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Synthesis, In vitro Antimicrobial and Cytotoxic Activities of Some Novel Bis- 1, 3, 4-oxadiazoles

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Abstract

A series of novel bis-1,3,4-oxadizaoles were synthesized by oxidative cyclisation of respective Schiff bases derived from dicarbohydrazide using ceric ammonium nitrate (CAN) as catalyst. The synthesized compounds were screened for *in vitro* antibacterial activity against *Staphylococcus aureus* (MTCC 87), *Escherichia coli* (MTCC 46) and antifungal activity against *Candida albicans* (NCIM 3471) by two fold serial tube dilution method. The compounds were evaluated for in vitro cytotoxicity activity against human lung carcinoma cells (A-549) by standard MTT assay method. The DNA cleavage analysis of three compounds (4b, 4g and 4j) was also performed.

Key words: *Bis-heterocycles, 1,3,4–oxadiazoles, Antimicrobial activity, Cytotoxicity activity, MTT assay, DNA Cleavage analysis.*

Introduction

The increasing number of neoplastic disease associated to the high mortality rates have stimulated an unparalleled level of research directed towards the development of new lead molecules for the treatment of cancer. The progress in the understanding of biochemical basis of the cancer expression also has resulted significant development of more selective and efficient anti neoplastic agents. Anti-neoplastic molecules generally target DNA and enzymes that are involved in replication and transcription and represent the rational development in the modern drug discovery [1]. Most anticancer drugs bind either reversibly or irreversibly to DNA and proteins indicating the relationship between their interaction and therapeutic effect.

One of the most interesting classes of anticancer molecules are DNA intercalators, characterized by the presence of planar aromatic heterocyclic pharmacophore [2]. Intercalation as a mode of the reversible binding of ligands to DNA was first described by Lerman for the acridine

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derivative proflavine. A very large number of compounds has been shown to be DNA intercalating agents, and many of these show cytotoxic activity. Because of the early structure activity relationship suggesting a positive correlation between cytotoxic potency and the strength of DNA binding, and because bis-intercalation would theoretically increase DNA binding, many dimeric compounds designed as bis-intercalators were evaluated as anticancer drugs. More recently, several series of dimers of more lipophilic chromophores have shown potent and broad-spectrum activity against a variety of human solid tumor cell lines, both in culture and as xenografts in nude mice [3-4].

Bis-heterocyclic compounds are gaining increased interest in the recent past as the dimeric analogues have proven to be having better and potential biological activity than the corresponding monomer. The bis-heterocyclic molecules were also shown to exhibit such diverse pharmacological activity as antimicrobial [5-7], antifungal [8], anti-inflammatory [9], anti-viral [10] and cytotoxicity [11-16].

Commercially available antimicrobial agents suffer from unreliable effectiveness due to the emergence of many resistant microorganisms like methicillin-resistant *Staphylococcus aureus* (MRSA), chloroquine resistant *Plasmodium falciparam*, Multi drug resistant *Mycobacterium tuberculosis* and vancomycin resistant *Enterococcus faecium* (VRE) [17]. Hence such type of infections continue to be the driving force for the search and discovery of novel, more potent and selective nontraditional antimicrobial agents with less likeliness of development of cross-resistance.

Targeting DNA is an important strategy not only for developing modern cytotoxic compound but also for potent antibacterial agents. We have earlier reported the synthesis of biologically active bis-heterocyclic molecules from our lab [18-21]. It was observed that many of these bis analogues have exhibited significant DNA binding ability which correlated very well with their cytotoxicity profile as well.

In addition 1,3,4 oxadiazole derivatives have been reported to possess wide biological activities [22-24] and also many oxadiazoles are being used clinically such as hypnotic drug fenadiazole and antiviral raltegravir [25].There have been number of synthetic methods reported for the preparation of 1,3,4-oxadiazoles . Most of them deal with either the cyclodehydration of carboxylic acid hydrazides or oxidation of hydrazones by using various oxidizing agents [26-28]. Acylation of tetrazoles and some solid phase synthesis and one pot synthesis of oxadiazoles using carboxylic acids and acyl hydrazides has also been reported [29-30]. In this paper we present preparation of bis heterocycles containing 1,3,4-oxadiazoles as dual antimicrobial and cytotoxic agents by the oxidative cyclization of respective hydrazones using ceric ammonium nitrate as oxidizing agent.

Experimental

The melting points were determined in open glass capillaries and are uncorrected. The follow-up of the reactions and checking the purity of the synthesized compounds was made by thin layer chromatography (TLC) on silica gel pre-coated aluminum sheets (Type $60GF_{254}$; Merck, Germany) and the spots were detected by exposure to UV lamp at λ_{254} for a few seconds. IR spectra were recorded on Shimadzu FT-IR 8400-S spectrophotometer by KBr pellet technique. Elemental analyses were performed and found values are within 0.4% of theoretical values unless otherwise noted. ^{1}H-NMR spectra was recorded on AMX-400 NMR spectrophotometer at 400 MHz using DMSO-d_6 as the solvent and tetra methyl silane (TMS) as internal standard. The chemical shifts are expressed in δ ppm. LCMS were recorded by using Shimadzu LCMS-2010A instrument by ESI.

Chemistry

The synthesis of bis-1, 3, 4 oxadiazoles was carried out as shown in the Scheme of synthesis (Figure 1). The adipoyl dihydrazide which served as starting material was prepared from adipic acid as per the reported method [18].

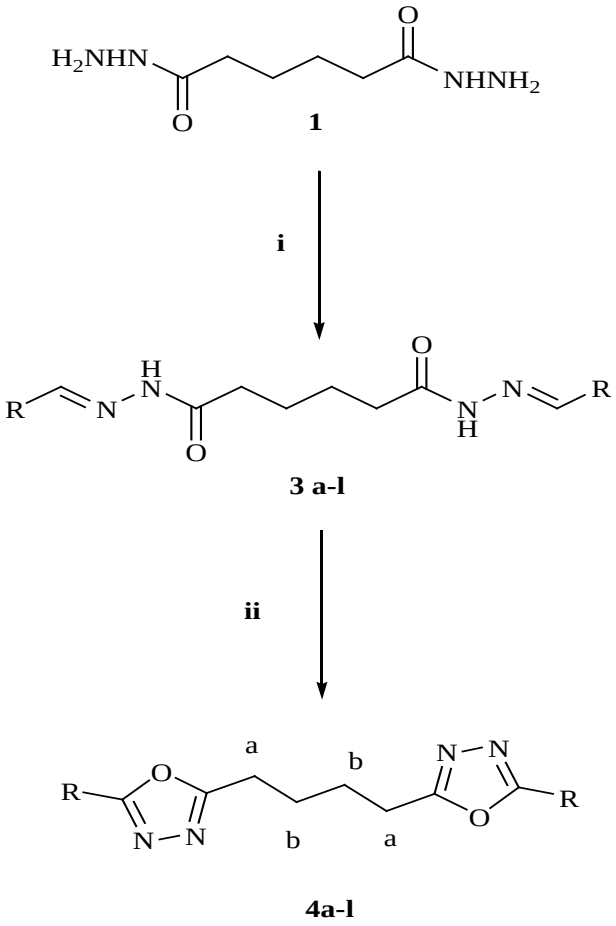

Figure 1. Scheme of synthesis, Reaction condition: i) RCHO/ AcOH, reflux ii) CAN, EtOH, reflux.

General procedure for the synthesis of diarylidene adipohydrazides (3a-l)

Conventional method

The mixture of 0.001 mole (0.174g) of adipoyl dihydrazide and 0.002 moles of appropriate aryl or heteroaryl aldehydes in 15ml of absolute alcohol and 1ml of glacial acetic acid was refluxed on water bath for 4-6 hours (monitored by TLC). After the completion of reaction the flask was cooled and the precipitate was washed with cold water, dried and then recrystallized from aqueous ethanol.

General procedure for the Synthesis of 1, 4-bis (5-substituted-1,3,4-oxadiazol-2yl) butane derivatives (4a-l)

Conventional method

The mixture of 0.001 mole of appropriate Schiff base (3a-i) and 0.0025 moles (1.1g) of ceric ammonium nitrate (CAN) in 10ml of absolute alcohol was refluxed on water bath for about 6-8 hours. The completion of the reaction was checked by TLC. The reaction mixture was poured into the crushed ice with vigorous stirring and the left at room temperature for 2 hours. The solid separated was filtered dried and crystallized from mixture of DMF and ethanol [22]. The physical data of the compounds synthesized are presented in Table 1. The spectral data of the compounds are summarized in Table 2.

Table 1. Physical data of prepared compounds.

Cpds	R	Molecular formula	Molecular weight	%yield	Melting point(^{0}C)	Elemental Analysis Calculated (found)		
						C	H	N
4a	Phenyl	$C_{20}H_{18}N_4O_2$	346	85	111	69.35 (70.01)	5.24 (5.20)	16.17 (16.23)
4b	4-methoxy phenyl	$C_{22}H_{22}N_4O_4$	406	75	193	65.01 (65.24)	5.46 (5.52)	13.78 (13.55)
4c	2-hydroxy phenyl	$C_{20}H_{18}N_4O_4$	378	69	187	63.48 (63.36)	4.79 (4.71)	14.81 (14.62)
4d	4-dimethyl amino phenyl	$C_{24}H_{28}N_6O_2$	432	80	262	66.65 (66.76)	6.53 (6.45)	19.43 (20.02)
4e	3-methoxy 4-hydroxy phenyl	$C_{22}H_{22}N_4O_6$	438	82	169	60.27 (60.41)	5.06 (5.13)	12.78 (12.49)
4f	2-chloro phenyl	$C_{20}H_{16}N_4O_2Cl_2$	415	75	226	57.84 (57.67)	3.88 (3.79)	13.49 (13.54)
4g	3-nitro phenyl	$C_{20}H_{16}N_6O_6$	436	70	144	55.05 (55.16)	3.70 (3.65)	19.26 (19.53)
4h	4-bromo phenyl	$C_{20}H_{16}N_4O_2Br_2$	502	85	212	47.04 (47.19)	3.20 (3.26)	11.11 (11.28)
4i	3,4-dimethoxy phenyl	$C_{24}H_{26}N_4O_6$	466	80	108	61.19 (61.23)	5.62 (5.68)	12.01 (12.20)
4j	2-thienyl	$C_{16}H_{14}N_4O_2S_2$	358	70	153	53.61 (53.76)	3.94 (4.03)	15.63 (15.78)
4k	3-indolyl	$C_{24}H_{20}N_6O_2$	424	82	169	67.91 (68.10)	4.75 (4.70)	19.80 (19.93)
4l	4-pyridyl	$C_{18}H_{16}N_6O_2$	348	78	187	62.06 (62.29)	4.63 (4.70)	24.12 (24.20)

Table 2. Spectral data of the synthesized compounds.

Cpds	Spectral Data
3f	**IR(cm^{-1}):** 3210(N-H);3080(Ar-CH_2);1730 (CONH); 1590(Ar C=C); **^{1}H-NMRδ(ppm):** 9.3 (s, CONH); 8.1(s,CH=N-); 7.58-8.41(m,Ar-CH,6H),2.55(t,CH_2(a),4H), 1.60(t,CH_2 (b),4H)
3g	**IR(cm^{-1}):**3200(N-H);3090(Ar-CH_2);1740 (CONH) **^{1}H-NMRδ(ppm):** 9.4 (s, CONH); 8.2(s,CH=N-); 7.37-7.49(m,Ar-CH,6H),2.55(t,CH_2(a),4H), 1.63(t,CH_2 (b),4H)
3h	**IR(cm^{-1}):**3190(N-H), 3098(Ar-CH_2); 1700 (CONH) **^{1}H-NMRδ(ppm):** 9.3 (s, CONH);8.1(s,CH=N-); 7.22-7.48(m, Ar-CH, 6H), 2.55(t, CH_2 (a),4H), 1.62(t,CH_2 (b),4H)
4a	**IR(cm^{-1}):** 3129(Ar-CH);1608(Ar C=C);1610(C=N) **^{1}H-NMRδ(ppm):** 7.22-7.48(m,Ar-H,10H), 2.55 (t,-CH_2,(a)4H), 1.6 (t,-CH_2,(b)4H). **LC-MS *m/z*:** 347 (M+H).
4b	**IR(cm^{-1}):** 3110 (Ar-CH);1604(Ar C=C);1620(C=N) **^{1}H-NMRδ(ppm):**6.83-7.37(m,Ar-H,8H),3.73(s,-OCH_3,6H);2.55(t,-CH_2,(a),4H), 1.62 (t,-CH_2,(b),4H). **LC-MS *m/z*:** 415 (M+H).
4c	**IR(cm^{-1}):** 3329(b,Ar-OH);1608(Ar C=C);1610(C=N) **^{1}H-NMRδ(ppm):**6.79-7.31(m,Ar-H,8H),5.0(s,Ar-OH,2H),2.55(t,-CH_2,(a)4H), 1.62 (t,-CH_2,(b)4H).
4d	**IR(cm^{-1}):** 3156(Ar-CH);1610(Ar C=C);1628(C=N) **^{1}H-NMRδ(ppm):**6.65-7.30(m,Ar-H,8H),2.85(s,Ar-N(CH_3)$_2$,12H), 2.55(t,CH_2,(a),4H) , 1.62 (t,-CH_2,(b),4H).
4e	**IR(cm^{-1}):** 3429(Ar-OH);1608(Ar C=C);1610(C=N) **^{1}H-NMRδ(ppm):**6.66-6.93(m,Ar-H,6H),5.0(s,-OH,2H), 3.73(s,-OCH_3,6H),2.55(t,-CH_2,(a),4H),1.62 (-CH_2,(b),4H).
4f	**IR(cm^{-1}):** 3090(Ar-CH);1600(C=N);1593(Ar C=C) **^{1}H-NMR δ(ppm):** 7.16-7.42(m,Ar-H,8H),2.55(t,-CH_2,(a),4H), 1.62(t,-CH_2,(b),4H).
4g	**IR(cm^{-1}):** 3190(Ar-CH);1608(Ar C=C);1610(C=N) **^{1}H-NMR δ(ppm):** 7.38-7.58(m,Ar-H,8H),2.55(t,-CH_2,(a),4H), 1.62(-CH_2,(b),4H). **LC-MS *m/z*:** 437 (M+H)
4h	**IR(cm^{-1}):** 3187(Ar-CH);1601(Ar C=C);1612(C=N) **^{1}H-NMR δ(ppm):** 7.37-7.49(m,Ar-H,8H),2.55(t,-CH_2,(a),4H), 1.62(-CH_2,(b),4H).

Table 2 Continued..

4i	**IR(cm^{-1}):**3189(Ar-CH);1610(ArC=C);1620(C=N) **^{1}H-NMRδ(ppm):**6.34-7.26(m,Ar-H,6H),3.73(s,Ar-OCH_3,6H); 2.55(t,-CH_2,(a),4H), 1.62(t,-CH_2,(b),4H). **LC-MS *m/z*:** 465 (M+H)
4j	**IR(cm^{-1}):** 3180(Ar-CH);1604(Ar C=C);1622(C=N) **^{1}H-NMR δ(ppm):** 7.0-7.2(m,Ar-H,6H),2.55(t,-CH_2,(a),4H); 1.62(t,-CH_2,(b),4H)
4k	**IR(cm^{-1}):** 3210(Ar-CH);1610(Ar C=C); 1623(C=N) **^{1}H-NMRδ(ppm):**10.1(d,Ar-NH,2H),7.0-7.6(m,Ar-H,10H), 2.55(t,-CH_2,(a),4H);1.62(t,-CH_2,(b),4H.
4l	**IR(cm^{-1}):** 3342(Ar-NH);3080(Ar-CH);1604(Ar C=C);1620(C=N) **^{1}H-NMR δ(ppm):** 7.64-8.65(m,Ar-H,8H),2.55(t,-CH_2,(a),4H); 1.62(t,-CH_2,(b),4H)

Biological activity

Antimicrobial activity

All the compounds were tested for *in vitro* antimicrobial activity against the following microorganisms: *Staphylococcus aureus* (Gram positive bacteria), *Escherichia coli* (Gram negative bacteria) and *Candida albicans* (fungi). The minimal inhibitory concentration (MIC) values for compounds tested, defined as the lowest concentration of the compound preventing the visible growth, were determined by using by serial dilution method [21]. The inocula were adjusted to 0.5 McFarland Standard (1.5 x 10^8 CFU/mL) were prepared from 24 h broth cultures and used for the study. The test compound dissolved in DMSO was first diluted to the highest concentration (100µg/ml) to be tested. Then serial two-fold dilutions were made in concentration ranging from 50µg/ml to 6.25µg/ml in 10ml sterile tubes. A prepared suspension of the standard microorganisms was added to each dilution in a 1:1 ratio. Growth (or its absence) of microorganisms was determined visually after incubation for 24h at 37°C for bacteria and for 48h at 24°C for fungi. A control test was also performed with test medium supplemented with DMSO at the same dilutions as used in the experiment in order to ensure that the solvent had no influence on bacterial growth. Ampicillin and fluconazole were used as standard drugs for antibacterial and antifungal activity respectively. The mean of the values of MIC obtained from three independent measurements is presented in Table 3.

In vitro cytotoxicity activity

The cytotoxicity of the compounds was evaluated *in vitro* against human lung carcinoma cells (A-549) using a standard

MTT assay [20]. The cell lines were procured from National Centre for Cell Sciences, Pune, India, and were plated in 96- well plates (10^4 cells/well in 100µl of medium) and incubated for 24 h for attachment. The test compounds were prepared prior to the study by dissolving in 0.1% DMSO and diluted with medium. The cells were then exposed to different concentration of test compounds (10, 20,50 and 100µM) in a volume of 100µl/well. After 72 h of exposure, the medium was removed and the cell cultures were incubated with 100µl of MTT reagent (0.1%) for 4h at 37 ^{0}C. the pink colored formazan was dissolved in 100µl of DMSO and absorbance of each well was read in an ELISA micro plate reader at 570 nm. The experiment was performed in triplicate and the percentage cytoxicity was calculated. The drug concentration that causes 50% cell growth inhibition after 72 h of continuous exposure to the test compounds (IC_{50}) was determined by plotting the graph of concentration of the drug against the percent cytotoxicity and performing the regression analysis. The IC_{50} values of the test compounds are shown in Table 3.

Table 3. Antimicrobial and cytotoxic activity of the synthesized compounds.

Compounds	Minimal inhibitory concentrations (MIC, in µg/ml)			Cytotoxicity IC_{50} (µM)
	S. aureus	***E. coli***	***C. albicans***	**A-549**
4a	50	12.5	50	31.18 ± 2.66
4b	6.25	6.25	12.5	12.25 ± 2.08
4c	100	12.5	25	13.31 ± 4.03
4d	12.5	50	50	69.82 ± 1.10
4e	100	50	100	82.31 ± 2.91
4f	50	25	50	57.31 ± 2.06
4g	12.5	6.25	6.25	84.31 ± 2.11
4h	50	100	100	87.31 ± 3.58
4i	50	12.5	50	60.31 ± 1.39
4j	25	12.5	6.25	14.31 ± 3.90
4k	ND	50	ND	ND
4l	ND	50	ND	ND
Ampicillin	6.25	6.25	-	-
Fluconazole	-	-	6.25	-
5-flurouracil	-	-	-	08.31 ± 0.90

ND= Not determined

DNA Cleavage Analysis

The compounds 4b, 4g and 4j were further evaluated for DNA cleavage assay using DNA isolated from *E. coli* to study the effect of these compounds on bacterial DNA [31]. The fresh bacterial culture of *E. coli* (1.5 ml) was centrifuged and the pellet obtained was dissolved in 0.5 ml of the lysis buffer

(100 mM tris pH 8.0, 50 mM EDTA, 50 mM lysozyme). This solution was incubated for 10 min at 55^{0}C and was centrifuged at 10,000rpm for 10 min and to the supernatant liquid 3 volumes of chilled absolute alcohol was added. The precipitated DNA was separated by centrifugation, dried the pellet and dissolved in Tris buffer (10 mM tris pH 8.0) and used for the analysis. The DNA was exposed to the samples separately (compounds **4b, 4g** and **4j** at 50 µg/ml and 100 µg/ml concentration) and were incubated at 37ºC for 2 h. the DNA exposed with samples was further subjected to agarose gel electrophoresis by loading 20 µl of DNA sample carefully into the wells, along with standard DNA marker and passing the constant 50 V of electricity for around 30 min. The gel was removed carefully and stained with ethidium bromide solution (10 µg/ml) for 10-15 min and the bands were observed under UV trans-illuminator (Figure 2).

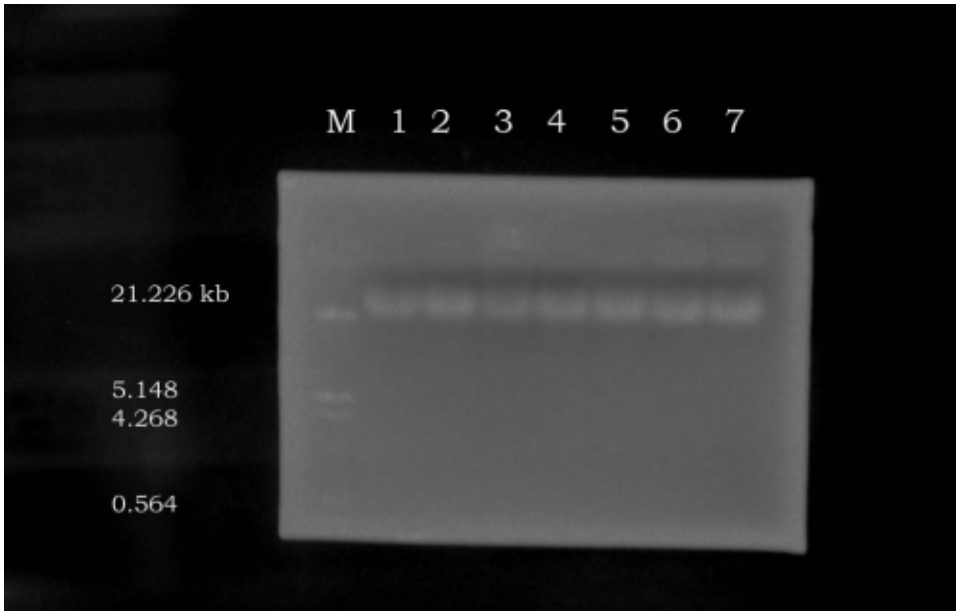

Figure 2. DNA Cleavage Analysis.

Note: M) Standard DNA marker; 1) Control *E. coli* DNA (untreated sample); 2) 4b (50µg); 3) 4b (100µg); 4) 4g (50µg); 5) 4g (100µg); 6) 4j (50 µg); 7) 4j (100µg).

Results and discussion

The synthesis of bis-1,3,4-oxadiazoles by oxidative cyclization of corresponding Schiff bases using ceric ammonium nitrate is presented. The Schiff bases **3a-l** were prepared from the reaction of adipoyl-di-hydrazide and different aryl/hetero aryl carboxaldehydes in alcoholic medium using glacial acetic acid as catalyst. All the Schiff bases were obtained in good yield (65-75%). The IR spectrum of compound **3g** showed absence of absorption due to amino group at

3330 cm^{-1}. The absorptions at 3090 cm^{-1} due to aromatic C-H str indicates the presence of aromatic ring system in the compound. The ^{1}H-NMR spectra of the compound **3g** showed the following signals. The signal at δ 8.2 ppm is attributed to –CH=N- group of the Schiff base. Aromatic protons resonated between δ7.37-7.49 ppm as multiplet. The C_1 and C_4 methylene protons of butyl chain resonated as triplet at δ 2.55 ppm while those of C_2 and C_3 methylene group appeared at δ1.63 ppm.

The Schiff bases are then cyclized using CAN as catalyst as per the reported method [22]. The bis-1,3,4-oxadiazoles were obtained in good yield (69-95%). The ^{1}H-NMR spectrum of compound **4g** showed the absence of signals due to -CONH and CH=N-groups at 9.4 and 8.2 ppm respectively. This indicates the cyclisation of the oxadiazole. Further the M+1 peak at 437 in mass spectrum confirmed the oxadiazole formation. Similarly other molecules were found to be in agreement with the proposed structures.

The in vitro anti bacterial activity was evaluated against *Staphylococcus aureus* and *Escherichia coli* while anti fungal activity was performed against *Candida albicans* by two fold serial dilution methods. The minimal inhibitory concentration (MIC) values expressed in µg/ ml for all the compounds is given in table 2. From the data presented in the table 2, it is clear that the compounds **4b**, **4g** and **4j** exhibited higher activity than the other compounds. The type of the substitution on the phenyl ring appears to influence the degree of anti microbial activity. The methoxy group at the *para* position confers higher activity as in compound **4b** however additional methoxy group at *meta* position seems to decrease the activity as evident from the data for compound **4e**. the strong electron withdrawing group like nitro functional group at meta position as in compound **4g** exhibited higher activity. Interestingly the halo substituted molecules **4f** and **4h** failed to exhibit significant anti microbial activity. Among the hetero aryl substituted oxadiazoles only thienyl containing derivative **4j** was found to be active.

The *in vitro* cytotoxicity activity of the oxadiazoles was evaluated by standard MTT protocol against A-549 human lung carcinoma cells. From the cytotoxicity data presented in Table 3, it is clear that the compounds **4b**, **4c** and **4j** exhibited higher cytotoxicity in comparison to 5-flurouracil. Thus compounds 4b and 4j can be considered as dual anti microbial and cytotoxic agents, however further studies are necessary to confirm the safety of these lead molecules.

The compounds **4b**, **4g** and **4j** were further studied for their effect on bacterial DNA by DNA cleavage analysis using DNA isolated from *Escherichia coli.* This test was done to know whether the bacterial DNA undergoes cleavage on being exposed to the drug sample.

The result of the analysis is depicted in Fig 2. The compounds were loaded at two different concentrations of 50 µg/ ml and 100 µg/ ml and the DNA exposed were run on agarose gel electrophoresis. From the Figure 2, it is clear that only compound 4b at 100 µg/ ml concentration exhibited a weak DNA cleavage potential while other molecules did not show any DNA cleavage activity. Thus we can conclude that the compounds are not having direct action on bacterial DNA and their antimicrobial property could have different mechanism of action. Further tests such as dihydro folate reductase (DHFR) enzyme inhibition assay are underway to elucidate the possible mechanism of action.

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