 (s, 1H), 10.76 (s, 1H), 10.87 (s, 1H)., ^{13}C NMR (DMSO-d_6, 22.4 MHz): δ 25.4, 26.3, 32.9, 108.3, 110.6, 115.9, 117.1, 118.0, 118.7, 119.6, 120.5, 121.2, 121.8, 122.3, 124.0, 125.7, 136.2, 137.8, 138.9, 140.3, 174.2., ESI-MS: *m/z* 367.15 $[M+Na]^+$., IR (KBr, cm^{-1}): 1244, 1623, 1646, 3329, 3396, 3542., Anal. Calcd. for $C_{21}H_{20}N_4O$: C, 73.23, H, 5.85, N, 16.27%, Found: C, 73.20, H, 5.87, N, 16.26%.

4.6.4. N′-((5-bromo-1H-indol-3-yl)methylene)-2-(1H-indol-3-yl)acetohydrazide ***(7d)***

Yield: 91%., Yellow solid., Mp: 156 °C., ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.74 (s, 2H), 7.19–7.71 (m, 9H), 8.19 (s, 1H), 8.41 (s, 1H), 9.92 (s, 1H), 10.14 (s, 1H)., ^{13}C NMR (DMSO-d_6, 100 MHz): δ 29.7, 108.0, 110.9, 114.7, 116.9, 117.3, 118.9, 119.7, 122.4, 123.4, 123.9, 126.1, 132.1, 133.2, 136.8, 137.4, 138.0, 149.7, 174.3., ESI-MS: *m/z* 395 $[M]^+$, 397 $[M+2]^+$., IR (KBr, cm^{-1}): 1229, 1621, 1657, 3382, 3410, 3527., Anal. Calcd. for $C_{19}H_{15}BrN_4O$: C, 57.74, H, 3.83, N, 14.17%, Found: C, 57.72, H, 3.84, N, 14.15%.

4.6.5. N′-((5-bromo-1H-indol-3-yl)methylene)-3-(1H-indol-3-yl)propanehydrazide ***(7e)***

Yield: 89%., Pale pink solid., Mp: 186 °C., ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.55 (t, J = 7.2 Hz, 2H), 3.01 (t, J = 8.0 Hz, 2H), 6.94–7.80 (m, 9H), 8.14 (s, 1H), 8.37 (s, 1H), 10.80 (s, 1H), 11.01 (s, 1H)., ^{13}C NMR (DMSO-d_6, 100 MHz): δ 21.3, 35.5, 111.7, 113.4, 114.3, 114.4, 118.6, 118.8, 122.4, 122.7, 124.5, 125.4, 126.4, 127.5, 131.6, 136.2, 136.7, 139.8, 143.0, 173.6. ESI-MS: *m/z* 409 $[M]^+$, 411 $[M+2]^+$., IR (KBr, cm^{-1}): 1251, 1616, 1654, 3293, 3423, 3540., Anal. Calcd. for $C_{20}H_{17}BrN_4O$: C, 58.69, H, 4.19, N, 13.69%, Found: C, 58.70, H, 4.17, N, 13.72%.

4.6.6. N′-((5-bromo-1H-indol-3-yl)methylene)-4-(1H-indol-3-yl)butanehydrazide ***(7f)***

Yield: 90%., Greenish yellow solid., Mp: 218 °C., ^{1}H NMR (DMSO-d_6, 400 MHz): δ 2.06–2.13 (m, 2H), 2.17 (t, J = 7.6 Hz, 2H), 2.75 (t, J = 7.6 Hz, 2H), 6.89–7.82 (m, 9H), 8.28 (s, 1H), 8.36 (s, 1H), 10.54 (s, 1H), 10.91 (s, 1H)., ^{13}C NMR (DMSO-d_6, 100 MHz): δ 24.8, 27.1, 33.1, 108.6, 111.2, 115.8, 117.4, 118.1, 118.5, 119.2, 120.9, 121.1, 122.3, 122.9, 123.6, 125.5, 136.7, 137.3, 138.7, 140.1, 173.2., ESI-MS: *m/z* 437 $[M^+]$, 439 $[M+2]^+$., IR (KBr, cm^{-1}): 1250, 1631, 1657, 3381, 3408, 3496., Anal. Calcd. for $C_{21}H_{19}BrN_4O$: C, 59.58, H, 4.52, N, 13.24%, Found: C, 59.60, H, 4.49, N, 13.25%.

4.7. General procedure for preparation of N′-((5-substituted-1-(3-methylbut-2-enyl)-1H-indol-3-yl)methylene)-n-(1H-indol-3-yl)acetohydrazide ***(8a–f)***

A mixture of indole-3-carboxylic acid hydrazides **(3)** (1 mmol) and 5-substituted-1-(3-methylbut-2-enyl)-1*H*-indole-3-carbaldehyde **(6)** (1 mmol) in ethanol and glacial acetic acid was refluxed for 20–45 min. As the reaction progresses, *N′*-((5-substituted-1-(3-methylbut-2-enyl)-1*H*-indol-3-yl) methylene)-n-(1*H*-indol-3-yl) acetohydrazide **(8a–f)** separates out as a solid in the reaction mixture. After completion of the reaction, the solid product was collected by simple filtration. The product was purified by column chromatography (silica gel 100–200 mesh) using 6:4 (Hexane: EtOAc) as eluents affording pure products **(8a–f)**.

4.7.1. 2-(1H-indol-3-yl)-N′-((1-(3-methylbut-2-enyl)-1H-indol-3-yl)methylene)acetohydrazide ***(8a)***

Yield: 95%., Yellow solid., Mp: 180 °C. ^{1}H NMR (DMSO-d_6, 200 MHz): δ 1.58 (s, 3H), 1.79 (s, 3H), 3.32 (s, 2H), 4.52 (d, J = 7.2 Hz, 2H), 5.31 (t, J = 7.2 Hz, 1H), 6.96–8.14 (m, 10H), 8.34 (s, 1H), 8.97 (s, 1H), 10.21 (s, 1H)., ^{13}C NMR (DMSO-d_6, 22.4 MHz): δ 17.9, 25.8, 31.3, 45.8, 108.5, 111.4, 111.6, 113.0, 116.2, 118.6, 118.8, 119.9, 120.8, 121.1, 122.1, 122.9, 124.7, 124.9, 127.8, 132.3, 132.1, 136.1, 140.5, 174.4., ESI-MS: *m/z* 385 $[M+H]^+$., IR (KBr, cm^{-1}): 1224, 1615, 1651, 3272, 3452., Anal. Calcd. for $C_{24}H_{24}N_4O$: C, 74.97, H, 6.29, N, 14.57%, Found: C, 74.94, H, 6.33, N, 14.55%.

Please cite this article in press as: Choppara, P. et al., Synthesis, characterization and cytotoxic investigations of novel bis(indole) analogues besides antimicrobial study. Arabian Journal of Chemistry (2015), http://dx.doi.org/10.1016/j.arabjc.2015.05.015

4.7.2. 3-(1H-indol-3-yl)-N′-((1-(3-methylbut-2-enyl)-1H-indol-3-yl)methylene) propane hydrazide **(8b)**

Yield: 87%., Cream coloured solid., Mp: 184 °C., ^{1}H NMR (DMSO-d_6, 200 MHz): δ 1.82 (s, 3H), 1.85 (s, 3H), 2.31 (t, J = 7.6 Hz, 2H), 2.82 (t, J = 7.6 Hz, 2H), 4.71 (d, J = 7.2 Hz, 2H), 5.39 (t, J = 7.2 Hz, 1H), 6.92–7.83 (m, 10H), 8.09 (s, 1H), 8.80 (s, 1H), 10.50 (s, 1H)., ^{13}C NMR (DMSO-d_6, 22.4 MHz): δ 17.5, 24.7, 26.1, 37.8, 45.4, 110.2, 111.0, 115.2, 117.4, 118.0, 118.9, 119.5, 120.5, 121.8, 122.1, 122.6, 124.1, 125.7, 127.4, 136.7, 137.2, 137.6, 139.0, 140.1, 175.7. ESI-MS: m/z 399 $[M+H]^+$., IR (KBr, cm^{-1}): 1071, 1620, 1671, 3321, 3467., Anal. Calcd. for $C_{25}H_{26}N_4O$: C, 75.35, H, 6.58, N, 14.06%, Found: C, 75.33, H, 6.60, N, 14.05%.

4.7.3. 4-(1H-indol-3-yl)-N′-((1-(3-methylbut-2-enyl)-1H-indol-3-yl)methylene)butane hydrazide **(8c)**

Yield: 87%., Cream coloured solid., Mp: 172–174 °C. ^{1}H NMR (DMSO-d_6, 200 MHz): δ 1.79 (s, 3H), 1.81 (s, 3H), 2.04–2.10 (m, 2H), 2.27 (t, J = 7.6 Hz, 2H), 2.76 (t, J = 7.6 Hz, 2H), 4.63 (d, J = 7.2 Hz, 2H), 5.39 (t, J = 7.2 Hz, 1H), 6.97–7.85 (m, 10H), 8.20 (s, 1H), 8.36 (s, 1H), 10.81 (s, 1H)., ^{13}C NMR (DMSO-d_6, 22.4 MHz): δ 17.9, 24.6, 25.7, 26.1, 33.8, 45.1, 110.1, 110.8, 115.5, 117.6, 117.9, 118.7, 119.2, 120.7, 121.7, 121.9, 122.4, 123.7, 125.3, 127.2, 136.6, 137.2, 137.4, 138.9, 140.1, 175.1., ESI-MS: m/z 451.18 $[M+K]^+$., IR (KBr, cm^{-1}): 1231, 1624, 1669, 3331, 3472., Anal. Calcd. for $C_{26}H_{28}N_4O$: C, 75.70, H, 6.84, N, 13.58%, Found: C, 75.69, H, 6.85, N, 13.57%.

4.7.4. N′-((5-bromo-1-(3-methylbut-2-enyl)-1H-indol-3-yl)methylene)-2-(1H-indol-3-yl)acetohydrazide **(8d)**

Yield: 89%., White solid., Mp: 178 °C., ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.52 (s, 3H), 1.71 (s, 3H), 3.29 (s, 2H), 4.52 (d, J = 7.2 Hz, 2H), 5.28 (t, J = 7.2 Hz, 1H), 7.02–8.17 (m, 9H), 8.41 (s, 1H), 8.99 (s, 1H), 10.35 (s, 1H)., ^{13}C NMR (DMSO-d_6, 100 MHz): δ 16.3, 24.2, 31.6, 44.9, 108.1, 111.2, 111.7, 113.2, 116.6, 118.7, 119.2, 119.5, 120.8, 121.3, 122.5, 123.6, 124.8, 125.8, 127.6, 132.6, 132.2, 135.0, 140.7, 173.2., ESI-MS: m/z 463 $[M]^+$, 465 $[M+2]^+$., IR (KBr, cm^{-1}): 1212, 1624, 1658, 3325, 3487., Anal. Calcd. for $C_{24}H_{23}BrN_4O$: C, 62.21, H, 5.00, N, 12.09%, Found: C, 62.20, H, 5.02, N, 12.06%.

4.7.5. N′-((5-bromo-1-(3-methylbut-2-enyl)-1H-indol-3-yl)methylene)-3-(1H-indol-3-yl)propanehydrazide **(8e)**

Yield: 94%., Cream coloured solid., Mp: 194 °C., ^{1}H NMR (DMSO-d_6, 200 MHz): δ 1.57 (s, 3H), 1.76 (s, 3H), 2.34 (t, J = 7.2 Hz, 2H), 2.80 (t, J = 7.2 Hz, 2H), 4.67 (d, J = 7.2 Hz, 2H), 5.43 (t, J = 7.2 Hz, 1H), 6.99–7.91 (m, 9H), 8.27 (s, 1H), 8.71 (s, 1H), 10.42 (s, 1H)., ^{13}C NMR (DMSO-d_6, 50 MHz): δ 16.9, 24.4, 27.1, 37.4, 45.9, 110.3, 111.5, 115.1, 117.9, 118.3, 119.3, 119.8, 120.3, 121.7, 122.3, 122.8, 124.8, 125.6, 127.2, 136.3, 137.1, 137.8, 139.3, 140.3, 176.2., ESI-MS: m/z 477 $[M]^+$, 479 $[M+2]^+$., IR (KBr, cm^{-1}): 1190, 1628, 1671, 3401, 3563., Anal. Calcd. for $C_{25}H_{25}BrN_4O$: C, 62.90, H, 5.28, N, 11.74%, Found: C, 62.88, H, 5.29, N, 11.73%.

4.7.6. N′-((5-bromo-1-(3-methylbut-2-enyl)-1H-indol-3-yl)methylene)-4-(1H-indol-3-yl)butanehydrazide **(8f)**

Yield: 90%., White solid., Mp: 182 °C., ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.65 (s, 3H), 1.74 (s, 3H), 2.02–2.11 (m, 2H), 2.26 (t, J = 7.2 Hz, 2H), 2.81 (t, J = 7.2 Hz, 2H), 4.51 (d, J = 7.6 Hz, 2H), 5.31 (t, J = 7.6 Hz, 1H), 7.12–7.85 (m, 9H), 8.13 (s, 1H), 8.28 (s, 1H), 10.72 (s, 1H)., ^{13}C NMR (DMSO-d_6, 100 MHz): δ 16.8, 25.2, 25.9, 27.4, 33.9, 45.3, 110.9, 111.4, 116.2, 117.7, 118.1, 118.8, 119.5, 120.8, 121.9, 122.4, 122.9, 123.8, 125.2, 127.9, 136.9, 137.5, 137.9, 138.7, 140.5, 174.7., ESI-MS: m/z 514 $[M+Na]^+$, 512 $[M+Na+2]^+$., IR (KBr, cm^{-1}): 1239, 1619, 1674, 3291, 3463., Anal. Calcd. for $C_{26}H_{27}BrN_4O$: C, 63.55, H, 5.54, N, 11.40%, Found: C, 63.57, H, 5.57, N, 11.39%.

4.8. Antibacterial assay

100 mL sterile conical flask of nutrient broth was inoculated with the test organisms and incubated at 37 °C overnight. By using a sterile pipette, 0.6 mL of the broth culture of each test organism was added to 60 mL of molten agar, mixed well and maintained at 45 °C. Sterile agar test plates of each test organism were prepared by pouring inoculated medium with uniform thickness. The agar was allowed to set and harden and wells of 4 mm diameter were cut at equidistant using a sterile cork borer. Agar plugs were removed. 100 μg/mL of test solutions **(7a–f)** and **(8a–f)** were prepared in DMSO and were introduced into the wells using micropipette. The plates were kept at room temperature for 2 h for better diffusion of solution into the medium. The plates were incubated for 24 h at 37 °C. After incubation the diameter of inhibitory zones formed around each well was measured in millimetre (mm) using antibiotic zone scale. The assay was carried out in duplicate. DMSO was used as control and the antibacterial activity of the test compounds was compared with standard "ciprofloxin".

4.9. Antifungal assay

Sterile molten potato dextrose agar (PDA) medium was inoculated with 50 μL of fungal spore suspension aseptically and maintained at 45 °C temperature. The inoculated medium was mixed well and poured immediately in sterilized petri plates. Then five wells of 6 mm diameter were punched using sterile borer and filled with 100 μg/mL of test compounds **(7a–f)** and **(8a–f)** as well as sterile DMSO 100% as negative control. Plates were incubated for 24 h at 37 °C. Antifungal activity was determined by measuring the zone of inhibition. The zones produced by the test compounds were compared with the ketoconazole (standard).

4.10. Anticancer activity

4.10.1. Cell lines and cell culture

The cell lines are (Colo 205) Human colon cancer cell line, Hep G2, hepatocellular carcinoma cell line which was derived from the liver tissue of a 15-year-old Caucasian American male, and HeLa (Hela or HeLa cell) the oldest and most commonly used human cell line (Rahbari et al., 2009). The cell line was derived from cervical cancer cells taken on February 8, 1951 (Scherer

et al., 1953) from Henrietta Lacks, a patient who eventually died of cancer on October 4, 1951. George Gey was able to isolate one specific cell, multiply it, and start a cell line. Gey named the sample HeLa, after the initial letters of Henrietta Lack's name. Colo 205 (Human colon cancer cell line), Hep G2 (Human liver carcinoma cell line) and HeLa (Cervical cancer cell line) cell lines were obtained from the National Centre for Cellular Sciences (NCCS), Pune, India. Cells were cultured in RPMI-1640 media, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM $NaHCO_3$, 2 mM -glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. All cell lines were maintained in culture at 37 °C in an atmosphere of 5% CO_2.

4.10.2. Test concentrations

Initially, stock solutions of each test substance were prepared in 100% Dimethyl Sulfoxide (DMSO, Sigma Aldrich) with a final concentration of 8 mg/mL. Exactly 50 μL of stock was diluted to 1 mL in culture medium to obtain experimental stock concentration of 400 μg/mL. This solution was further serially diluted with media to generate a dilution series of 10 μg to 200 μg/mL. Precisely, 100 μL of each test concentration was added to 100 μL of cell suspension (total assay volume of 200 μL, and efficacy of the derivatives was evaluated with three different set of experiments) and incubated for 24 h at 37 °C in 5% CO_2.

4.10.3. Cytotoxicity

The cytotoxicity of novel bis(indole) analogues **(7a–f)** and **(8a–f)** was screened on human colorectal cancer (Colo-205), human hepatocellular liver carcinoma (Hep G2), and human cervical cancer (HeLa) cell lines using etoposide as positive control by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay, according to the method of Mosmann (1983). Briefly, the cells (2×10^4) were seeded in each well containing 100 μL of medium in 96 well plates. After overnight incubation at 37 °C in 5% CO_2, exactly 100 μL of different test concentrations (10 μg–200 μg/mL) was added to the cell suspension, which is equivalent to 2–40 μg per 200 μL of assay volume. The viability of cells was assessed after 24 h, by adding 10 μL of MTT (5 mg/mL) per well and incubated at 37 °C for additional three hours. The medium was discarded and the formazan blue, which formed in the cells, was dissolved in 100 μL of DMSO. The intensity of colour formation was measured at 570 nm in a spectrophotometer (Spectra MAX Plus, Molecular Devices, supported by SOFTmax PRO-5.4). The percent inhibition of cell viability was determined with reference to the control values (without test compound). The data were subjected to linear regression analysis and the regression lines were plotted for the best straight-line fit. The IC_{50} (inhibition of cell viability) concentrations were calculated using the respective regression equation and expressed in μM.

Acknowledgement

We are grateful to DRDO, New Delhi, for providing financial assistance through a project ERIP/ER/1003916/M/01/1354 and UGC, New Delhi, for the award of UGC-BSR JRF to the author P.C. The authors BM and JVR are grateful to the Director, Indian Institute of Chemical Technology, Hyderabad, India, for the encouragement.

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Please cite this article in press as: Choppara, P. et al., Synthesis, characterization and cytotoxic investigations of novel bis(indole) analogues besides antimicrobial study. Arabian Journal of Chemistry (2015), http://dx.doi.org/10.1016/j.arabjc.2015.05.015