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RESEARCH ARTICLE

Synthesis and biological applications of (E)-4-Methoxy-N′-(2,3,4-trimethoxybenzylidene)benzohydrazide monohydrate

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Abstract

A new, efficient method to biologically and pharmaceutically important (E)-4-methoxy-N′-2,3,4-trimethoxybenzylidene)benzohydrazide monohy drate (1c) was prepared by reacting 4-methoxybenzohydrazide (1a) and 2,3,4-trimethoxybenzaldehyde (1b) in the presence of a concentrated hydrochloric acid as an efficient catalyst. The synthesized compound was evaluated for antimicrobial activity against Gram-positive pathogen *Staphylococcus aureus*, Gram-negative pathogen *Escherichia coli*, and *Aspergillus niger* as *fungi*. The compound exhibited good anti-bacterial and antifungal activity at Minimum Inhibitor Concentration level. The antioxidant activity was determined by DPPH method and that compound possesses best antioxidant activity. Further, the synthesized compound was evaluated for their cytotoxicity against breast cancer cell line (MCF-7). The result showed that the synthesized compound is non-toxic in nature.

Keywords

4-methoxybenzohydrazide

Antimicrobial activity

DPPH

MCF-7 cell line

Introduction

Recent literatures discuss about the use of benzohydrazide derivatives in organic and organometallic [1-2] synthesis, as it possesses potential application in analytical and medicinal chemistry [3-4]. The substituted benzohydrazones, which can be readily obtained by condensation of benzohydrazide and aromatic aldehydes or ketones in presence of acid catalyst [5] represent a class of azomethine compound. Many researchers work interestingly in the synthesis of benzohydrazone using different methods. However, the most valuable property of 4-methoxybenzohydrazone lies in their great physiological activity owing to the presence of the azomethine group and provides application in medicinal and pharmaceutical field with various biological applications [6]. It is well known that the substituted benzohydrazone group plays an important role for the antibacterial [7] and antifungal activity [8]. Benzohydrazones are being synthesized in order to combat diseases with minimal toxicity and maximal efficiency. These predictions have provided a therapeutic pathway to develop new effective biologically active hydrazones was reviewed by Rollas and kucukguzel, showed their antidepressant [9] and antiviral

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activities [10]. A number of benzohydrazide derivatives have been reported to exhibit notably, anticonvulsant [11], anti-inflammatory [12] anti-tubercular [13] and anticancer activities [14]. The emergence of resistance to the major classes of antibacterial agent is recognized as a serious health problem. Particularly, in recent years much attention has been focused on the multi-drug resistant microbes and fungi resulting from the widespread use and misuse of classical antimicrobial drugs. These resistant strains, for instance, methicilin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) are capable of acting as antibiotics [15-16]. Conversely, statistics of chronic infections, such as otitis media, tonsillitis, adenoiditis, over and above device-related disease are caused by biofilm-forming mucosal pathogens of *S. aureus* and *S. epidermis* [17-18]. Various structural modifications improve the biological effectiveness of a compound [19]. Literature survey reveals that inclusion of halogen atom(s) within the particle is one of the most effective strategies to enhance its biopotency, bioavailability, and lipophilicity Suresh et al., prove that fluoro-containing arylthiourea compounds show better activity as compared to other analogues [20], however fluoro-methyl, methyl or methoxy substituent on the benzene ring also improve antimicrobial potency. According to other author findings [21-24], the antibacterial and antifungal efficiency depends on the occurrence of such electron-withdrawing substituent at C-2 and C-4 position of the phenyl ring. Now in the present study, 4-methoxybenzohydrazone was synthesized and biological evaluation such as antimicrobial activity; antioxidant activity and cytotoxicity were carried out.

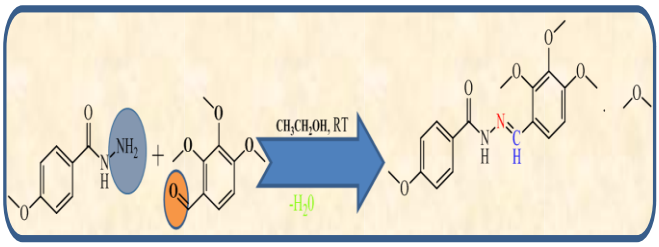
Experimental

Materials

All the required chemicals were purchased from Merck and Sigma-Aldrich Co (USA) and used without further purification. Melting point was determined in open capillary on a Guna-Mac apparatus and uncorrected.

Synthesis

Synthesis of (E)-4-methoxy-N′-(2,3,4-trimethoxybenzylidene)benzohydrazide monohydrate 4-methoxybenzohydrazide (1a) (0.001 mmol, 0.17 g) was dissolved in ethanol (10 mL) and 2,3,4-trimethoxybenzaldehyde (1b) (0.001 mmol, 0.145 g) was added slowly, followed by adding Con. HCl (0.2 mmol), and continuously stirred for 30 min. at room temperature (37 ^{0}C). The yellow colour precipitate formed was filtered, washed with petroleum ether (40-60 %) and dried over in a vacuum desiccator. Finally, it was recrystallized from alcohol. The compound (1c) is shown in Scheme 1. Melting point was determined in open capillary on a Guna-Mac apparatus and uncorrected. M.P:165^{0}C, yield (92%).



Scheme 1

Solubility

In the present work, the (E)-4-methoxy-N′-(2,3,4-trimethoxybenzylidene) benzohydrazide monohydrate (1c) was synthesized from 2,3,4-trimethoxybenzaldehyde (1b) and 4-methoxybenzohydrazide (1a) using various reaction condition. Initially, we attempted to syntheses using various organic/inorganic acids and bases as catalysts (Table 1) and different types of solvents (water, methanol, and ethanol) at room temperature (Table 2). The inorganic acids/bases (HCl, H_2SO_4 and HNO_3/NaOH, KOH and Na_2CO_3) catalyzed reactions gave fascinating results but the reactions using organic acids/bases (HCOOH, CH_3COOH/ $(CH_3)_3N$, $(C_2H_5)_3N$) gave moderate yield. Finally the use of Con. HCl as efficient catalyst and ethanol as solvent gave fascinating results. The effect of catalyst and solvents are shown in Tables 1 & 2 respectively.

Table 1. The effective catalysts on 1a with 1b solvents

S. NO	Catalyst	Yield (%)
1.	HCl	92
2.	H_2SO_4	81
3.	HNO_3	72
4.	HCOOH	39
5.	CH_3COOH	45
6.	KOH	66
7.	NaOH	65
8.	Na_2CO_3	57
9.	$(C_2H_5)_3N$	34
10.	$(CH_3)_3N$	38

Table 2. The effect of solvent on 1a with 1b

S. No	Solvents	Yield (%)
1	Ethanol	92
2	Methanol	61
3	Water	60
4	Acetone	57
5	Dimethylsulfoxide	69
6	Chloroform	40
7	Diethylether	73
8	Tetrahydrofuran	52
9	Ethylacetate	76

Results and Discussion

Antimicrobial activity

The nutrient agar medium was prepared and uncontaminated using autoclaving at 121^{0}C, 15 lbs pressure for 15 min. The petri plate was allowed to harden. The pathogen broth culture was swabbed on this Petri plates using sterile buds. The organic solvent DMSO was dissolved in the test compound. The solvent (DMSO, 1 mg/mL) was placed on agar plate seeded with the appropriate test organisms. Benzohydrazone was tested for their *in vitro* growth inhibitory activity against *S. aureus* as gram positive pathogen and *E. coli* as gram negative pathogen strains and *in vitro* antifungal perspective against *A. niger* strain. The Petri plates were incubated, at 37 ^{0}C for 24 h for gram-positive, gram-negative microorganisms and 48 h for fungi. After incubation, the plates were observed for the zone of inhibition. The antimicrobial activity of the synthesized compound was compared with Erythromycin and Gentamycin as standard. The results obtained were tabulated shown in Fig 1. The synthesized compound showed antimicrobial activity against one positive (*S. aureus*) bacteria, gram negative (*E. coli*) bacteria, and *A. niger* was evaluated using Erythromycin and Gentamycin as standard drugs. The compound (1c) showed good antimicrobial activity with the methoxy group at an ortho, para and meta position of an aromatic ring and found to be most effective against gram-negative *E. coli* bacteria with a zone of high inhibition. So the compound (1c) is most effective showing antimicrobial activity against Erythromycin and Gentamycin. The result is shown in Table 3.

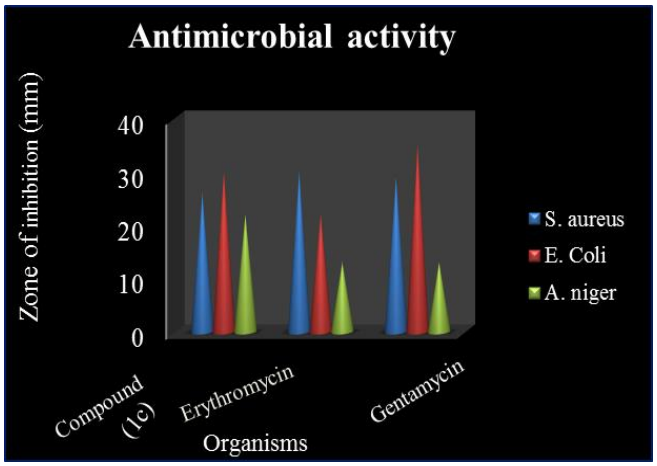


Fig 1. Antimicrobial activity of compound (1c)

Table 3. Antimicrobial activity of compound (1c).

S. No	Organism	*S. aureus (mm)*	*E. coli (mm)*	*A. niger (mm)*
1	1c	26	30	29
2.	Erythromycin	30	22	35
3.	Gentamycin	22	13	13

Antioxidant activity

The scavenging activity of the compound was measured according to the method of [31]. The DPPH radical is a stable free radical having λ_{max} at 517 nm. A fixed concentration of the experimental compound was added to a solution of DPPH in methanol at different concentrations (20, 40, 60, 80 µM, 4 mL) and the final volume was made up to 4 mL with increasing distilled water. The solution was incubated at 37ºC for 30 min., in the dark. The decrease in absorbance of DPPH was measured at 517 nm using UV-Visible Perkin Elmer spectrophotometer. The methoxy (OCH_3) radical scavenging activities of the compound have been investigated using the assay [32]. The antioxidant activity of the synthesized compound was evaluated against DPPH radical assay [33]. For the antioxidant assay described above, the test was run in triplicate and various concentrations of the synthesized

compound were used to fix a concentration at which the compound showed around 50% activity. In addition, the percentage of activity was calculated using the following formula:

$$\text{Radical scavenging activity } (\%) = \frac{[A_c - A_s]}{A_c} \text{ X } 100$$

(A_C and A_S are the absorbance value of blank and presence of the compound tested, respectively). The 50% activity (IC_{50}) can be calculated using the percentage of activity results. Ascorbic acid is used as positive control. The free radical-scavenging activity of the synthesized compound (1c) along with those of the standard ascorbic acid, was determined using a 1,1-diphenyl-2-picrylhydrazyl assays. DPPH radical scavenging activity valuation is a convenient assay for screening the antioxidant activity of compound (1c) and can be observed from Table 4 and Fig 2. The product showed excellent DPPH radical scavenging activity compared to synthetic commercial antioxidant ascorbic acid with IC_{50} values ranging from 44.61± 3.53 µg/mL. The corresponding value for the standard antioxidant ascorbic acid, by contrast, was 34.37 ± 4.17 µg/mL. We also observed that the presence of an electron releasing group (OCH_3) group on the substituted benzene ring increased the scavenging ability.

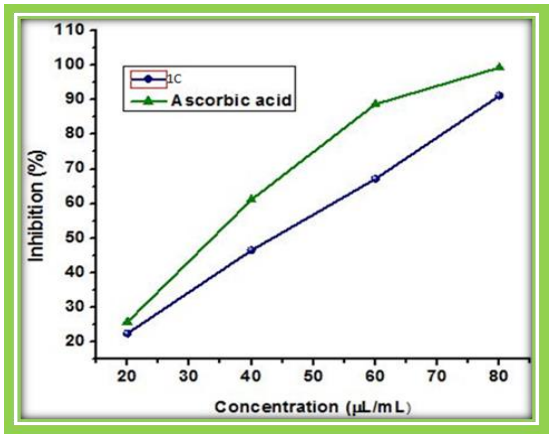


Fig. 2. Antioxidant activity of synthesized compound (1c).

Table 4. Antioxidant activity of compound (1c).

DPPH (µl/ml)	Compound (1c)	Ascorbic acid
20	22.3 ± 0.38	25.65 ± 1.78
40	46.4 ± 2.37	61.12 ± 4.23
60	67.1 ± 4.41	88.75 ± 6.19
80	91.2 ± 5.08	99.38 ± 6.89
IC_{50}	44.61 ± 3.53	34.37 ± 4.17

Cytotoxic activity of Compound (1c)

The cytotoxic activity of the synthesized compound was determined against MCF-7 human breast cancer cell line using tetrazolium colourless salt to a blue formazan in mitochondria (MTT) assay and IC_{50} values (concentration required to achieve 50% inhibition of cancer cell) of the standard and test compound as presented in Table 5 and Fig. 3(a-b). The cell viability assay was determination of MTT test which is used to assess the cytotoxicity of the compound based on the capacity for viable cells to metabolize a tetrazolium colorless salt to a blue formazan in mitochondria. In brief, T2m-bl cells were seeded in 96 well tissue culture plates (6000 cells/well) and incubated in 5% CO_2 incubator at 37^0C for 24 h. Following incubation, cells were treated with varying concentration of the test compound for 48 h at 37^0C. The appropriate solvent was added as a negative control. After incubation, 20 mL of MTT reagent (5 mg/mL in 50 mM PBS) was added to the wells and the culture plate was left for further incubation at 37^0C for 4h. After incubation, cells were cleaned off from the spent medium and 120 µL of MTT solvents [20 % SDS and 50 % dimethyl formamide in 50 MM of PBS] was added to each well and culture plate was left for an overnight incubation at 37^0C. The absorbance was taken at 570 nm with 690 nm reference filter using ELISA reader. Percent viability can be given as formula and is given in Table 5.

$$\% \text{ of Cell viability} = \frac{\text{Absorbance of the test sample treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

The result obtained in the present study is encouraging. However, to ratify the non-toxic nature of the compound and to inflate the therapeutic application of (E)-4-methoxy-N′-(2,3,4-trimethoxybenzylidene) benzohy drazide monohydrate, test on various other cell lines needs to be conducted.

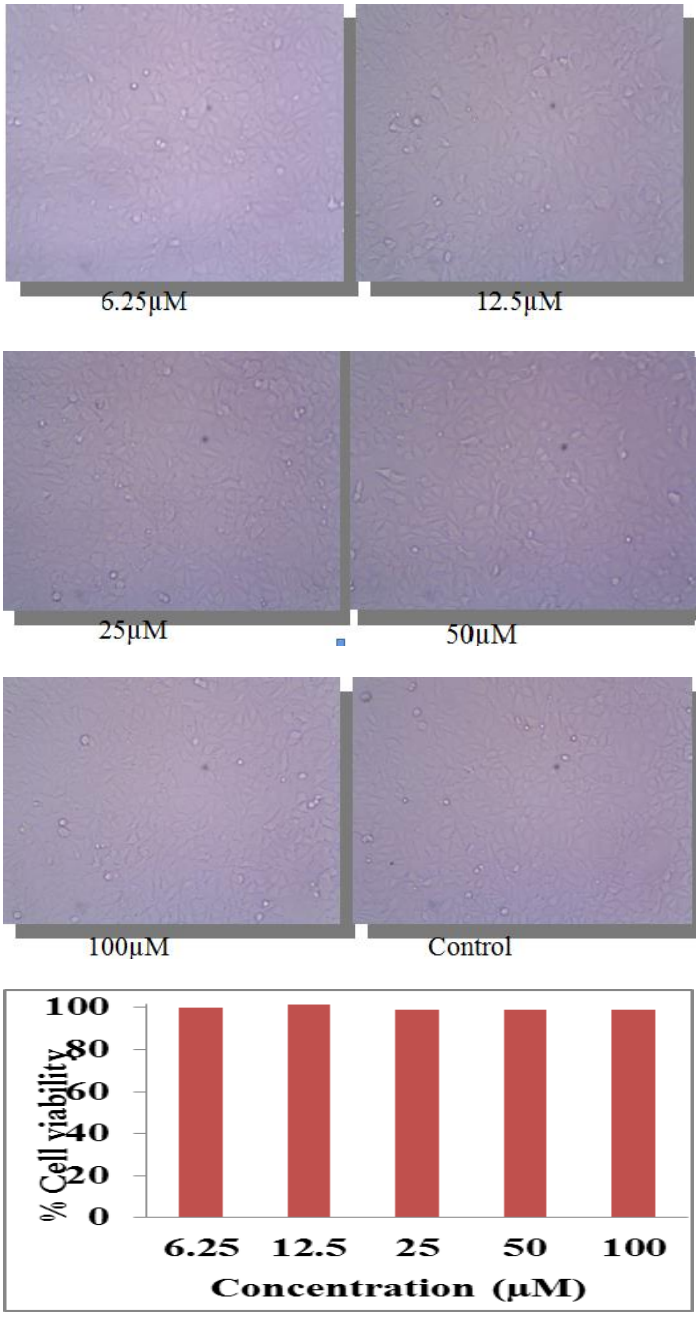


Fig. 3a. Cytotoxicity of compound (1c) Cell viability inhibition of compound.

Table 5. Cytotoxic activity of compound (1c).

S. No	Concentration(µm)	Cell viability (%)	Average
1.	6.25	100.0907	0.368
2.	12.5	101.1786	0.372
3.	25	99.0027	0.364
4.	50	99.0027	0.363
5.	100	98.7307	0.367

Conclusions

In the present study, (E)-4-methoxy-N′-(2,3,4-trimethoxybenzylidene) benzohydrazide monohydrate (1c) was synthesized using 4-methoxybenzohydrazide (1a) with 2,3,4-trimethoxybenzaldehyde (1b). The reaction occurred very fast, under mild condition using reasonable reagent and solvents and the yield is also higher. Compound (1c) was effectively screened against gram positive *S. aureus*, gram negative *E. coli* and *A. niger* bacterial and fungal strain. The antioxidant ability of the synthesized compound (1c) showed good activity which increases with increasing number of the methoxy group. Cytotoxic activity result indicated that the synthesized compound was non-active than the control against MCF-7 cancer cell line. The investigated compound showed a range of cytotoxic activity that could be categorized as non-toxic in nature. So the compound can be utilized in drug designing.

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