International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.7, No.5, pp 2520-2531, 2014-2015

A Study on the Antimicrobial and Antioxidant Activities of Some New 1, 3, 4-Thiadiazole Derivatives

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Abstract: The various derivatives of thiadiazole were prepared by the reaction of *m*-chlorophenyl isocyanate and *p*-chlorophenyl isocyanate with 5-(substituted phenyl) -2-amino-1, 3, 4-thiadiazole in dry acetonitrile. Elemental analysis, IR, ^{1}H NMR, mass spectral data confirmed the structure of the newly synthesized compounds. All the derivatives of these moieties were evaluated for *invitro* antimicrobial and *invitro* antioxidant activity. Most of the synthesized compounds showed potent antimicrobial activity at 100 and 50 µg/ml and antioxidant scavenging avtivity. Compounds showed most significant antibacterial activity against gram positive test organism *S.aureus* and most significant antifungal activity against test organisms *Aspergillus niger* and *Candida albicans.* All the compounds were screened for *in vitro* antioxidant activity by various methods as Scavenging of DPPH, Scavenging of nitric oxide radical inhibitory activity.
Keywords: Synthesis; 1, 3, 4-Thiadiazole; Antimicrobial activity, Antioxidant activity, Scavenging, Hydrogen peroxide, Nitric oxide radical.

1. Introduction

Heterocyclic Compounds are the cyclic compounds having as ring members atoms of at least two different elements, e.g. quinolone, 1, 2-thiazole, bicycle [3.3.1] tetrasiloxane[1].Usually they are indicated as counterparts of carbocyclic compounds, which have only ring atoms from the same element. Although heterocyclic compounds may be inorganic, most contain within the ring structure at least one atom of carbon, and one or more elements such as sulfur, oxygen, or nitrogen[2]. Since non-carbons are usually considered to have replaced carbon atoms, they are called heteroatoms. The structures may consist of either aromatic or non-aromatic rings. The biological activity of the compounds is mainly dependent on their molecular structures[3]. A recent literature survey revealed that the 1, 3, 4-thiadiazole moiety have been widely used by the medicinal chemist in the past to explore its biological activities. It has been shown that 1, 3, 4-thiadiazole derivatives have multiple biological activities including anticancer [4-6], antibacterial [7-9],antifungal[10], anti-inflammatory [11-13] and antidepressant[14] activity. In fact the structure of 'N–C–S' in 1, 3, 4-thiadiazole derivatives work as active centre. Motivated by these findings it is proposed to synthesize and investigate the antioxidant activity of a new series of molecules having amide backbone to diazole nucleus which will definitely contribute substantially in increasing pharmacological activity. Numerous 1, 3, 4-thiadiazoles have been synthesized and reported to be biologically versatile compounds having bactericidal, fungicidal, muscle relaxant properties[15]. Keeping in view, the development of novel compounds containing 1, 3, 4-thiadiazole with improved profile is still a necessity. Hence, in the present work we have synthesized some new 1, 3, 4-thiadiazole derivatives in association with the other molecules like chlorophenyl isocyanate with different functional groups to get new series of compounds.

2. Experimental

2.1. Materials and Methods:

2.1.1. Measurements:

Melting points were determined in open capillary method and are uncorrected. The IR spectra were obtained by potassium bromide pellets using Thermo Nicolet iS 10 FT-IR spectrometer (Thermo scientific). ^{1}H-

NMR spectra were recorded on Bruker 400using TMS as an internal standard. The mass spectra of 1a-3a and 1b-3b were measured on Shimadzu 2010A LC- MS. Elemental analysis apparatus was Euro EA Elemental Analyser. Purity of all synthesized compounds was checked by thin layer chromatography technique (0.2 mm thickness of silica gel GF plates) and iodine was used as visualizing agent.

2.1.2. Synthesis:

2.1.2.1.1. General procedure for the synthesis of 5-(2-hydroxyphenyl)-2-amino - [1, 3, 4]-thiadiazole (a, b, c)[16,17]:

A mixture of thiosemicarbazide (0.1 mole), aryl carboxylic acid (0.1 mole) and conc.sulphuric acid (5ml) in 50 ml of ethanol was refluxed for 2-3hour. Reaction was monitored by TLC using mobile phase Chloroform: methanol (4:1). After completion of the reaction the reaction mixture was poured on to crushed ice. The solid separated out was filtered, washed with cold water and recrystallized from ethanol to give colourless crystals, Yield 90%, m.p.183-186^{o}C.

IR (KBr) ν/(cm^{-1}): 3514.22 (O-H, st.), 663.92, 688.65 (C-S-C, st.), 3428.26 (NH_2,N-H,st.), 1616.23 (C=N, st.), 1425.76 (Aryl C=C, st.), 3018.75(Aryl C-H, st.); ^{1}H NMR (400MHz, DMSO-*d*6) δ 6.89-7.20(m, 4H, ArH), 10.32 (s, 1H, OH), 2.60-2.65 (bs, 2H, NH_2), Mass spectrum m/z: 193 (M^+).

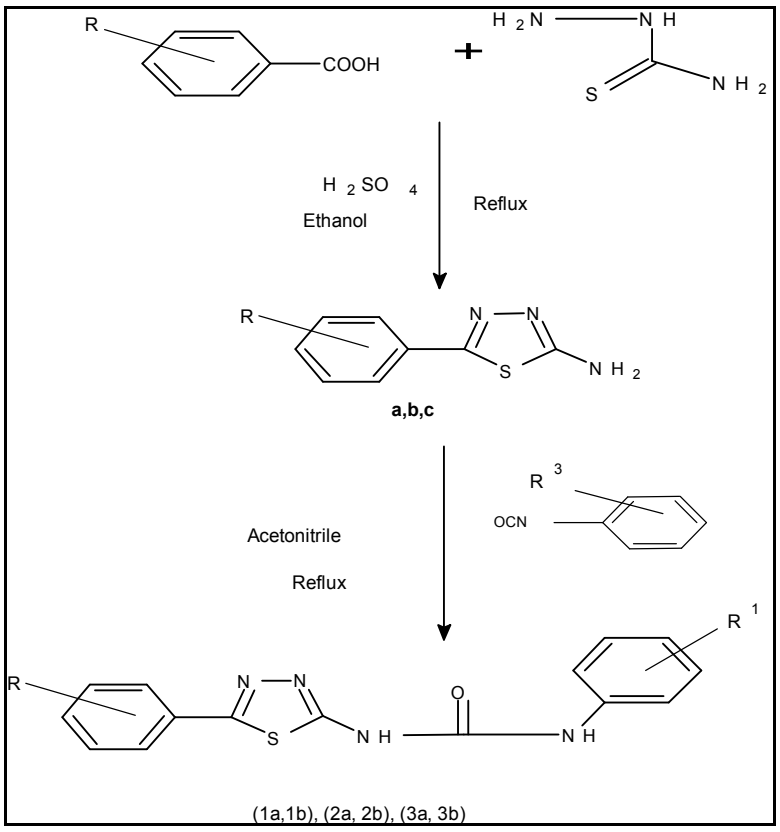
2.1.2.1. 2. Synthesis of 5-(3-chlorophenyl) -2-amino - [1, 3, 4]-thiadiazole (b):

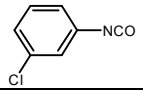
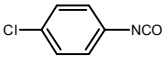
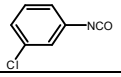
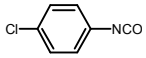
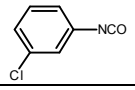
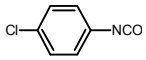
Yield 79%, m.p.165-167^0 C ; IR (KBr) ν/(cm^{-1}): 762.35 (C-Cl, st.), 682.35 (C-S-C, st.), 3447.21 (NH_2, N-H,st.), 1647.11 (C=N, st.),1491.70(C-N, st.), 1425.79 (Aryl C=C, st.),3025.25(Aryl C-H, st.); ^{1}H NMR (400MHz, DMSO-*d*6) δ 6.95-7.35 (m, 4H, ArH), 2.48 (bs, 2H, NH_2), Mas spectrum m/z :211(M^+).

2.1.2.1.3. Synthesis of 5-(4-nitrophenyl) -2-amino - [1, 3, 4]-thiadiazole (c):

Yield 88%, m.p.225-227^{0}C ; IR (KBr) ν/(cm^{-1}): 1375.53 , 1545.11, (NO_2, st.), 687.73 (C-S-C, st.), 34.50.78 (NH_2, N-H,st.), 1649.95 (C=N, st.), 1416.45 (Aryl C=C, st.),3087.72(Aryl C-H, st.); ^{1}H NMR (400MHz, DMSO-*d*6) δ 7.30-7.73(m, 4H, ArH), 2.59 (bs, 2H, NH_2), Mass spectrum m/z: 222 (M^+).

2. 1. 2. 2. Synthesis of 1-(substituted phenyl)-3-[5-(2-substitutedphenyl)-1, 3, 4-thiadiazol-2yl] urea 1(a, b), 2(a, b), 3(a, b):



Compd	R	R^1
1a	2OH	
1b	2OH	
2a	4Cl	
2b	4Cl	
3a	$2NO_2$	
3b	$2NO_2$	

1-(substituted phenyl)-3-[5-(2-substitutedphenyl)-1, 3, 4-thiadiazol-2yl] urea were obtained to scheme 1. *m* & *p*-chlorophenyl isocyanate (2 mmol) was slowly added to a well stirred solution of 2-amino-5(substituted phenyl)-1,3,4-thiadiazole(2 mmol), (a, b &c) in dry acetonitrile (12 ml). After complete addition, the reaction mixture was kept for 30 min at room temperature; the mixture was heated on water bath under reflux until completion (TLC detection) of the reaction. The residue was cooling; the precipitated product was separated and recrystallized from ethanol to give the crystalline product. Purity of the compounds was analyzed by n-Hexane: methanol (9:1) as mobile phase. The physical data and spectral study of the synthesized compounds were shown in the **Table 1 and 2**.

Table 1: Physical data of the synthesized compounds

Compd	Mol. formula	Mol.Wt	m.p. ^{0}C	Yield %	Rf	Elemental analysis					
						Calculated			Found		
						C	H	N	C	H	N
1	$C_8H_7N_3OS$	193.22	185-186	90	0.71	49.73	3.65	21.75	49.63	3.85	21.71
2	$C_8H_6ClN_3S$	211.67	165-167	79	0.72	45.39	2.86	19.85	45.12	2.67	19.80
3	$C_8H_6N_4O_2S$	222.22	225-227	88	0.70	43.24	2.72	25.21	43.20	2.67	25.20
1a	$C_{15}H_{11}ClN_4O_2S$	346.79	202-204	78	0.81	51.95	3.20	16.16	50.95	3.12	16.67
1b	$C_{15}H_{11}ClN_4O_2S$	346.79	212-214	85	0.83	51.95	3.20	16.16	51.78	3.22	16.06
2a	$C_{15}H_{10}Cl_2N_4OS$	365.23	170-172	90	0.79	49.33	2.76	15.34	49.30	2.96	15.14
2b	$C_{15}H_{10}Cl_2N_4OS$	365.23	245-248	85	0.78	49.33	2.76	15.34	48.73	2.60	14.94
3a	$C_{15}H_{10}ClN_5O_3S$	375.78	224-216	66	0.80	47.94	2.68	18.64	47.34	2.98	17.66
3b	$C_{15}H_{10}ClN_5O_3S$	375.78	230-232	78	0.84	47.94	2.68	18.64	46.94	1.98	18.60

Table 2: ^{1}H- NMR, FT-IR and MASS data of compounds (1a-3a and 1b-3b)

Cpd No.	Spectral data
1a	^{1}H NMR (400MHz, DMSO-*d*6) δ 6.88-7.31 (m, 4H, ArH); 7.47-7.79 (m, 4H, ArH); 10.10 (s, 1H, OH); 5.89 (s, 2H, CH_2-NH); 5.84 (s, 1H, CONH) ; IR *v*/(cm^{-1}): 3500.02 (O-H, st.), st.), 3424.54 (N-H, st.), 1653.93 (C=N, st.), 1745.65 (C=O, st.), 3272.44 (CON-H, st.) 1440.52 (Aryl C=C, st.), 771.20 (C-Cl) ; Mass *m/z* : 346(M^+)
1b	^{1}H NMR (400MHz, DMSO-*d*6) δ 7.30-7.56 (m, 4H, ArH); 7.91-7.94 (m, 4H, ArH); 10.15 (s, 1H, OH); 6.15 (s, 2H, CH_2NH) ; 6.13 (s, 1H, CONH) ;IR *v*/(cm^{-1}): 3510.17 (O-H, st.), 3411.75 (N-H, st.), 1624.11 (C=N, st.), 1491.79 (C-N, st.) 1701.17 (C=O, st.), 3295.76 (CON-H, st.), 775.30 (C-Cl) ; Mass *m/z* : 347 (M^++1)$^+$
2a	^{1}H NMR (400MHz, DMSO-*d*6) δ 7.00-7.31 (m, 4H, ArH); 7.52-7.94 (m, 4H, ArH); 6.00 (s, 2H, CH_2NH); 5.90 (s, 1H, CONH) ; IR *v*/(cm^{-1}): 763.27, (C-Cl), 683.56 (C-S-C, st.), 1652.42 (C=O, st.), 3289.59 (CON-H, st.) ; Mass *m/z* : 365(M^+)
2b	^{1}H NMR (400MHz, DMSO-*d*6) δ 7.45-7.55 (m, 4H, ArH); 7.90-7.94 (m, 4H, ArH); 5.54 (s, 2H, CH_2NH); 5.53 (s, 1H, CONH) ;IR *v*/(cm^{-1}): 712.80, 762.25(C- Cl), 640.03, 682.10 (C-S-C, st.), 3372.75 (N-H, st.), 1681.45 (C=O, st.), ; Mass *m/z* : 364 (M^+-1)$^+$

3a	^{1}H NMR (400MHz, DMSO-*d*6) δ 7.60-8.45 (m, 4H, ArH); 7.00-7.31 (m, 4H, ArH); 5.99 (s, 2H, CH_2N<u>H</u>); 5.78 (s, 1H, CON<u>H</u>) ;IR *v*/(cm^{-1}) : 790.03 (C-Cl), 687.92 (C-S-C, st.), 3424.01 (N-H, st.), 1700.38 (C=O, st.), 3158.73 (CON-H, st.),1535.07 (NO_2) ; Mass *m/z* : 374 $(M^+-1)^+$
3b	^{1}H NMR (400MHz, DMSO-*d*6) δ 7.03-7.62 (m, 4H, ArH); 7.70-8.29 (m, 4H, ArH); 6.12 (s, 2H, CH_2N<u>H</u>); 5.02 (s, 1H, CON<u>H</u>) ;IR *v*/(cm^{-1}): 752.52, 776.42 (C-Cl), 624.66, 691.03 (C-S-C, st.), 3423.95 (N-H, st.), 1490.78 (C-N, st.), 1600.81 (C=N, st.), 1690.50 (C=O, st.), 3159.61 (CON-H, st.) 1438.89 (Aryl C=C, st.), 2979.91 (Aryl C-H, st.), 1535.91 (NO_2) ; Mass *m/z* : 375(M^+), 374$(M^+-1)^+$

2.2. Assessment of *In-Vitro* Antimicrobial Activity:

2.2.1. Evaluation of antimicrobial activity:

Evaluation of antimicrobial activities of the test compounds was performed by Cup plate method using muller –Hinton agar medium. The cups of 9 mm diameter were made by scooping out medium with a sterilized cork borer in Petridis which was streaked with the organisms and The solutions of each test compound (0.05 ml, 50 µg/ml & 0.1 ml, 100 µg/ml) were added separately in the cups using a micropipette. The plates were incubated at 37^{0}C for bacteria and 26^{0}C for fungi. Following incubation the plates were observed for zones of inhibition. The inhibition zone around the cup as calculated edge to edge zone of confluent growth which is usually corresponds to the sharpest edge of the zone and to be measured diameter in millimeter.

2.2.1.1. Zone of inhobition[18-20]:

All synthesized compounds were screened for their The *in vitro* antimicrobial activity of all the compounds and standard drugs were assessed against two representatives of gram-positive bacteria *viz Bacillus subtilis* (ATCC11774), *Staphylococcus aureus* (NTCC-6571), two gram-negaitive bacteria *viz Escherichia coli* (TG14) , *Pseudomonas aeruginosa* (ATCC9027) and two fungi *viz*. *Aspergillus niger* and *Candida albicans* by cup plate method. The inhibition zone was measured in mm using Amoxycillin,Ofloxacin and Amphoteracine B as standards in dimethyl sulphoxide (DMSO). DMSO showed no inhibition zone. Each compound and standard drugs were diluted obtaining 1000 µg/ml concentration, as a stock solution. All the compounds were tested at a concentration of 50 µg/ml and 100 µg/ml. Each experiment was repeated twice and the average of the two determinations was recorded. **Then** the potency of the compounds was calculated by using the following formula as described by Edwin and Marion[21].

$$Potence = Antilog\left[2 + d\left(\frac{(U2+U1)-(S2+S1)}{(U2-U1)+(S2-S1)}\right)\right]$$

In the above equation, 2 is the factor for converting to percent and *d* is the log of the ratio of the stronger concentration to the weaker. This ratio between dilutions must be the same for both the standard and the compound being assayed.

U2 is zone of inhibition of compound at 100µg/ml, U1 is zone of inhibition of compound at 50µg/ml, S2 is zone of inhibition of standard at 100µg/ml, S1 is zone of inhibition of standard at 50µg/ml. The results were reported in **Table 3** (Figure 1, 2), **Table 4** (Figure 3, 4) and **Table 5** (Figure 5, 6).

Table 3: *In vitro* antibacterial (gram + ve) activity: Zone of inhibition in (mm)

Compds	*S. aureus*		Amoxycilline		%Potency	*B. subtilis*		Amoxycilline		%Potency
	U2	U1	U2	U1		U2	U1	U2	U1	
1a	20	17	29	23	**59.97**	18	10	27	22	**61.80**
1b	22	15	29	23	**86.69**	21	12	27	22	**87.49**
2a	20	12	29	23	**69.98**	19	11	27	22	**68.39**
2b	22	14	29	23	**84.52**	20	11	27	22	**77.80**
3a	21	18	29	23	**69.18**	18	9	27	22	**63.24**
3b	21	14	29	23	**76.38**	20	11	27	22	**77.04**
DMSO	----	----	----	----	----	----	----	----	----	----

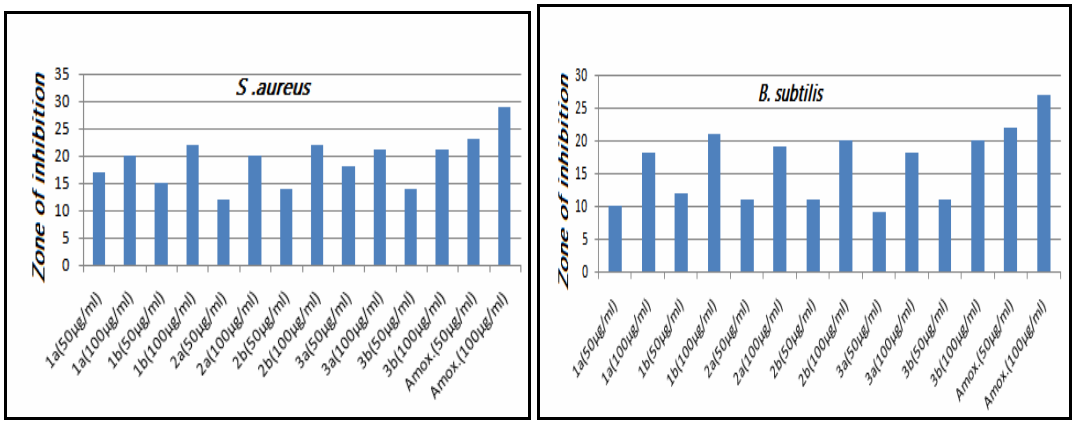


Figure 1 &2: Zone of Inhibition of Synthesized compounds against gram (+ve) organosms

Table 4. In *vitro* antibacterial (gram -ve) activity: Zone of inhibition in (mm)

Compds	*E. coli*		Ofloxacin		%Potency	*P. aeruginosa*		Ofloxacin		%Potency
	U2	U1	U2	U1		U2	U1	U2	U1	
1a	**11**	**9**	17	13	**59.97**	**11**	**9**	19	14	**53.09**
1b	**12**	**10**	17	13	**74.98**	**12**	**10**	19	14	**67.92**
2a	**11**	**10**	17	13	**55.46**	**11**	**8**	19	14	**57.01**
2b	**12**	**11**	17	13	**71.28**	**12**	**9**	19	14	**63.09**
3a	**10**	**7**	17	13	**53.82**	**10**	**7**	19	14	**49.88**
3b	**11**	**9**	17	13	**60.11**	**11**	**7**	19	14	**59.97**
DMSO	----	----	----	----	----	----	----	----	----	----

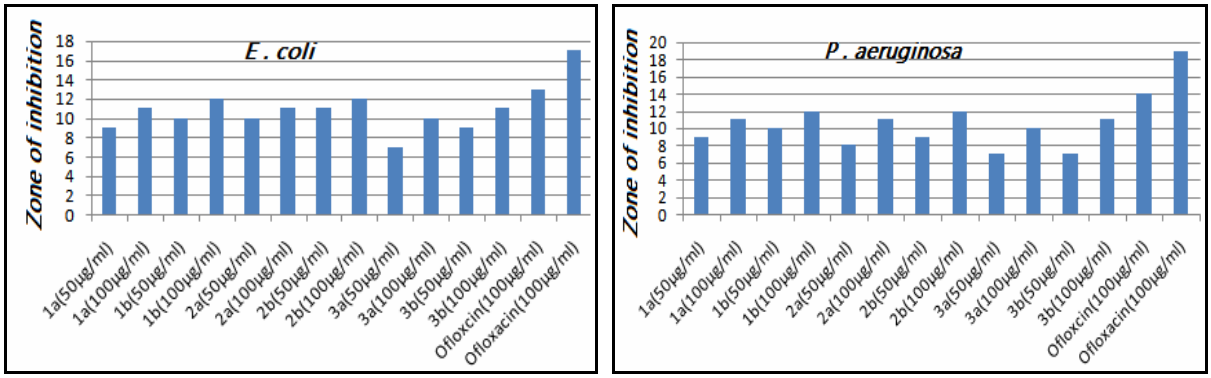


Figure 3 &4 : Zone of Inhibition of Synthesized compounds against gram (-ve)organisms

Table 5: *In vitro* antifungal activity: Zone of inhibition in (mm)

Compounds	*A. niger*		Amph. B		%Potency	*C.albicans*		Amph. B		%Potency
	U2	U1	S2	S1		U2	U1	S2	S1	
1a	10	9	18	12	**63.53**	9.4	6.3	16	11	**71.61**
1b	11	8	18	12	**82.41**	10.2	7.8	16	11	**82.60**
2a	9	7	18	12	**64.12**	9.6	8.1	16	11	**69.82**
2b	11	9	18	12	**79.98**	10	9	16	11	**74.13**
3a	9	7	18	12	**57.01**	9.1	7.2	16	11	**64.41**
3b	10	9	18	12	**66.52**	9.4	6.3	16	11	**71.61**
DMSO	----	----	----	----	**----**	----	----	----	----	----

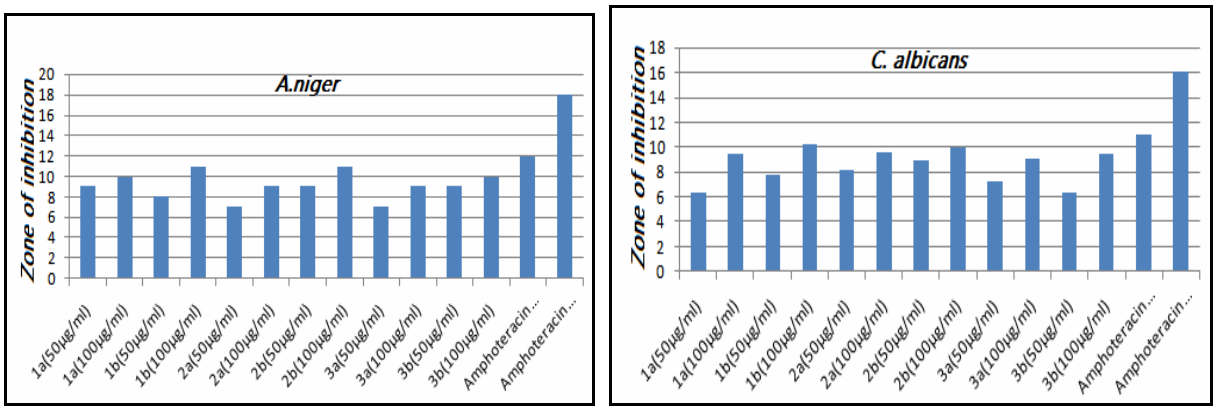


Figure 5 &6 : Zone of Inhibition of Synthesized compounds against Fungi

2.2.1.2. Minimum inhibitory concentration (MIC) [22] :

The lowest concentration (highest dilution) of compound preventing the appearance of turbidity is considered to be the minimal inhibitory concentration (MIC). At this dilution the compound is known to be bacteriostatic. Bacterial strains were primarily inoculated into Mueller-Hinton agar for overnight growth. A number of colonies were directly suspended in saline solution until the turbidity matched the turbidity of the McFarland standard (approximately10^8 CFU ml^{-1}), i.e., inoculum size for test strain was adjusted to10^8 CFU ml^{-1} (Colony Forming Unit per milliliter) per well by comparing the turbidity (turbidimetric method). Similarly, fungi were inoculated on Sabouraud Dextrose broth and the procedures of inoculum standardization were similar. DMSO was used as diluents/vehicle to get desired concentration of the synthesized compounds and standard drugs to test upon standard microbial strains, i.e., the compounds were dissolved in DMSO and the solutions were diluted with a culture medium.

Each compound and standard drugs were diluted obtaining 2000 µg/ml concentration, as a stock solution. By further progressive dilutions with the test medium, the required concentrations were obtained for primary and secondary screening. In primary screening, 0.1ml of culture (bacterial and fungal) was added to the solution of 1000, 500 and 250 µg/ml concentrations of the synthesized compounds were tested.The active compounds found in this primary screening were further diluted to obtain 200, 100, 62.5, 50, 25, 12.5 and 6.250 µg/ml concentrations for secondary screening to test in a second set of dilution against all microorganisms. Eight MIC tubes were taken and labeled it as number 1-8. Briefly, the control tube (8^{th} tube) containing no antibiotic is immediately sub cultured [before inoculation] by spreading a loopful evenly over a quarter of plate of medium suitable for the growth of the tested organism. The tubes are then put for incubation at 37 ^{0}C for 24 h for bacteria and 48 h for fungi. Growth or a lack of growth in the tubes containing the antimicrobial agent was determined by comparison with the growth control, indicated by turbidity. The lowest concentration that completely inhibited visible growth of the organism was recorded as the minimal inhibitory concentration (MIC, µg/ml), i.e., the amount of growth from the control tube before incubation (which represents the original inoculum) is compared. A set of tubes containing only seeded broth and the solvent controls were maintained under identical conditions so as to make sure that the solvent had no influence on strain growth. The result of this is much affected by the size of the inoculums. The test mixture should contain 10^8 CFU ml^{-1} organisms. The interpretation of the results was based on Amphotericin B break points for the fungi and also on amoxicillin and ofloxacillin for bacterial pathogens. The protocols were summarized in **Table 6** as the minimal inhibitory concentration (MIC, µg/ml).

Table 6: Minimum inhibitory concentration (MIC, µg/ml)

Compd.	Gram-positive bacteria		Gram-negative bacteria		Fungi	
	S.aureus	B. subtilis	E. coli	P. aeruginosa	A. niger	C. albican
1a	250	500	250	500	500	250
1b	50	200	50	200	200	100
2a	200	500	250	500	500	500
2b	50	250	100	250	200	200
3a	250	500	500	500	500	500
3b	100	250	250	250	250	250

Amoxicillin	50	250	----	----	----	----
Amphotericin B	----	----	----	----	250	100
Ofloxacin	----	----	100	250	----	----

***(----) Showed no activity.*U2: Zone of inhibition of compound at 100 µg/ml; U1: Zone of inhibition of compound at 50 µg/ml; S2: Zone of inhibition of standard at 100 µg/ml; S1: Zone of inhibition of standard at 50 µg/ml. Amph.- Amphotericin B.**

2.2.2. Assessment of *In-Vitro* Antioxidant Screening*:*

2.2.2.1. DPPH radical scavenging activity:

The nitrogen centered stable free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) has often been used to characterize antioxidants. It is reversibly reduced and the odd electron in the DPPH free radical gives a strong absorption maximum at λ517 nm using SPECORD® 50 plus (analytic jena) spectrophotometer, which is purple in colour. This property makes it suitable for spectrophotometer studies. A radical scavenging antioxidant reacts with DPPH stable free radical and converts it into 1, 1-diphenyl-2-picrylhydrazine. The resulting decolorization is stoichiometric with respect to the number of electrons captured. The change in the absorbance produced in this reaction has been used to measure antioxidant properties.

The hydrogen atom or electron donation ability of the compounds was measured from the bleaching of the purple colored methanol solution of DPPH radical. The spectrophotometric assay uses the stable radical DPPH as a reagent. To 4 ml of 0.004% (w/v) methanol solution of DPPH, 1 ml of various concentrations of the test compounds (4, 8, 10 μg/ml) in methanol were added. After a 30 min incubation period at room temperature, the absorbance was read against blank at λ 517 nm[23]. Ascorbic acid was used as the standard. The percent of inhibition (I %) of free radical production from DPPH was calculated by the following equation

$$I\% = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of the control reaction (containing methanolic DPPH and ascorbic acid), A_{sample} is the absorbance of the test compound (containing methanolic DPPH and test compound).Tests were carried out in triplicate. The results were reported in **Table 7** (Fig. 7).

Table 7 - %DPPH Radical Scavenging activity.

Compound No.	% inhibition (Mean± S.D)		
	DPPH scavenging (%)		
	4µg/ml	8µg/ml	10µg/ml
1a	63.914±0.069	68.559±0.145	70.254±0.106
1b	64.594±0.088	68.922±0.154	70.784±0.104
2a	60.796±0.025	66.075±0.138	68.466±0.090
2b	61.384±0.028	66.451±0.063	68.859±0.112
3a	62.400±0.035	67.154±0.073	69.585±0.108
3b	63.415±0.148	67.783±0.140	69.885±0.152
Standard	73.15± 0.045	80.954±0.039	83.826±0.081
Blank	-----	-----	-----

Values are mean ± SEM (n=3); Standard = Ascorbic acid ;(----) Showed no scavenging activity.

2.2.2.2. Nitric oxide scavenging activity:

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 mL), phosphate buffer saline (pH 7.4, 1 ml) and test samples or standard, ascorbic acid solution in dimethyl sulphoxide (1 mL) at various concentrations (4, 8, 10 µg/ ml) was incubated at 25^0C for 150 min. After incubation, 0.5 mL of reaction mixture containing nitrite ion was removed, 1 ml of sulphanillic acid reagent was added to this, mixed well and allowed to stand for 5 min for completion of diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min in diffused light. A pink colored chromophore was formed. The absorbance was measured at λ 640 nm[24] using SPECORD® 50 plus (analytic jena) spectrophotometer. Ascorbic acid was used as standard. NO scavenging activity was calculated by the following equation-

% of scavenging=$[(A_{control} - A_{sample}) / A_{control}] \times 100$

where $A_{control}$ is the absorbance of the control reaction (containing all reagents and Ascorbic acid), A_{sample} is the absorbance of the test compound (containing all reagents and test compound). Tests were carried out in triplicate. The results were reported in **Table 8** (Figure 8)

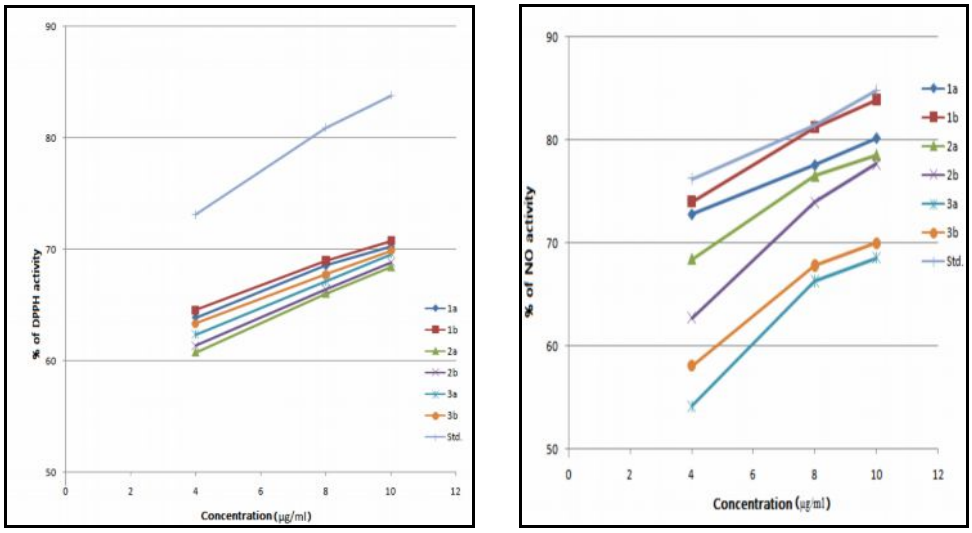


Figure 7& 8: Free radical scavenging activity of compound (1a-3a and 1b-3b) by DPPH and NO method .

Table 8: %NO Radical Scavenging activity.

Compound No.	% inhibition (Mean± S.D)		
	Nitric oxide radical (NO) scavenging (%)		
	4 µg/ml	8µg/ml	10µg/ml
1a	72.812±0.090	77.564±0.080	80.160±0.083
1b	74.043±0.082	81.225±0.122	83.859±0.154
2a	68.453±0.026	76.542±0.054	78.557±0.160
2b	62.751±0.072	73.908±0.175	77.651±0.183
3a	54.170±0.126	66.287±0.067	68.555±0.152
3b	58.108±0.145	67.851±0.065	70.001±0.075
Standard	76.246±0.017	81.460±0.137	84.794±0.080
Blank	-----	-----	-----

Values are mean ± SEM (n=3); Standard = Ascorbic acid ; (----) Showed no scavenging activity.

3. Results and Discussion:

3.1. Chemistry:

Treatment of aryl carboxylic acid in absolute ethanol with thiosemicarbazide afforded the corresponding 2-amino-5(substituted phenyl)-1, 3, 4-thiadiazole (a, b and c). Molecular formula of the compounds (Table 1) derived from elemental analyses data are supported by their molecular weight. The IR spectrum of a showed characteristic absorption bands at 3428 cm^{-1} characteristic due to NH_2 functions in addition to the -OH absorption band at 3514 cm^{-1} .Its ^1H NMR spectrum revealed the characteristic signal at δ 10.32 assigned to OH protons, two characteristic signals at δ 2.60 and 2.65 assigned to NH_2 protons which is exchangeable with D_2O, confirming the formation of thiadiazole. Also, its mass spectrum showed the molecular ion peak at *m/z* 193 [M^+] . IR data of synthesized (1a , 1b, 2a, 2b, 3a and 3b) thiadiazole analogues clearly shows C=N stretching band around 1600-1653 cm^{-1} and C–S-C absorption band around 624-687 cm^{-1} which indicates ring closure of 1,3,4-thiadiazole ring. All the final compounds have strong absorption around 2985-3093, cm^{-1} which was evidence for the presence of aromatic C–H bonds. Presence of aromatic N-H bonds was confirmed by presence of absorption band at around 3372- 3424 cm^{-1}. The most characteristic FTIR bands of the synthesized compounds (1a , 1b, 2a, 2b, 3a and 3b) were appeared at 1745,1701,1652,1681,1700 and 1690 cm^{-1} for C=O and 3500 ,3510 , 763,662,1535 and 1536 cm^{-1} for OH, C-Cl and NO_2 functions, respectively. IR data also confirms the presence of specific functional groups present in the all six synthesized

compounds. In ^{1}HNMR spectra, NH proton at C-2 of the thiadiazole moiety were seen at δ 5.89, 6.15, 6.00, 5.54, 5.99 and 6.12, respectively, for all six compounds. The ^{1}H-NMR and mass spectra of synthesized compounds were in conformity with the assigned structure. The mass spectra of these compounds showed molecular ion peaks corresponding to their molecular formula.

3.2. Antimicrobial Activity:

3.2.1. Zone of Inhibition:

The results of antibacterial activity are shown in the Table 3 4 and 5 of inhibition zones measurements at conc. 50 µg/ml and 100 µg/ml. The investigation of the structure–activity relationship of antibacterial screening revealed that the compounds 1b (86.52 and 87.49%, respectively), with *o*-hydroxyphenyl substituent at the 5-position of the nucleus were found to be highly active against *Staphylococcus aureus* and *Bacillus subtilis*. Against *Staphylococcus aureus* and *Bacillus subtilis* compound 2b (84.52% and 77.80%, respectively) containing chloro substituted phenyl ring in thiadiazole were found to be less potent in comparison to 1b but it was exhibited better result than 3b (76.38 and 77.04%, respectively), *o*-nitro substituted phenyl ring in thiadiazole moiety . So these compounds were found to be active in order as 1b>2b>3b against *Staphylococcus aureus* and *Bacillus subtilis*. In addition compound 2a and 3a exhibited good inhibitory activities (69.98%, 68.39% and 69.18% ,63.24 respectively) with *m*-chloro substituted phenyl ring on C2 position of the thiadiazole have exhibited better activity against *Staphylococcus aureus* and *Bacillus subtilis*. Whereas compound 1a (61.80%) with o-hydroxy substituted phenyl at C5 and *m*-chloro substituted phenyl ring on C2 position of the thiadiazole exhibited better result against *Bacillus subtilis* but poor activity against *Staphylococcus aureus.*

This finding suggests that the presence of a *p*-chloro substituted phenyl ring at linked to the (C2) 1, 3, 4-thiadiazole moiety promotes increased biological activity.

3.2.2. Minimum Inhibitory Concentration (MIC):

The examination of the data (Table 6) reveals that most of the compounds showed antibacterial and antifungal activity when compared with standard drugs amoxicillin, ofloxacin and amphotericin B. Compounds 1b and 2b were found to be highly potent against most of the employed strains to inhibit the growth of organism in comparisons to amoxicillin against *Staphylococcus aureus* (MIC =50 µg/ml). In particularly it was found to be compound 3b possess the significance activity against employed strains (MIC =100 µg/ml). It was revealed that the presence of *p*-chloro group in the phenyl ring linked to the 1, 3, 4-thiadiazole moiety promotes increased biological activity. The compounds 1b was found to be more potent to amoxicillin, towards the employed strains to inhibit the growth of organism (MIC = 200 µg/ml). In addition to compound 2b and 3b possess the significance activity against employed strains (MIC =250 µg/ml). Whereas all the remaining compounds were found to be less potent to amoxicillin, towards *Bacillus subtilis* (MIC > = 500 µg/ml). Towards Gram-negative strain compounds 1b showed most promising activity (MIC <100 µg/ ml), whereas 2b was found to be equally potent, to ofloxacin, *Escherichia coli* (MIC= 100 µg/ml).The compound 1b showed significance inhibitory activity (MIC <250 µg ml^{-1}) 2b and 3b were found to exhibit comparable activity to ofloxacin towards *Pseudomonas aeruginosa* (MIC=250 µg/ml). Against fungal pathogen *Aspergillus niger,* compounds1b was found to be highly potent (MIC <250 µg/ml) .Compounds 3b was found to exhibit comparable activity to amphotericin B towards *Aspergillus niger* (MIC=250 µg/ml). Compounds 1b were found to be equally efficient (MIC= 100 µg/ml) and in addition compound 2b exhibited comparable activity to amphotericin B against *Candida albicans* (MIC <200 µg/ ml). The remaining compounds showed moderate to good activity to inhibit the growth of microbial pathogens and are all less effective than standard drugs. The investigation of the structure–activity relationship of antibacterial screening revealed that the compounds 1b , 2b and 3b with *p*-chloro group in the phenyl ring linked to the 1,3,4-thiadiazole moiety at C2 position and *o*-hydroxyl group in the phenyl ring at C5 position of 1,3,4-thiadiazole ring promotes increased better results against *Staphylococcus aureus* , *Bacillus subtilis* , *Escherichia coli*. Antifungal evaluation results revealed that compound 1b and 3b with *o*-hydroxyl group in the phenyl ring at C5 and *p*- chloro group in the phenyl ring linked to the 1, 3, 4-thiadiazole moiety at C2 position promotes increased *Aspergillus niger* and *Candida albicans.*

3. 3. Anti-oxidant activity (*In-Vitro*):

The compounds 1a-3a and 1b-3b were tested for anti-oxidant property by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide methods at three different concentrations 4 µg/ml, 8µg/ml and 10µg/ml.

The observed data on the anti-oxidant activity of the compounds controlled drug were shown in (Table 7 & 8, Figure: 7,8).

3.3.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method:

All the synthesized compounds were tested for antioxidant activity against nitric oxide free radical. Compounds 1b, 1a, 3b and 3a were exhibited highest percentage of inhibition (70.784, 70.254%, 69.885% and 69.585%, respectively) at 10µg/ml due to presence of *o*-hydroxy phenyl and *o*-nitro phenyl group on the thiadiazole moiety when compared to the standard ascorbic acid (83.826%). Presence of chloro group at *para*-position of the phenyl ring on the C5 position of the 1, 3, 4-thiadiazole nucleus make the compounds 2a (68.46%), 2b (68.85%) were less potent. An increase in concentration results in an increase in DPPH• scavenging activity.

3. 3. 2. Nitric oxide scavenging method:

Among the compounds tested for antioxidant activity, N-(4-chlorophenyl)-3-[5-(2-hydroxyphenyl)-1,3,4-thiadiazol-2-yl]urea (1b) exhibited the highest antioxidant activity with the % Inhibition value of 83.85, while % Inhibition of reference compound ascorbic acid was found to be 84.79. Other moderately active compounds, N-(3-chlorophenyl)-3-[5-(2-hydroxyphenyl)-1,3,4-thiadiazol-2-yl]urea (1a) and N-(4-chloro phenyl)-3-[5-(4-chlorophenyl)-1, 3, 4-thiadiazol-2-yl]urea (2b) showed the % inhibition values of 80.16 and 79.65, respectively.

From the result it has been reveals that the electron-donating functional group likes –OH having the ability to bind with free radical (1b and 1a) was responsible for the potency. That was, the compounds having electron donating moiety on the aromatic ring displayed slightly high activity

The significant inhibition concentration by compounds 3b and 3a may be decrease due to the presence of strong electron-withdrawing nitro-group at *o*-position. In compound 1b and 1a replacement of nitro by electron donating hydroxyl group increases the inhibitory concentration. The presence of 1, 3, 4-thiadiazole-2-yl nucleus may be responsible for the moderate significant inhibition shown by all other compounds. Also the results of *in-vitro* scavenging activity suggested that the presence of a substituted phenyl ring linked to the 1, 3, 4-thiadiazole-2yl moiety promotes increased radical scavenging activity

The compounds showed radical scavenging activity which is comparable with standard drugs, increasing order of OH > Cl > NO_2 for nitric oxide (NO) scavenging activity and increasing order of OH > NO_2 > Cl for DPPH scavenging activity. The activity increasing as the electron withdrawing ability of the substituent decreased.

4. Conclusions:

The antimicrobial data given for the compounds allowed us to state that the variation of antimicrobial activity may be associated with the nature of tested microorganisms and also is due to the chemical structure of the tested compounds. Performed SAR observation has showed the importance of electronic environment on antimicrobial activity. The presence of hydroxyl (OH) and halogens (especially chloro) substituents on the aromatic ring as well as shorter intermediate chain have increased the activity of the compounds compared to those with other substituents which may be due to the presence of the versatile pharmacophore which might increase the lipophilic character of the molecules and thus facilitate the crossing through the biological membrane of the microorganisms and thereby inhibit their growth.

An increase in concentration results in an increase in DPPH• scavenging activity. Among the different derivatives, **1b** exhibited highest activity followed by 1a, 3b, 3a, 2b and 2a . Though, ascorbic acid showed much better DPPH• scavenging effect. Nitric oxide radical scavenging effect of all the synthesized compounds were tested and compared with ascorbic acid. Compound **1b** produced better NO• scavenging activity compare to standard ascorbic acid. The thiadiazole derivatives exhibited greater activity due to the presence of electron donating hydroxyl substituent on the aromatic ring enhanced the activity. Thus the results exemplified that the hydroxyl substituted N-(4-chlorophenyl)-3-[5-(2-hydroxyphenyl)-1, 3, 4-thiadiazol-2-yl] urea (1b) is the most powerful antioxidant agent.

5. Acknowledgement:

The authors are thankful to the Dr. N. Dutta (CMD), Assam down town university, Dr. B. K. Dey (Principal), Assam down town University, for providing laboratory facilities. The authors are also thankful to Dr. M. G Borthakur , Central Analytical Instrument Facility (CAIF), Guwahati Biotech Park, Guwathati, also Dr. A. Azome, JSS college of pharmacy, Ooty, India, for providing spectral data.

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