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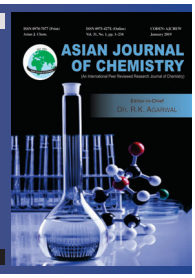

A Green Synthesis, Molecular Docking and Cytotoxicity of (*E*)-1-(4-(Difluoromethoxy)-2-Hydroxybenzylidene) Semicarbazide

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A green, one-pot three-components reaction of (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide has been designed, synthesized and characterized by elemental, FT-IR, ^{1}H and ^{13}C NMR spectral data. The cytotoxic activities of the synthesized compound were evaluated by MTT assay in human cancer cell lines. Docking studies of 3-substituted indolin-2-one scaffolds on vascular endothelial growth factor receptor 2 (VEGFR-2) involved in cell proliferation and angiogenesis was performed. Based on the ligand efficiency indices, Schiff bases may be regarded as efficient candidates for further molecular developments of anticancer agents. The molecule is useful in sensing and drug-carrying applications.

Keywords: Green synthesis, Schiff base, Docking studies, Cytotoxicity.

INTRODUCTION

In recent years, Schiff bases are one of the most widely used organic compounds and unveil a broad array of antimalarial, antiproliferative [1-4], biological activities [5-7], antibacterial [8,9] including antifungal [10,11] and antipyretic activities. Imine group also appears in many natural products [12] those are very important for their biological activity [13,14]. The identification and synthesis of Schiff bases that can be used for selectively and accurately detecting as well as monitoring environmentally and biologically hazardous substances is crucial. According to the World Cancer Report published by the International Agency for Research on Cancer, the number of cancer cases worldwide doubled between 1975 and 2000; this number is expected double again and almost triple by 2020 and 2030, respectively [15]. Therefore, various research initiatives are being undertaken worldwide for the treatment of cancer and the objective is the discovery of novel potent and effective antineoplastic agents, particularly those that specifically interact with unique biological targets.

Large-scale screening of natural products by the National Cancer Institute has helped in identifying quinone as a key pharmacophoric moiety owing to its cytotoxic activity [16-18]. A literature survey revealed that various naturally occurring and synthetic Schiff bases have been receiving considerable attention for their anticancer activity [19-31]. Schiff bases have also become eminent owing to their physiological and pharmacological activities. Compounds with the azomethine group (-C=N-) in their structure are known as Schiff bases. They are usually synthesized by a condensation reaction between primary amines and active carbonyl groups. Various Schiff bases have been found to exhibit anticancer activity [32,33].

The interactions between a small molecule and a protein at the atomic level can be modeled using a molecular docking approach. Modeling facilitates the characterization of the behaviour of small molecules in the binding site of target proteins; it also elucidates fundamental biochemical processes underlying the interaction. Docking comprises two main steps: (1) predicting ligand conformation, ligand position and ligand orientation within binding sites (usually referred to as "pose") and (2) assessing binding affinity [34]. These two steps are associated with sampling methods and scoring schemes, respectively. The docking efficiency increases significantly if the location of the binding site is known before docking. In many cases, the binding sites are known before ligands are docked into them. Furthermore the information about the sites can be obtained

by comparison of the target protein with a family of proteins that share a functional similarity or with proteins that cocrystallize with other ligands. When knowledge regarding the binding sites is not available before docking, cavity detection programs or online servers, such as GRID, POCKET, SurfNet, PASS and MMC can be used to identify presumed active sites within target proteins. Docking without any assumptions about the binding site is called blind docking.

Cytotoxicity studies serve as an initial step in the determination of the potential toxicity of a test substance, including those of plant-based biologically active compounds. Cellular toxicity studies are crucial and mandatory to ensure no or minimal toxicity of pharmaceutical or cosmetic preparations. The successful development of drugs and cosmetics depends on the results of cytotoxicity studies [35]. The concept of basal cytotoxicity, which refers to recording the deleterious effects of products on structures and functions common to all human cells, is relevant when the association between acute toxicity and cytotoxicity is considered. The selectivity index is a critical measure for identifying substances with high biological activity and negligible cytotoxicity.

Therefore, these results mentioned above prompted us to continue our investigation towards the synthesis of Schiff bases after introducing amino group in difluoromethoxy ring system in order to achieve new lead compound for future development as anticancer agents and docking studies. Docking of Schiff bases with VEGFR-2 Inhibitors protein (PDB code: 2OH4) has been focused to investigate the most preferred binding mode and hence the mechanism of compound (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene)semicarbazide (DHS). Molecular docking was performed by Glide module implemented in Maestro version 9.3.5 of Schrödinger software suite, 2011. Herein, we report the synthesis, characterization of Schiff-based, ((*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide as well as cytotoxicity, living cell image and molecular docking.

EXPERIMENTAL

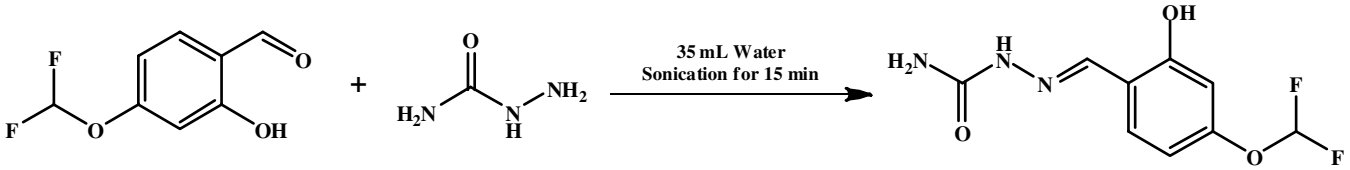
All the reagents and chemicals were purchased from Aldrich Chemicals Ltd. and used without further purification. SIDILU Indian make sonic bath working at 35 kHz (constant frequency, maintained at 28 ºC) was used for ultrasonication reactions. Melting point was determined by a melting point apparatus using an open capillary method and are uncorrected. Elemental analysis was carried out on a VARIOMICRO V2.2.0 CHN analyzer. ^{1}H and ^{13}C NMR spectra obtained on a Bruker Avance III 400 MHz spectrometer respectively DMSO-d_6 as solvents using TMS as an internal standard. Bright field and live cell images descriptions were captured at 40x magnifications with a microscope (Olympus FV1000-LX81.z) using Camedia software and processed using Adobe Photoshop version 10.0.

Synthesis of compound (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide (DHS): A 20 mL conical flask was charged with semicarbazide (1 mmol), 4-(difluoromethoxy)-2-hydroxybenzaldehyde (1 mmol) and water (25 mL). The reaction mixture was sonicated for 15 min (35 kHz, constant frequency) at 28 ºC. The reaction was followed by TLC using hexane:benzene (6:4) as an eluent. After the completion of the reaction, the mixture was poured onto the crushed ice. The solid thus precipitated was collected by filtration (**Scheme-I**). Further purification was accomplished by recrystallization from ethanol and water in 4:6 ratio. Yield : 89.65 %; yellow colour solid; m.p: 193-197 ºC; m.w. 508.38; Elemental analysis calcd (found) (%) of $C_{18}H_{12}N_6O_7F_4$: (%): C: 42.53 (42.27), H: 3.97 (3.88), N: 16.53 (16.49). ^{1}H NMR (DMSO-d_6, 400 MHz): 12.14 (s, 1H), 9.92 (s, 2H), 8.31-8.29 (t, 2H), 8.07-7.93 (t, 2H), 7.26 (s, 2H), 7.28-7.19 (m, 1H), 6.60-6.58 (d, 2H). ^{13}C NMR (DMSO-d_6, 100 MHz): 164.68, 160.94, 152.89, 141.37, 139.65, 136.50, 131.50, 130.65, 130.14, 129.57, 127.68, 123.54, 121.90, 120.63, 120.31, 115.21 ppm. IR (KBr, ν_{max}, cm^{-1}): 3064 (Ar-CH), 1642 (C=O), 1615 (C=N), 1493-1422 (C=C), 3384 (C=OH), 3255 (NH), 1231-962 (C-H), 845-722(C-H).

Protein structure preparation: For compound (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide (DHS), molecular docking was performed using the Glide module provided in the Maestro version 9.3.5 of Schrödinger software suite, 2011. The ligand was prepared using the Ligprep application. The conformers were generated using a rapid torsion angle search approach and the subsequent minimization of each generated structure by using the optimized potential for liquid simulations (OPLS)-2005 force field. The protein data bank was accessed to obtain the 3D coordinates of the crystallographic structure of the protein. The protein complex was preprocessed and prepared using the protein preparation wizard module of Maestro version 9.3.5 of the Schrödinger software suite, 2011. The minimization of the complex was continued using the OPLS-2005 force field until the root-mean-square deviation was 0.3 Å. The molecular docking studies of the ligands and protein were performed using the Glide module. The Glide module provides three levels of docking precision, namely high throughput virtual screening, standard precision and extra precision. We carried out our calculations in XP mode. The molecules that exhibited the best fit with the protein were ranked according to their Glide scores.

Cell culture: The human cancer KB cells (NCCS, Pune, India) were cultured in Dulbecco's modified Eagle Medium supplemented with 10 % FBS and antibiotics (penicillin-100 µg/mL; streptomycin-50 µg/mL). The cells were cultured at 37 ºC in 95 % air and 5 % CO_2 in an incubator.

Anticancer activity and cell viability assay: The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of DHS on the KB



Scheme-I: Synthetic way of compound (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide

cells. The cells were seeded into a 96-well plate at a density of 1.5×10^4 cells/well and incubated in medium containing CYGD at concentrations ranging from 1.5 to 500 µM for 48 h. For each treatment, triplicate wells were maintained; 100 µL of MTT was added to each well. The plate was incubated at 37 ºC for 4 h to facilitate the intracellular formation of formazan crystals because of the reaction between MTT and metabolically active cells. The medium containing MTT was carefully removed from the wells. Next, 100 µL of dimethyl sulfoxide was added to each well to dissolve the intracellular formazan crystals, and the plates were shaken for 10 min. Using an enzyme-linked immunosorbent assay reader, the absorbance was measured at 511 nm. The cell were examined using a fluorescence microscope. The percentage survival of the cells was calculated using the following formula:

$$\text{Survival } (\%) = \frac{\text{Live cell number (test)}}{\text{Live cell number (control)}} \times 100$$

RESULTS AND DISCUSSION

In the synthetic progress of (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide, water plays a significant role in the field of organic synthesis and many organic reactions have been carried out in aqueous media. Therefore, water has become a useful medium for the synthesis of organic compound, not only for the reward concerning the avoidance of expensive catalysts and organic solvents but also for some characteristic reactivity and selectivity in the formation of the products. Our success in this effort has resulted in the development of a novel, green, one-pot pseudo two components reaction for the synthesis of DHS form a molecule of a semicarbazide and 4-(difluoromethoxy)-2-hydroxybenzaldehyde in water under ultrasonication. The synthesized target was characterized and confirmed by FT-IR, ^{1}H and ^{13}C NMR spectra analyses.

Prediction of pharmacokinetic properties: The 2D structures of DHS were subjected to a computational program using Qikprop module of Schrödinger software for *in silico* determination of pharmacokinetic properties. The Lipinski's rule of five factors of DHS and statistical parameters of the pharmacokinetic properties of DHS showed in Tables 1 and 2. The pharmacokinetic properties predictions of DHS indicate the compound was endowed with a drug like properties. The results revealed that there is no violation of an agreement with the rule of five. The molecular weight of compound range from 508.38 a.m.u. The number of hydrogen bond donor is zero whereas the hydrogen bond acceptor values vary from 4. Also, the partition coefficient values of DHS are less than seven. The tested compound has a maximum percentage of human oral absorption DHS which have less than 100%. The aqueous solubility (QPlogS) parameter and IC_{50} values of HERG K+ channel blockage (QP logHERG) of the tested DHS possess permissible parameters. The prediction of blood-brain barrier permeability (QPlogBB) for the tested DHS was assessed and the DHS were predicted to have acceptable values range from -1.374. The CYGD whereas displayed negative value.

TABLE-1
LIPINSKI'S RULE OF FIVE FACTORS OF (*E*)-1-(4-(DIFLUOROMETHOXY)-2-HYDROXYBENZYLIDENE)SEMICARBAZIDE

mol_MW (< 500)	Donor HB (< 5)	Accpt HB (< 10)	QP log Po/w (< 5)	Rule of five
245.185	4	2.75	0.577	0

TABLE-2
PHARMACOKINETIC PROPERTIES OF (*E*)-1-(4-(DIFLUOROMETHOXY)-2-HYDROXYBENZYLIDENE)SEMICARBAZIDE

Percent human oral absorption[a] (> 100 high, < 25 poor)	QP log S[b] (–6.5 to 0.5)	QP log HERG[c] (below –5)	QP log BB[d]
61.86	-1.363	-3.276	-1.374

[a]Percentage of human absorption, [b]Predicted aqueous solubility; S in mol/L, [c]Predicted IC_{50} value for blockage of HERG K+ channels, [d]Predicted blood-brain barrier permeability.

In a recent study, it is reported that hydroxybenzylidene derivatives play a crucial role as potent and selective inhibitors of VEGFR-2. Therefore, the additional biological importance of receptor DHS was examined by performing molecular docking (MD) simulations to examine the binding modes with the active site of VEGFR-2. The MD simulation was performed on the ligand-docked structure of VEGFR-2 inhibitor complex by using the computational code of Schrödinger (Fig. 1).

The DHS of VEGFR-2 (PDB code: 2OH4) and the docking predicted conformation of the compound was prepared individually before carrying out MD simulations. The docking study

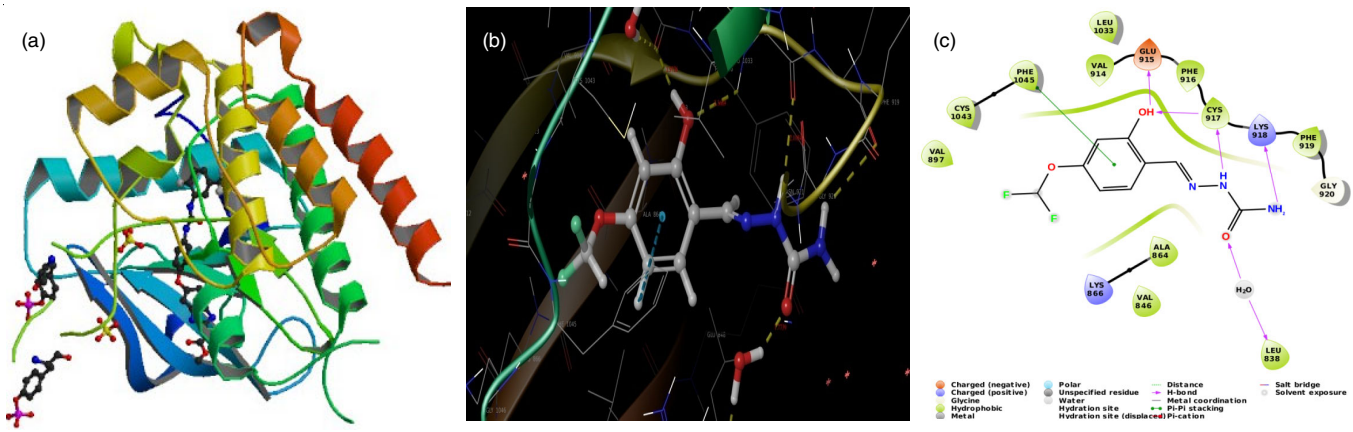


Fig. 1. (a) Molecular docking study of VEGFR-2 (PDB code: 2OH4) with (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide and (b,c) the various non-covalent interactions between the DHS and VEGFR-2

TABLE-3
MOLECULAR DOCKING STUDIES OF (*E*)-1-(4-(DIFLUOROMETHOXY)-2-HYDROXYBENZYLIDENE) SEMICARBAZIDE.

Glide gscore	Glide evdw	Glide ecoul	Glide energy	Interacting residues
-8.441	-29.808	-10.645	-40.453	PHE1045, GLU915, CYS917, LYS918, LEH838, H_2O

Glide evdw = van der Waals interaction energies, Glide ecoul = Coulomb interaction energies

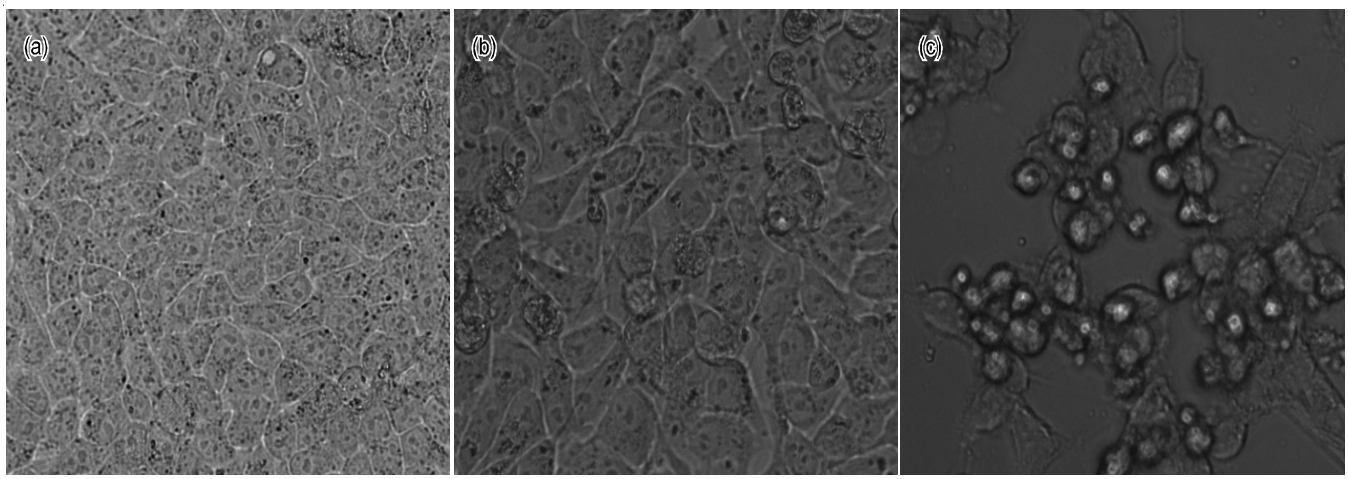


Fig. 2. Live cell images of (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide: (a) before and (b and c) after treatment with DHS examined by fluorescence microscopy

of VEGFR-2 with DHS has exhibited well established non-covalent bonds with amino acids in the VEGFR-2 active pocket (Fig. 1) and showed a relatively good binding affinity (-362.99 kcal/mol). The multiple hydrogen bonds and electrostatic interactions with the various amino acids of VEGFR-2 with DHS were shown in Table-3.

Cytotoxicity: The cytotoxicity responses toward different concentrations of DHS were studied using cellular imaging. Hence, the cellular imaging results clearly showed that DHS efficiently monitored changes in the intracellular concentration under specific biological conditions. Moreover, based on the results of MTT assay, which involved the treatment of KB cells with various concentrations of DHS for up to 5 h, DHS exhibited acceptable cytotoxicity. As shown in Fig. 3, at 20 μM DHS, significant cytotoxic effects were not observed on the KB cells for at least up to 4 h. The synthesized DHS was tested for cytotoxic activity on the KB cells by using the MTT test, which enables the assessment of the effects of complexes on cellular mitochondrial metabolism. The cells were tested for 2 days with increasing concentrations of the test compounds. The microscopic images of the control cancer cells and apoptotic morphological changes in DHS-treated KB cells are provided in Fig. 2.

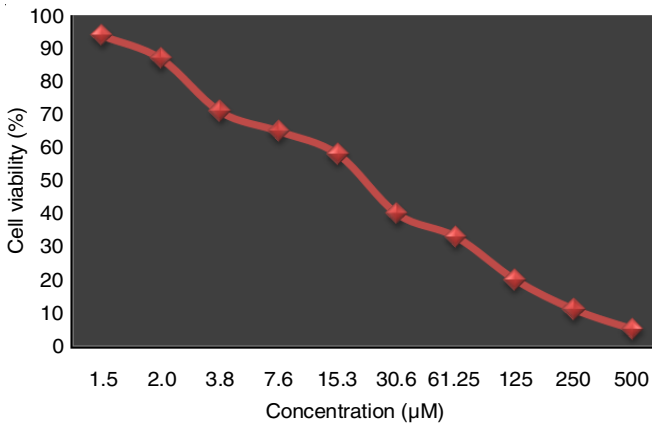


Fig. 3. IC_{50} values of (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene)-semicarbazide against KB cell lines

The results showed that DHS causes minimum cell death in normal cells. DHS treatment inhibited 83 % of KB cells when treated with IC_{50} values. The IC_{50} values of DHS (Fig. 3) suggested that DHS possessed a more potent inhibitory effect against the cancer cells than against normal cells. The form of DHS with the OH group in the meta position exhibited the highest IC_{50} value, which suggested that the electronic effect may be one of the factors determining the anticancer activities of DHS. The IC_{50} values of DHS against KB cells are listed in Table-4.

TABLE-4
IC_{50} VALUES OF (*E*)-1-(4-(DIFLUOROMETHOXY)-2-HYDROXYBENZYLIDENE)SEMICARBAZIDE AGAINST KB CELL LINES

Concentration (μM)	Viability (%)	Concentration (μM)	Viability (%)
0	100	31.20	50.04
1.95	92.39	62.50	43.36
3.90	86.05	125	29.54
7.80	74.96	250	18.1
15.60	61.27	500	4.71

Conclusion

A successful attempt for design and synthesize an easy-to-make green, one-pot pseudo-three-component (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide (DHS) is made and characterized by FT-IR, ^1H and ^{13}C NMR spectral analysis. The docking results provided potent and valuable information for the future fabrication of more useful VEGFR-2 inhibitors. The semicarbazide has potent *in vitro* cytotoxic against KB cell line, living cells images. On the

establishment of these results, in worth mentioning, it is believed that present protocol receptor (*E*)-1-(4-(difluoromethoxy)-2-hydroxybenzylidene) semicarbazide will be a valuable addition in academia for its drug properties.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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