Synthesis of 8–benzoyl–3–(substituted)–benzo[4,5]imidazo[2, 1–d][1, 2, 3, 5] tetrazin–4(3H)–one and evaluation of their antimicrobial and antiproliferative activity

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Abstract—In the present study, a number of new benzimidazo [2,1–d][1,2,3,5]tetrazine–4(3H)–one derivatives have been prepared for the purposes of evaluating antimicrobial and anticancer activity studies. Comparing and *in vitro* study of inhibitory effects of anti gram positive and gram negative bacteria, also in anti fungal studies by well dish method technique, remarkable activity was observed. Furthermore, anticancer activities of these compounds have been investigated against some breast (MCF–7, T47D & BT–549), prostate (PC–3 & DU–145), lung (H1975) and colon (HCT–116 & HCT–15) human cancer cell lines. All the derivatives were efficiently synthesized by five steps process. The structures of the newly synthesized compounds were elucidated by their ^{1}H & ^{13}C NMR, LC–MS/MS, IR spectral data and elemental analysis. The detailed synthesis, spectroscopic and biological evaluation data are reported.

Index Terms—Benzimidazole derivatives, Imidazotetrazinones, Biological activity, Anticancer activity.

1 INTRODUCTION

Azolo–tetrazinones constitute an interesting class of pharmacologically active compounds which have been extensively studied during the past three decades [1]. The molecules have 'evolved' from simple bicyclic polyazaheterocycles bearing bridgehead nitrogen atoms which were synthesised in the 1970s [2], with the first examples of the imidazo[5,1–*d*][1,2,3,5]tetrazine ring system being reported in 1984 [3]. In particular, large numbers of papers on pyrrolo–, pyrazolo–, indazole– and imidazotetrazinones [4], [5], [6], [7] have been published because these compounds show remarkable antitumor, antibacterial activity [8] and herbicidal properties [7]. The imidazotetrazine ring possesses valuable pharmaceutical properties such as acid stability [9], oral availability, CNS penetration, and even tumor localization. Mitozolomide, the first azolo–tetrazinone to show remarkable anticancer activity,[11a] but its severe delayed toxicity, revealed in the phase II clinical trials, compromised its clinical use [10], [11], [12]. Substitution of the 3–(2–chloroethyl) group on the 3 position of the 1,2,3,5–tetrazine moiety, with a methyl substituent led to temozolomide (2), which proved to be less potent but also less toxic than its 2–chloroethyl congener, being now in the market with the trade name of Temodal and used against malignant melanoma, mycosis fungoides and brain tumours [13] due to this property.

There is always a need for new and effective anticancer, antifungal and antibacterial agents with broad spectrum anticancer, antibacterial and antifungal activities. It was decided to exploit this interest by ascertaining the molecules features essential for activity and utilizing them to develop a new class of drugs.

Prompted by the various biological activities of imidazotetrazinones and its substituted derivatives, we envisioned our approach towards the synthesis of biologically active *benzimidazotetrazinone* derivatives derived from 5–benzoyl–2–aminobenzimidazole with a series of substituted isocyanates. The synthesized compounds were structurally characterized with the help of various spectral and analytical studies. Moreover, the *in vitro* biological activities of the synthesized compounds were tested against some pathogenic bacterial and fungal strains by modified well diffusion method using agar medium. Anticancer activities of these compounds have been investigated against some breast (MCF–7, T47D & BT–549), prostate (PC–3 & DU–145), lung (H1975) and colon (HCT–116 & HCT–15) human cancer cell lines.

2 EXPERIMENTAL METHODS

2.1 Materials and physico–chemical measurements

All the chemicals and solvents used in this work were extra pure analytical grade and purchased from Sigma Aldrich and Fluka (Puriss) products without further purification. The solvents used for the preparation and physical measurements were purified according to the literature methods [14]. Melting point (m.p.) of the ligands was determined on Buchi–Tottoti open glass capillary tube and is uncorrected. Micro analytical (C, H & N) data were performed on Elementar Vario EL III CHNS analyzer. Molar conductance (Λ_m) of the ligands (1×10^{-3} mol solution in DMSO) was measured using an Elico CM 180 conductivity bridge by

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using KCl solution as calibrant. The IR spectra were recorded on KBr discs on a Perkin Elmer 100 FT–IR spectrometer (400–4000 cm^{-1}). The proton (^1H) and carbon (^{13}C) NMR spectral observation of the ligands were recorded on a Perkin Elmer R–32 spectrometer in $CDCl_3$ and DMSO–d_6 at 400MHz & 100 MHz with TMS as internal reference. The LC mass spectra of the ligands were determined on Agilent–1100 series mass spectrometer in ESI–MS mode. All the chemical reactions were performed under N_2 atmosphere using standard techniques**.** Column chromatography was performed with silica gel 230–400 mesh (Merck, India). Yield reported is the isolated yield after purification of the compounds.

2.2 Synthesis of 5–benzoyl–2–aminobenzimidazole (or) 5–benzoyl–1H–benzimidazole–2–amine (II)

For A suspension of Methyl(5–benzoyl–1H–benzo[d]imidazol–2–yl)carbamate **I. (**1 g, 3.23 mmol) dissolved with 10ml n–butanol and KOH was added in (2g, 35.6 mmol). Then reaction mixture heat to reflux for 8hrs.and reaction mixture was then concentrated in vacuo and quenched with water and extract with ethyl acetate, the crude product was purified by column chromatography using n–hexane/ethyl acetate (95:5) as eluent to afford the expected 5–benzoyl–2–aminobenzimidazole **L**. Yield: 78%. IR (KBr υ_{max} cm^{-1}): 3333.33, 3436.71(NH_2 and NH); ^1H NMR (400 MHz,DMSO–d_6) δ (ppm): 5.0 (s,1H,N$\underline{H}_2$), 7.39–7.50 (m, 4H, ArH), δ 7.69–7.74 (m, 3H, ArH), 7.92–7.93 (s,1H, ArH),10.73 (b,1H, NH): LCMS (ESI) m/z: 249.24[M+1].

2.3 Synthesis of 5–benzoyl–2–diazo–2H–benzimidazole (or) 5–benzoyl–2–diazobenzimidazole (III)

To a solution of (1g, 4.21 mmol) 5–benzoyl–2–aminobenzimidazole **II** in glacial acetic acid (6 ml) a solution of sodium nitrite (0.29g,4.21mmole) in a small amount of water (1ml) was added drop wise at –10˚c under nitrogen atmosphere. The mixture was neutralized at –10˚ to 0˚c with saturated Na_2CO_3 and the yellow solid precipitated was filtered off and dried under vacuum and in the dark. The crude product, quickly shaken in cyclohexane and filtered off, gave 5–benzoyl–2–diazobenzimidazole **III** brown solid. Yield (40–60 %). IR (KBr υ_{max} cm^{-1}):2191.42 (N_2^+).

2.4 General procedure for the synthesis of 3–substituded–4–oxo–8–benzoylbenzimidazo[2,1–d][1,2,3,5]tetrazine (IVa–e)

To a solution of 5–benzoyl–2–diazo–2H–benzimidazole **III** (1g,4.03 mmol) in anhydrous dichloromethane (10ml),the suitable isocyanate (4.03 mmol) in anhydrous dichloromethane (10ml) was added drop wise keeping the temperature at –10˚C.The reactions were allowed to reach at room temperature in the dark under a nitrogen atmosphere. The reaction mixture was stirred for 24 hrs. Then the solvent was evaporated under reduced pressure. The crude product (**Scheme**) was purified by column chromatography to afford the expected imidazotetrazinones give **IVa–e** (Yield: 40–85%).

2.4.1 8–Benzoyl–3–phenylbenzo[4,5]imidazo[2,1–d][1,2,3,5] tetrazin–4(3H)–one (IVa)

Pale yellow solid, Yield: 83%; mp209–210˚C; IR (KBr υ_{max} cm^{-1}): 1746.21 (CO), 1643.60 (Ar–CO–Ar); ^1H NMR (400 MHz,DMSO–d_6) δ (ppm): 7.26–7.37 (m, 4H,ArH), 7.43–7.46 (t, 4H,ArH), 7.68–7.69 (m, 2H,ArH), 7.83–7.87 (m,1H,ArH),7.92–7.96 (m,2H,ArH); ^{13}C NMR (100 MHz,DMSO–d_6) δ (ppm): 197.04, 153.38, 147.79, 145.32, 138.95, 136.34, 132.42, 130.55, 129.99, 129.94, 129.13, 128.77, 127.19, 124.11, 122.85, 115.02, 112.19; MS Calculated for $C_{21}H_{13}N_5O_2$: 367.36 Found: LCMS (ESI) m/z: 368.00 (M+1). Elemental Analysis: Calculated: C, 68.66; H, 3.57; N, 19.06. Found: C, 68.77; H, 3.67; N, 19.16.

2.4.2 8–Benzoyl–3–(2–chloroethyl)benzo[4,5]imidazo[2,1–d][1,2,3,5]tetrazin–4(3H)–one (IVb)

Off–white solid, Yield: 68%; mp167–168˚C(decom); IR (KBr υ_{max} cm^{-1}): 1743.09 (CO), 1626.34 (Ar–CO–Ar); ^1H NMR (400 MHz,DMSO–d_6) δ (ppm): 3.22–3.25 (t, 2H,N–CH_2–), 3.62–3.65 (t,2H,CH_2–Cl), 7.44–7.50 (m, 3H,ArH), 7.66–7.70 (m, 3H,ArH), 7.85 (s, 1H,ArH), 8.23–8.24 (d,2H,ArH); ^{13}C NMR (100 MHz,DMSO–d_6) δ (ppm):197.05, 153.38, 146.23, 145.32, 136.34, 132.42, 130.55, 129.99, 129.94, 128.77, 122.85, 115.02, 112.19, 45.18, 41.65; MS Calculated for $C_{17}H_{12}ClN_5O_2$: 353.76 Found: LCMS (ESI) m/z: 355.85 (M+2). Elemental Analysis: Calculated: C, 57.72; H, 3.42; N, 19.80. Found: C, 57.45; H, 3.79; N, 19.56.

2.4.3 8–Benzoyl–3–(2–chlorophenyl)benzo[4,5]imidazo[2,1–d][1,2,3,5]tetrazin–4(3H)–one (IVc)

White solid, Yield: 79%; mp218–220˚C(decom); IR (KBr υ_{max} cm^{-1}): 1745.57 (CO), 1646.21 (Ar–CO–Ar); ^1H NMR (400 MHz,DMSO–d_6) δ (ppm): 7.17–7.24 (m, 2H,ArH), 7.42–7.52 (m,4H,ArH), 7.64–7.71 (m,3H,ArH), 7.90–8.08 (m,2H,ArH), 8.09–8.11 (m,1H,ArH); ^{13}C NMR (100 MHz,DMSO–d_6) δ (ppm): 197.04, 153.38, 147.56, 145.32, 136.72, 136.34, 132.42, 132.25, 130.55, 129.99, 129.94, 129.81, 129.69, 129.24, 128.77, 122.85, 115.02, 112.20; MS Calculated for $C_{21}H_{12}ClN_5O_2$: 401.81 Found: LCMS (ESI) m/z: 402.93 (M+1). Elemental Analysis: Calculated: C, 62.77; H, 3.01; N, 17.43. Found: C, 62.98; H, 3.40; N, 17.22.

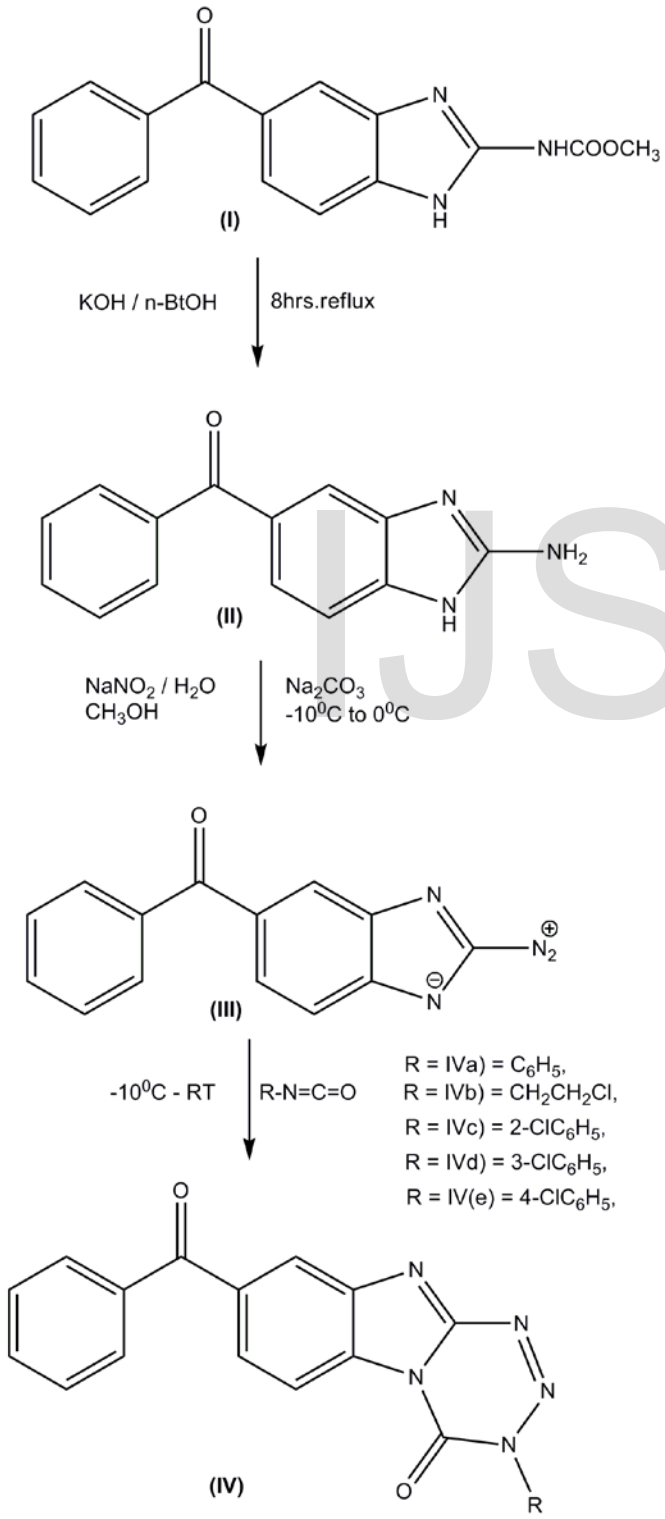
2.4.4 8–Benzoyl–3–(3–chlorophenyl)benzo[4,5]imidazo[2,1–d][1,2,3,5]tetrazin–4(3H)–one (IVd)

Off–white solid, Yield: 76%; mp228–229˚C(decom); IR (KBr υ_{max} cm^{-1}): 1747.78 (CO), 1657.42 (Ar–CO–Ar); ^1H NMR (400 MHz,DMSO–d_6) δ (ppm): 7.23–7.26 (m, 1H,ArH), 7.40–7.43 (m, 2H,ArH), 7.45–7.50 (m, 3H,ArH), 7.68–7.71 (m,2H,ArH), 7.78–7.80 (m,2H,ArH), 7.82–8.10 (m,1H,ArH), 8.14–8.21 (m,1H,ArH); ^{13}C NMR (100 MHz,DMSO–d_6) δ (ppm): 197.04, 153.38, 147.79, 145.32, 140.26, 136.34, 132.91, 132.42, 132.05, 130.55, 129.99, 129.94, 128.77, 127.98, 123.88, 122.85, 121.55, 115.02, 112.19; MS Calculated for $C_{21}H_{12}ClN_5O_2$: 401.81 Found: LCMS (ESI) m/z: 403.05 (M+1). Elemental Analysis: Calculated: C, 62.77; H, 3.01; N, 17.43. Found: C, 63.02; H, 3.36; N, 17.26.

2.4.5 8–Benzoyl–3–(4–chlorophenyl)benzo[4,5]imidazo[2,1–d][1,2,3,5]tetrazin–4(3H)–one (IVe)

White solid, Yield: 74%; mp240–241˚C(decom); IR (KBr υ_{max} cm^{-1}): 1741.19 (CO), 1659.36 (Ar–CO–Ar); ^1H NMR (400 MHz,DMSO–d_6) δ (ppm): 7.29–7.31 (d, 2H,ArH), 7.42–7.50 (m, 3H,ArH), 7.60–7.62 (d, 1H,ArH), 7.68–7.71 (m,2H,ArH), 7.81–

7.88 (m,3H,ArH), 8.03–8.05 (d,1H,ArH); ^{13}C NMR (100 MHz,DMSO–d_6) δ (ppm): 197.04, 153.38, 147.79, 145.32, 138.63, 136.34, 132.42, 131.86, 130.55, 129.99, 129.94, 128.77, 128.29, 125.63, 122.85, 115.02, 112.19; MS Calculated for $C_{21}H_{12}ClN_5O_2$: 401.81 Found: LCMS (ESI) m/z: 402.89 (M+1). Elemental Analysis: Calculated: C, 62.77; H, 3.01; N, 17.43. Found: C, 62.96; H, 3.28; N, 17.52.



Scheme. Synthesis of benzimidazotetrazinones (IVa-e).

2.5 Biological Activity

In vitro biological activities of the compounds (3×10^{-3} M) in DMSO medium were tested against three Gram–positive bacterial species: *Bacillus subtilis*, *Staphylococcus saphyphiticus* and *Staphylococcus aureus*, two Gram–negative bacterial species: *Escherichia coli* and *Pseudomonas aeruginosa* using Muller Hinton nutrient agar (NA) and three fungal species: *Aspergillus niger, Enterobacter species* and *Candida albicans* using potato dextrose agar (PDA) as medium were studied by modified well diffusion technique [15]. All the blank petri discs were moistened with the same solvent. All the investigations were made in three replicates for each and the detailed procedure for measuring the zone of inhibition as described earlier [16], [17]. The mean value obtained for two holes was used to calculate the zone of growth inhibition (in mm) of each sample and these values were compared with tetracycline (for antibacterial) and nystatin (for antifungal) control drugs (3×10^{-3} M) respectively.

2.6 Antiproliferative Activity

In vitro antiproliferative activity of the synthesized benzimidazotetrazinone derivatives (**IVa–e**) were screened against human cancer cell lines like non small lung cancer cell (H1975) prostate cancer cell (PC-3, DU-145), breast cancer cell (MCF-7, T47D, and BT549) and colon cancer cell (HCT-116, HCT-15) by the MTT assay method [18]. In this assay method, to analyzes the ability of living cells to reduce the yellow dye of 3–(4, 5–dimethyl–2–thiazolyl)–2,5–diphenyl–2H–tetrazolium bromide (MTT) to purple formazan product. The potential cytotoxicities of all the compounds were determined by measuring the percentage of cancers cells death/inhibition. The selected compounds were tested in various concentrations of 0, 1, 5, 10, 20, 40, 80,100 µM against human cancer cell lines to determine Growth inhibition was determined as compared to untreated cells (%).

3 RESULT AND DISCUSSION

The synthesised benzimidazotetrazinone derivatives (**IV a-e**) were stable, non–hygroscopic and soluble in common organic solvents but readily soluble in DMF, $CDCl_3$ and DMSO. The structure of the newly synthesized compounds was elucidated by their ^{1}H NMR,^{13}C NMR, LCMS,CHN and IR spectral data analysis The obtained elemental (C, H & N) analysis, various physico–chemical properties with its molecular formula and other spectral data values are summarized in the experimental part.

3.1 Physico–chemical properties of benzimidazotetrazinone compounds (IV a-e)

The analytical data of synthesised compounds (**IV a-e**) are well agreed with the calculated values. The observed low molar conductance values confirm the non–electrolytic in nature [19] with the absence of any counter ions. Fast atomic bombardment mass spectrum (FAB–MS) of the synthesized compounds show the molecular ion (m/z) peaks confirm the stoichiometry which is further confirmed by the observed analytical and their spectral

data of the ligands.

3.2. Infrared spectra with mode of bonding

The characteristic IR spectral data (KBr pellet, cm^{-1}) of the synthesised benzimidazotetrazinone ligands (**1Va–i**) were represented in the experimental part. From the IR spectral data, the characteristic band at 1720–1748 cm^{-1} in all the compounds is due to the carbonyl (C=O) group of the tetrazine moiety [20].

3.3 ^{1}H & ^{13}C NMR spectra

Both the ^{1}H & ^{13}C NMR spectra of the synthesized benzimidazotetrazinone derivatives (**IVa-e**) were recorded in $CDCl_3$ and DMSO–d_6 medium, tetramethylsilane (TMS) as internal standard (δ, in ppm) at room temperature. The representative ^{1}H NMR spectrum of compound **IVb** was shown in **Fig. 1.** All the protons and carbons atoms in the structure are established to be in their predictable regions [21].

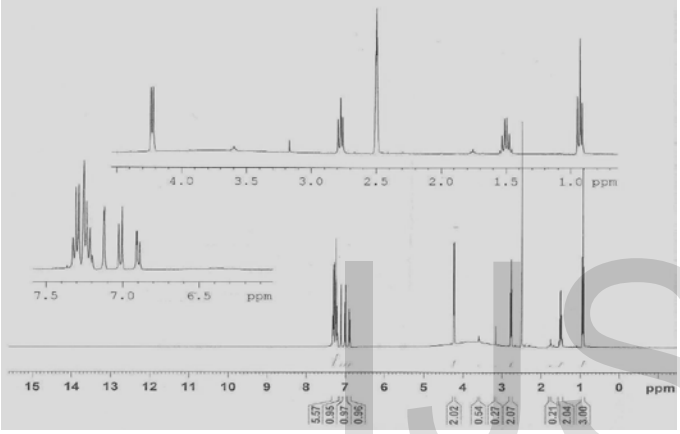


Figure 1. ^{1}H NMR spectra of compound (IVb).

3.4 *In vitro* biological activity

In vitro biological activities of the compounds in DMSO medium were tested against five pathogenic bacteria and three fungal strains by well diffusion method using agar as nutrient. Measured zone of inhibition (in mm) against the growth of bacterial and fungal for the above systems are shown in **Fig. 2**. On comparing the biological activities of the compounds with the commercially available standard drugs like tetracycline (antibacterial control) and nystatin (for antifungal) are used as control. The biological activity of the synthesized benzimidazotetrazinone derivatives (**IVa, IVb and IVe**) show moderate activity against different types of microorganism as compared to the standard control drugs. Variations in the effectiveness of different biocidal species against microorganisms depend on the impermeability of the cell of the microbes or on differences in ribosome of microbial cells. Higher inhibition zones of synthesized molecules have been explained on the basis of Overtone's concept and Tweedy's chelation theory [22], [23], [24].

According to Overtone's concept of cell permeability, the lipid membrane that surrounds the cell favors the passage of only lipid soluble materials only since, the liposolubility is an important factor that controls the antimicrobial activity. Complexation of antimicrobial active organic part with metal(II) ions reduce the polarity of M(II) ions significantly because of partial sharing of its positive charge with donor groups and delocalization of π–electrons over the whole chelate ring resulting in high lipid solubility. The increasing lipid solubility character of the metal chelate favors its permeation through lipid layer of the microorganism which probably leads to break–down of permeability barrier of cell process. During this process, increase in the lipophilic nature of the central metal(II) ions which favors its permeation more efficiently through the lipoid layer of the microorganisms, thus making the chelate compounds more antimicrobial activity. Also, the formation of chelation with metal ions gives some important properties to the organic compounds that also play important role in their biological activity such as low dissociation constant, special redox potential, electron distribution and increasing the cell permeability. Also, the characteristic property of the investigated ligand could be applied secure and sound in the treatment of infections caused by any of these particular strains. In addition, it was established that the pathogenic bacterial species like *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* show remarkable activities.

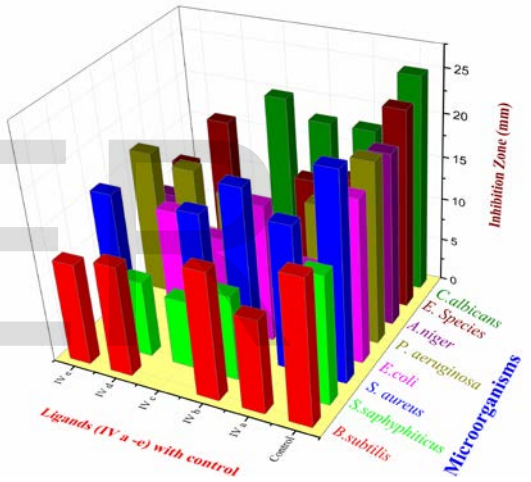


Figure 2. Microbial activity of ligand (Iva-e) with different microorganisms at 37 ^{o}C by well diffusion method.

3.5 Antiproliferative activities

In the present study, the antiproliferative activity of the synthesized five benzimidazotetrazinone derivatives (**IVa-e**), were selected for *in-vitro* disease-oriented anticancer screenings against the eight human tumor cell lines, including non small lung carcinoma (H1975) prostate carcinoma (PC-3, DU-145), breast carcinoma (MCF-7, T47D, and BT549) and colon carcinoma (HCT-116, HCT-15) by the MTT assay. The potential cytotoxicities of all compounds were determined by measuring the percentage of cell survival, as summarized in **Table 1**. The data suggested that at the concentration of 40 μg/mL, the 2-chloroethyl **IVb** whose viabilities were all below 10% displayed powerful inhibition against all eight tested tumor cell lines. The other compounds showed moderate inhibition against all cell lines. Among the other compounds derivative **IVc & IVd** showed better inhibition against H1975 non small lung cancer cells and BT549 breast cancer cells (cell viability was 41.85%, 30.33% and 41.39, 53.93%

respectively), compound **IVe** showed more potent inhibition aganist BT549 Breast carcinoma cells and HCT-15 colon carcinoma cells (cell viability was 36.20% and 37.55% respectively) and some of the benzimidazotetrazinone derivatives showed no significant activity.

Table 1. The substituents and cytotoxicity of the title compounds IVa-e.

% of Survival[a]				
Compound	**H1975**	**PC-3**	**DU145**	**MCF-7**
IVa	87.29±0.59	113.09±0.85	97.28±0.12	76.9±0.72
IVb	8.83±0.23	9.17±0.57	9.48±0.27	8.19±0.61
IVc	41.85±0.56	103.67±0.67	79.79±0.41	69.95±0.38
IVd	30.33±0.48	63.1±0.32	60.04±0.38	87.09±0.29
IVe	50.49±0.65	65.3±0.41	101.9±0.51	95.68±0.56
% of Survival[a]				
Compound	**T47D**	**BT549**	**HCT116**	**HCT-15**
IVa	95.64±0.29	91.2±0.12	87.21±0.54	86±0.21
IVb	9.57±0.24	9.1±0.39	9.13±0.45	10.24±0.74
IVc	100.37±0.36	41.39±0.82	74.01±0.48	80±0.26
IVd	79.00±0.92	53.93±0.61	78.92±0.25	69.21±0.34
IVe	84.48±0.81	36.2±0.69	43.22±0.31	37.55±0.12

[a] Survival percent (%) at 40 µg /mL of tested compounds on eight human tumor cell lines. Data shown are means ± SD of three independent experiments.

4 CONCLUSION

In conclusion we have developed a highly efficient synthesis of the new ring system benzimidazo [2, 1-*d*][1,2,3,5]tetrazine-4(3H)-one, by cycloaddition of isocyanates to the 5-propylthio-2-diazo-2H-benzimidazole. The tested some compounds exhibited promising inhibitory activity in prostate, breast, lung, & colon cancer cells. The antimicrobial screening suggests that all the newly synthesized compounds, showed moderate to good activity against the tested organisms. Among the newly synthesized compounds also showed the most promising antibacterial and antifungal activity. Hence the fact that the compounds prepared in this study are chemically unrelated to the current medication, suggests that further work with similar analogues is clearly warranted.

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