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Synthesis, antimicrobial and antimycobacterial evaluation of star shaped hydrazones derived from 1,3,5-triazine

Shrinath S. Machakanur[a], Basavaraj R. Patil[a], Aishakhanam H. Pathan[a], Ganesh N. Naik[a], Shashikala G. Ligade [b], Kalagouda B. Gudasi[a]*

[a]*Department of Chemistry, Karnatak University, Dharwad, Karnataka, India*
[b]*Department of Botany, Karnatak Science college, Dharwad, Karnataka, India*

ABSTRACT

Trisubstituted s-triazine hydrazones [N_3C_3(-OC_6H_4-p-CH=N-NH-C(O)-C_6H_4-p-X)$_3$] (X = H, Br, Cl, F) were prepared in excellent yields by the reaction of p-substituted N'-(4-hydroxy-benzylidene)-hydrazides with cyanuric chloride. The structures were elucidated by elemental analysis, FT-IR, ^{1}H, ^{13}C NMR and MALDI-TOF mass spectrometry. These s-triazine hydrazone derivatives were evaluated for their in vitro antimicrobial and antimycobacterial activity using serial dilution method.

Keywords: Triazine; Cyanuric chloride; Hydrazide; Hydrazone; Antimicrobial, Antituberculosis.

INTRODUCTION

Triazine compounds constitute an important class of heterocyclic chemistry and have been studied intensively [1, 2]. Cyanuric Chloride is an inexpensive, commercially available reagent used for the preparation of variety of *s*-triazine derivatives. The ease of displacement of chlorine atoms in cyanuric chloride by various nucleophiles enhances the utility of this reagent for the preparation of mono-, di- and tri substituted 1,3,5-triazine derivatives under controlled temperature conditions [3, 4].

The triazine scaffold has provided the basis for the design of compounds with a wide variety of properties useful in medicinal and agricultural applications [5-7]. Substituted s-triazine derivatives constitute an important class of compounds having antimalarial [8], antiviral [9], anticancer [10, 11] and estrogen receptor modulators [12]. Also, it was reported that some of these compounds possess potent antibacterial and antifungal activities [13-17].

Hydrazones represent one of the most biologically active classes of compounds [18]. Besides being utilizable for a wide range of pharmaceutical important derivatives, hydrazones are also important intermediates in organic synthesis [19]. Hydrazone linkage is extensively utilized for pH-dependent release of drugs from polymer-drug conjugates [20]. Many hydrazone derivatives have been claimed to possess, among others, antibacterial [21, 22] and antifungal [23, 24] activities. Looking at the antimicrobial importance of hydrazone moiety and triazine derivatives it would be worthwhile to design, synthesize some new triazine derivatives bearing hydrazone pharmacophore group and to investigate their possible antibacterial and antifungal activities.

We have previously described the synthesis of star shaped triazine hydrazones by condensation of tri-aldehyde core with aromatic hydrazides [25]. In this article, we illustrate the synthesis of similar triazine hydrazones by the reaction of cyanuric chloride with N'-(4-hydroxybenzylidene)-4-substituted-benzohydrazides. Thus prepared *s*-triazine hydrazones were evaluated for their antimicrobial and antituberculosis activity.

MATERIALS AND METHODS

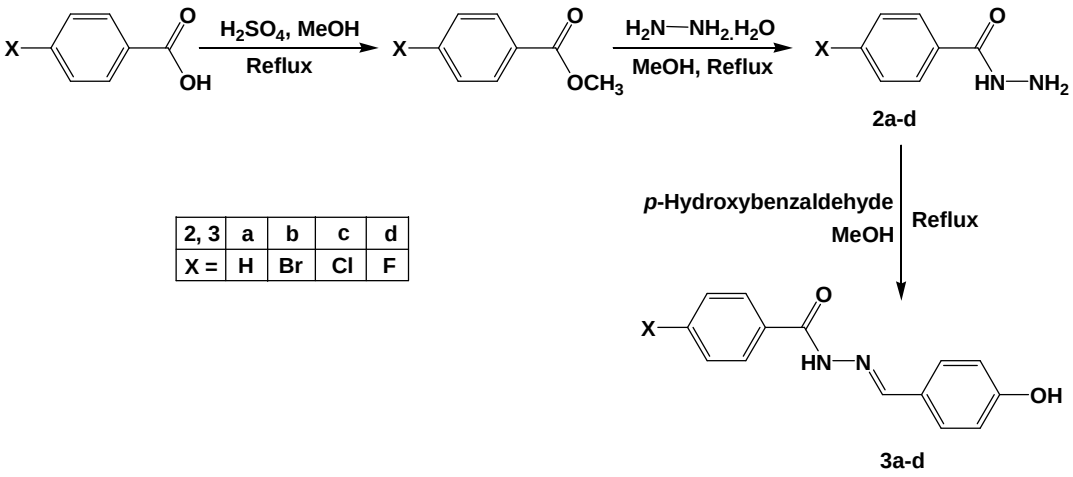
Materials and measurements

The ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 solvent with TMS as an internal standard at δ=0 ppm using a BRUKER AV-500 MHz High Resolution Multinuclear FT-NMR Spectrometer at room temperature. IR spectra were recorded on Impact-410 Nicolet (USA) FT-IR spectrometer in 4000–400 cm^{-1} range in a KBr matrix. The mass spectrum was obtained on Shimadzu GCMS-QP2010S and MALDI-TOF mass spectrum was measured with α-cyano-4-hydroxycinnamic acid as the matrix on a Voyager-DE STR spectrometer. Leco Model Truespec CHNS Analyser was used for Elemental (C, H, and N) analyses. Melting points were determined in an open capillary on a Gallenkamp melting point apparatus and are uncorrected.

Solvents were purified by standard methods [26]. Cyanuric chloride (Aldrich) and all other chemicals (sd fine chemicals, India) were used as received. All compounds were routinely checked by thin-layer chromatography (TLC) on aluminum-backed silica gel plates.

Synthesis of p-substituted benzoic acid hydrazides (2a-d)

p-substituted benzoic acid hydrazides were synthesized according to the literature method [25].



2, 3	a	b	c	d
X =	H	Br	Cl	F

Scheme 1. Synthesis of 4-substituted N'-(4-hydroxy-benzylidene)-hydrazides

Synthesis of 4-substituted N'-(4-hydroxy-benzylidene)-hydrazides (3a-d)

Reports dealing with the synthesis of N'-(4-hydroxybenzylidene)benzohydrazide (**3a**) [27], N'-(4-hydroxybenzylidene)-4-bromobenzohydrazide (**3b**), N'-(4-hydroxybenzylidene)-4-chlorobenzohydrazide (**3c**) [28], N'-(4-hydroxybenzylidene)-4-fluorobenzohydrazide(**3d**) [29], are well documented. Due to the non-availability of the IR and NMR spectral data in some of these reports, 4-substituted-benzoic acid (4-hydroxy-benzylidene)-hydrazides (**3a-d)** were synthesized as shown in scheme 1 by the following general procedure and their spectral data is presented.

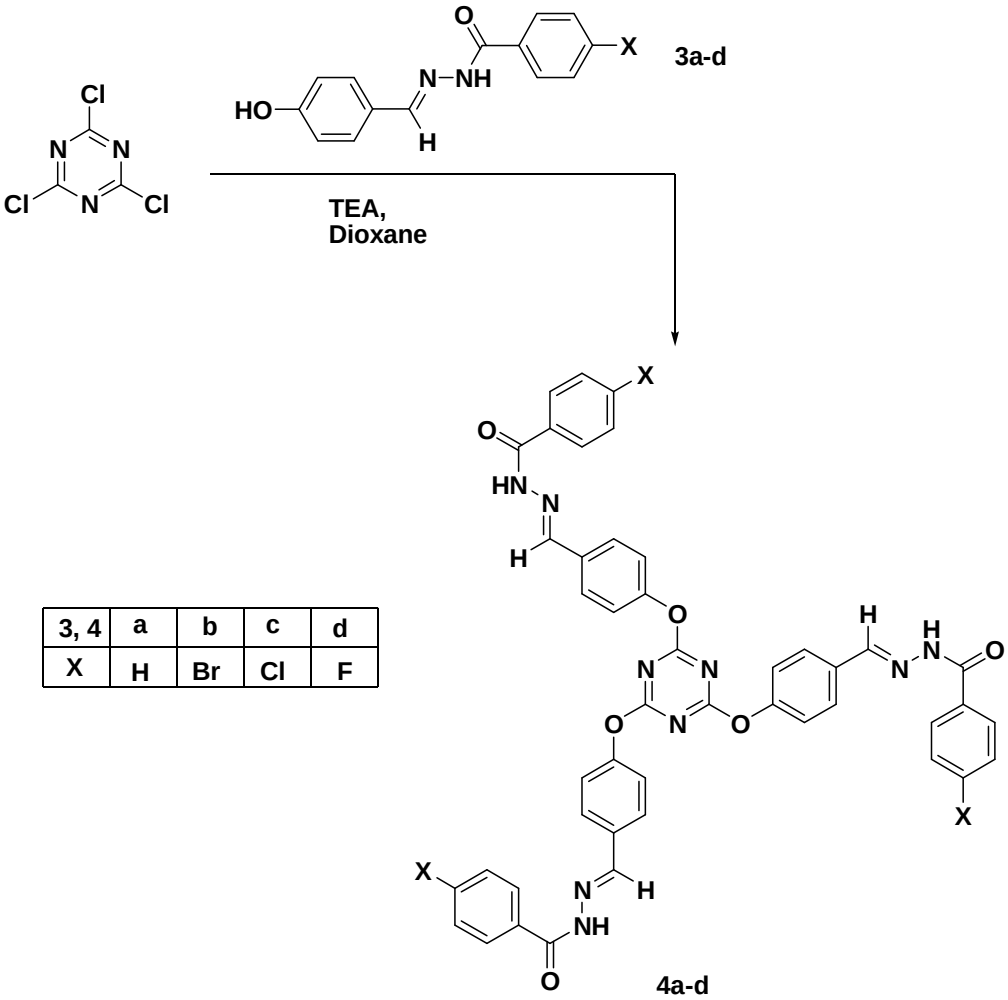
p-Hydroxylbenzaldehyde (1 mmol) was added to a solution of benzoic acid hydrazide (1 mmol) in 50 mL of methanol. The mixture was stirred at refluxing temperature for 3 h and then concentrated under vacuum. The solid product was collected by filtration under suction, and dried.

N'-(4-Hydroxybenzylidene)benzohydrazide (3a)

Yield: 86.04 %. IR (KBr) cm^{-1}: 3435 υ (O-H), 3303 υ (N-H), 1653 υ (C=O), 1608 υ (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 6.80 (d, 6H, $C^{2}H$), 7.32 (d, 6H, $C^{3}H$), 7.71 (d, 6H, $C^{9}H$), 7.74 (d, 6H, $C^{8}H$), 8.38 (s, 3H, $C^{5}H$) 10.08 (s, 3H, OH), 11.73 (s, 3H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 115.91 (C^{2}), 124.02 (C^{8}), 127.05 (C^{9}), 128.36 (C^{3}), 131.15 (C^{4}), 132.06 (C^{10}), 132.85 (C^{7}), 145.86 (C^{5}), 159.54 (C^{1}), 162.88 (C^{6}). MS m/z: 240 (M^{+}). Anal. Calcd. For $C_{14}H_{12}N_2O_2$: C, 69.99; H, 5.03; N, 11.66%. Found: C, 69.90; H, 4.98; N, 11.56%.

N'-(4-Hydroxybenzylidene)-4-bromobenzohydrazide (3b)

Yield: 80.23%. IR (KBr) cm^{-1}: 3438 υ (O-H), 3302 υ (N-H), 1658 υ (C=O), 1604 υ (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 6.84 (d, 6H, $C^{2}H$), 7.74 (d, 6H, $C^{3}H$), 7.78 (d, 6H, $C^{9}H$), 7.85 (d, 6H, $C^{8}H$), 8.34 (s, 3H, $C^{5}H$) 9.95 (s, 3H, OH), 11.34 (s, 3H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 115.69 (C^{2}), 125.49 (C^{10}), 128.43 (C^{8}), 129.63 (C^{3}), 129.89 (C^{4}), 131.14 (C^{9}), 133.35 (C^{7}), 146.68 (C^{5}), 159.94 (C^{1}), 162.37 (C^{6}). MS m/z: 319 (M^{+}). Anal. Calcd. For $C_{14}H_{11}BrN_2O_2$: C, 52.69; H, 3.47; Br, 25.04; N, 8.78%. Found: C, 52.61; H, 3.53; Br, 25.10; N, 8.73%.



3, 4	a	b	c	d
X	H	Br	Cl	F

Scheme 2. Synthetic route for1,3,5- triazine hydrazones

N'-(4-Hydroxybenzylidene)-4-chlorobenzohydrazide (3c)
Yield: 80.66 %. IR (KBr) cm^{-1}: 3444 υ (O-H), 3304 υ (N-H), 1652 υ (C=O), 1602 υ (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 6.86 (d, 6H, C^2H), 7.54 (d, 6H, C^3H), 7.74 (d, 6H, C^9H), 7.81 (d, 6H, C^8H), 8.42 (s, 3H, C^5H) 10.17 (s, 3H, OH), 11.74 (s, 3H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 114.93 (C^2), 123.62 (C^8), 128.82 (C^3), 129.61 (C^4), 133.37 (C^9), 134.16 (C^7), 136.29 (C^{10}), 145.33 (C^5), 160.66 (C^1), 162.66 (C^6). MS m/z: 274 (M^+). Anal. Calcd. For $C_{14}H_{11}ClN_2O_2$: C, 61.21; H, 4.04; Cl, 12.91; N, 10.20%. Found: C, 61.29; H, 4.10; Cl, 12.85; N, 10.15%.

N'-(4-Hydroxybenzylidene)-4-fluorobenzohydrazide (3d)
Yield: 84.09 %. IR (KBr) cm^{-1}: 3304 υ (O-H), 3305 υ (N-H), 1653 υ (C=O), 1605 υ (C=N). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 6.86 (d, 6H, C^2H), 7.34 (d, 6H, C^9H), 7.68 (d, 6H, C^3H), 7.94 (d, 6H, C^8H), 8.37 (s, 3H, C^5H) 10.13 (s, 3H, OH), 11.70 (s, 3H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 114.98 (C^9), 115.36 (C^2), 128.18 (C^8), 129.57 (C^3), 130.09 (C^4), 131.87 (C^7), 146.43 (C^5), 160.15 (C^1), 163.18 (C^6), 166.13 (C^{10}). MS m/z: 258 (M^+). Anal. Calcd. For $C_{14}H_{11}FN_2O_2$: C, 65.11; H, 4.29; F, 7.36; N, 10.85%. Found: C, 65.07; H, 4.30; F, 7.31; N, 10.88%.

Synthesis of triazine hydrazone **[N_3C_3(-OC_6H_4-*p*-CH=N-NH-C(O)-C_6H_4-*p*-X)$_3$]**
[X=H (4a); X=Br (4b); X=Cl (4c); X=F (4d)].
Cyanuric chloride (0.06 g, 1 mmol) was added to a mixture of N'-(4-hydroxy-benzylidene)-hydrazide (**3a)** (0.242 g, 0.32 mmol) and triethylamine in 50 mL of dioxane. The mixture was refluxed for 36 h and then concentrated under vaccum. The solid was filtered under suction and washed with water and then with minimum amount of warm THF. The resulting solid (**4a**) was dried in vacuo at 40°C for 4 h. The same general procedure was followed for the compounds **4b-d** with 36-40 h at refluxing temperature**.** The spectral and analytical data of (**4a-d**) are in good agreement with literature values for similar compounds reported in our earlier communication [25].

Biological study

Protocols for Antimicrobial activity

The synthesized compounds were assayed for their *in vitro* antimicrobial activity against Gram-positive bacteria *Staphylococcus aureus* (ATCC 9144), Gram-negative bacteria *Escherichia coli* (ATCC 87261) and fungi *Candida albicans* (ATCC 2091). Minimum inhibitory concentrations (MIC) for test compounds as well as for reference standards were determined using serial dilution method as per the standard protocols [30-31]. Ciprofloxacin and Flucinozole were used as reference clinical standards for the antibacterial and antifungal activities, respectively. Test compounds and reference drugs were dissolved in 0.5 mL DMSO. Final concentrations of 100 to 0.2 µg/mL were prepared by serial two fold dilutions with Muller-hinton broth for the bacteria and Potato Dextrose Media for the fungi. The final DMSO concentration was adjusted to 5%. Preliminary experiments demonstrated that DMSO had no effect on the microorganism in the concentrations studied. Each of the 10 test tubes was inoculated with a suspension of microorganism to be tested and incubated for 24-48 h at 35 ºC in an ambient air incubator. At the end of the incubation period, the tubes were visually examined for the turbidity. The lowest concentration of the test compound that inhibited visible growth of microorganisms after incubation at 35 ºC for 24 h for bacteria or 48 h for fungi was taken as the MIC value. The antimicrobial activity tests were run in triplicate.

Protocols for Anti-tubercular activity

Test compounds were evaluated for *in vitro* anti-mycobacterial activity. The anti mycobacterial activity of compounds were assessed against M. tuberculosis using microplate Alamar Blue assay (MABA). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with propotional and BACTEC radiometric method. Briefly, 200µl of sterile deionzed water was added to all outer perimeter wells of sterile 96 wells plate to minimize evaporation of medium in the test wells during incubation.

The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds were made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs [32]. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink. Test compounds were compared with reference drugs isoniazid.

RESULTS AND DISCUSSION

Synthesis of tri-arm 1,3,5-triazine hydrazones

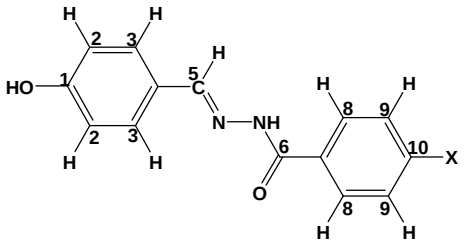
Tri-arm star shaped hydrazones with different substitutions at para positions emanating from 1,3,5-triazine core were synthesized as shown in Scheme 2. The condensation of benzoic hydrazide **2a**–**d** with 4-hydroxybenzaldehyde in tetrahydrofuran affords the corresponding hydrazones **3a-d**. The hydrazones **3a–d** bearing phenolic hydroxy functionality were then treated with cyanuric chloride in dioxane in presence of triethylamine for 36-40 h at refluxing temperature to obtain tri-arm 1,3,5-triazine hydrazones **4a–d.** The target compounds were obtained in high yields and were characterized by FT-IR, ^{1}H, ^{13}C spectroscopy, elemental analysis and MALDI-TOF mass spectrometry.

IR spectral studies

The formation hydrazones **3a-d** is confirmed by the appearance of imine C=N stretching frequencies in 1602-1608 cm^{-1} region. A broad band at 3214-3251 cm^{-1} is ascribed to N-H stretching frequency of the amide (-NH-C=O) moiety. A strong band at 1652-1658 cm^{-1} is due to the amide carbonyl (C=O) stretching frequency. The OH vibration observed at 3404–3444 cm^{-1} in **3a**–**d** are absent in **4a–d**. C_T-O-Ar absorption is observed as a distinct band at 1363-1374 cm^{-1} in **4a–d**, this is attributed to the involvement of carbon atom of triazine ring in ether linkage. Furthermore, triazine derivatives show another important band in the region 1567 and 1573 cm^{-1} ascribed to C=N stretching vibrations of *s*-triazine ring. Thus, the IR spectral data provide strong evidences for the formation of the 1,3,5-triazine hydrazones.

NMR investigations

The numbering used for NMR assignments is presented in figure 1. According to the NMR spectral data, all the triazine hydrazone (**4a-d**) molecules appear to have symmetric structures in solution.



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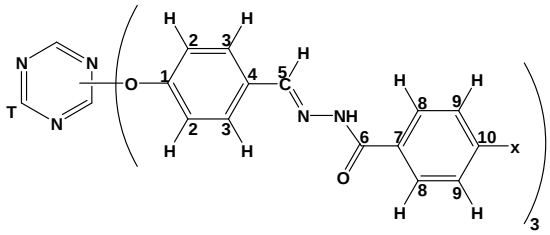

II

Figure 1. Numbering pattern for NMR assignments of (I) 3a-d and (II) 4a-d

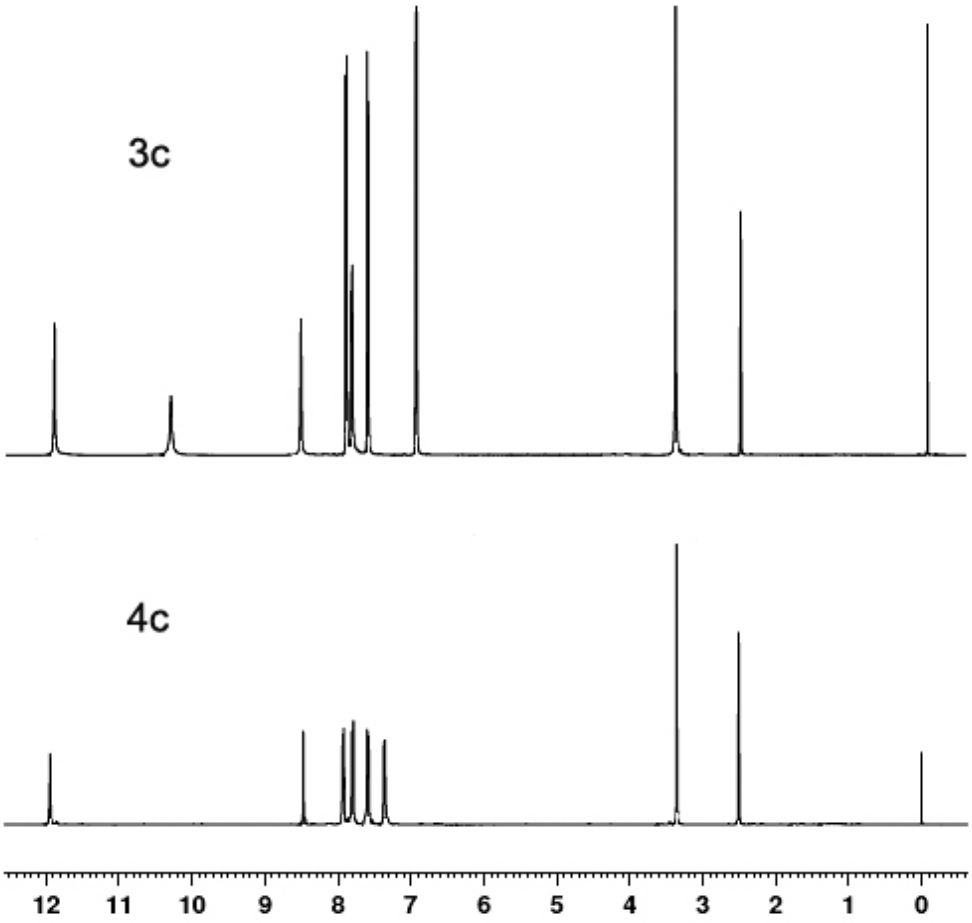

Figure 2. ^{1}H Nmr spectra of 3c and 4c

The appearance of a singlet for azomethine proton (-N-N=C***H***-) in the range 8.34-8.38 ppm confirms the formation of hydrazones **3a-d**. This is further supported by the resonance for hydrazide (-CO-N***H***-N=C-) protons as a broad singlet in the downfield region 11.34-11.76 ppm. The resonances due to aromatic protons appear in the range 6.80 to 7.99 ppm. A singlet at 9.95–10.13 ppm assigned to the phenolic hydroxy group of **3a-d** is absent in **4a-d** indicating the formation of ether linkage in the latter. This is further confirmed by a considerable change in the resonances of $C^{2}H$ protons. Other protons of **4a–d** have appeared in their usual pattern but with a slight variation in their chemical shift compared to the corresponding hydrazones **3a**–**d**. ^{1}H NMR spectra of **3c** and **4c** are given at Figure 2.

^{13}C NMR spectra of hydrazones **3a-d** exhibit resonances in the range 146.80-147.17 ppm due to the carbons (C^5) of the azomethine (-N-N=***C***H-) functionality. The amide carbonyl carbon (C^6) signal is observed in the region 161.92-163.09 ppm.

The resonance of C^1 carbons are observed at 159.42–160.15 ppm while in case of **4a-d** these resonances are observed in the range 152.22–152.44 ppm. The resonances of C^2 carbons of **4a-d** are observed downfield compared to the corresponding signal at 115.36-115.91 ppm in **3a**–**d**. Resonances of other carbons of **4a–d** exhibited slight variation in their chemical shift compared to the hydrazones **3a**–**d**. ^{13}C NMR spectrum of **3b** and **4b** are displayed in Figure 3.

Mass spectrometry

The single molecular nature of the hydrazone triazines **4a-d** was also checked by MALDI-TOF mass spectrometry, which confirmed the expected chemical structures with m/z values corresponding to $(M+Na)^+$ ion.

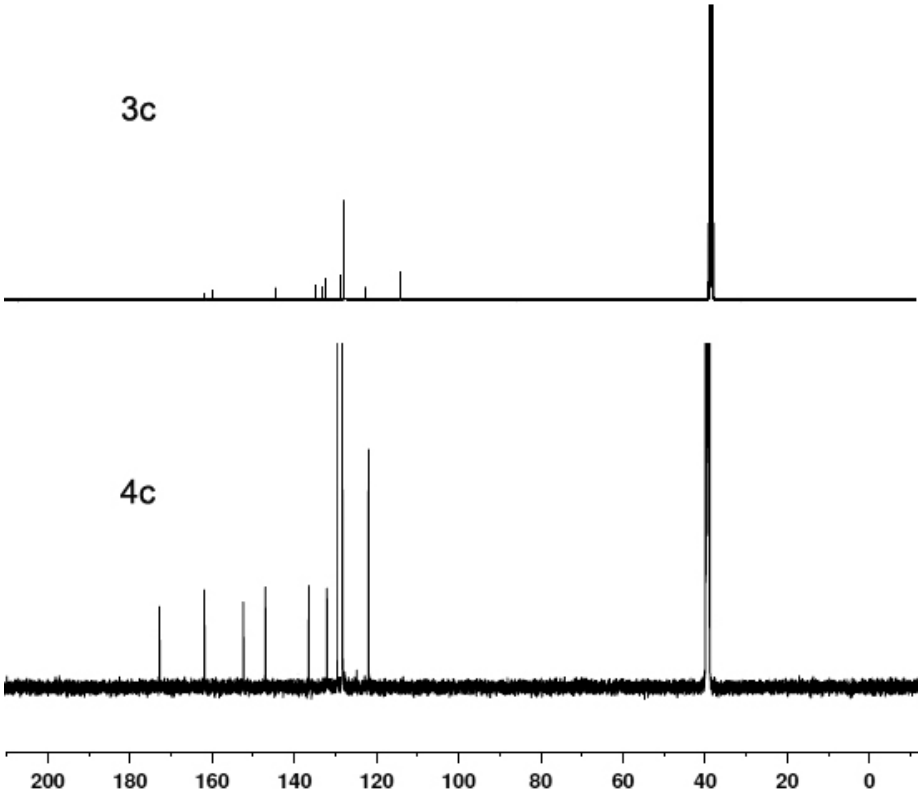

Figure 3. ^{13}C Nmr spectra of 3c and 4c

Biological activity

In order to determine the *in vitro* antimicrobial activity, the synthesized compounds 4a-d were screened against Gram-positive bacteria *Staphylococcus aureus* (ATCC 9144), Gram-negative bacteria *Escherichia coli* (ATCC 87261) and a fungal strain *Candida albicans* (ATCC 2091) using a two fold serial dilution technique. The investigation of antibacterial and antifungal screening data (Table 1) reveal that all the tested compounds exhibit moderate inhibition against the strains used. Antibacterial activity of compounds **4b**, **4c** is higher than other two compounds against *S.aureus*, while **4c**, **4d** have shown good activity against *E.coli*. Compounds **4a**, **4b**, **4c** were found to be more active against *C.albicans* fungal strain. Compound **4c** is found to be active against all the tested strains. However, activity exhibited by all synthesized compounds is less compared to the standard drugs used in the study.

Table 1. *In-vitro* antimicrobial and antimycobacterial activities of 4a-d (MIC in µg/mL)

Compounds	Bacterial		Fungal	Antituberculosis
	S. aureus	*E. coli*	*C. albicans*	*$H_{37}Rv$*
4a	12.5	25	0.8	25
4b	1.6	12.5	0.8	12.5
4c	1.6	1.6	0.8	6.25
4d	6.25	1.6	6.25	6.25
Ciprofloxacin	0.2	0.2	--	--
Flucanazole	--	--	0.4	--
Isoniazide	--	--	--	0.8

The anti-mycobacterial activity of synthesized compounds of **4a-d** were assessed against M. tuberculosis ***$H_{37}Rv$*** at several µg concentrations. The Minimum Inhibitory Concentrations (MIC) of compounds was compared with Isoniazid, the standard anti-TB drug. All the tested compounds have shown poor inhibition compared to the standard used. Among the compounds studied **4b** and **4c** have shown comparatively better inhibition.

CONCLUSION

An alternative method for the synthesis of tri-arm star shaped molecules bearing hydrazone functions is reported. 2,4,6-Tris(4-formylphenoxy)-1,3,5-triazine (**1**) possessing three reactive terminal aldehydic functions on the substituents could be readily elaborated to the trifunctionalized hydrazones by condensation with the benzoic hydrazides. IR as well as ^{1}H, ^{13}C NMR spectral characteristics of triazine hydrazones are consistent with their proposed structures. Microanalysis and MALDI-TOF mass spectrometry also proved the proposed chemical structures. The tri-arm star shaped hydrazones were evaluated *in vitro* for their antimicrobial and antimycobacterial activity using a two-fold serial dilution method and exhibited moderate inhibition against the strains. The compounds **4a-d** have been identified as fully substituted symmetric triazines and are important as synthetic and structural models for the reactions and molecular structure of the analogous triazine dendrimers. Efforts on the synthesis and characterization of higher generation triazine hydrazones are in progress and will be reported in future communications.

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