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ULTRASONIC SYNTHESIS AND *IN VITRO* EVALUATION OF SOME NEW BISCHALCONES AS POTENTIAL CYTOTOXIC AGENTS

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Abstract:

Despite the availability of various classes of chemotherapy agents for the treatment of the complicated disorder, cancer, developing most effective cytotoxic agents with high potency and least drawbacks being major concern in the field of medicinal chemistry. Therefore, the demand for novel molecules to treat cancer efficiently through multiple mechanisms is increasing. It is very much evident that bischalcones being the global research focus to compensate the demand. Fortunately, the development of the most appropriate bischalcone derivatives with high potency and binding affinity still not been addressed. Hence, emphasizing eco-friendly technological shift, in this research, ultrasonic technique was used to synthesis series of bischalcones derivatives, ***RVD1-RVD4****. The potential cytotoxicity of the compounds was confirmed through in-vitro evaluation using Brine Shrimp (Artemia salina) Lethality Assay. Among the compounds tested, compound* ***RVD3*** *and* ***RVD4*** *has showed significant cytotoxicity at LD50 values* ***13.18 µg/mL ±0.12*** *and* ***13.80 µg/ml ±0.11*** *respectively. Consequently, in silico molecular docking studies have also been performed to evaluate the possible underlying mechanism of action of the compounds against Dihydrofolate Reductase enzyme (DHFR) anticancer drug target. Molecular docking results revealed that the highly potent bioactive bis-chalcone* ***RVD3*** *is less selective towards inhibition of DHFR.*

Keywords: *Cancer, bis-chalcones, cytotoxic agent, ultrasonic, in-vitro brine shrimp (Artemia salina) lethality assay, molecular docking, Dihydrofolate Reductase (DHFR)*

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INTRODUCTION:

Drug, the chemical that interacts with a biological system and produces a biological response, is discovered through iterative process which essentially comprises a few discrete stages. Medicinal chemistry involved in fundamental steps of a drug discovery, essentially in the phases of lead discovery, lead optimization, building structure-activity relationships (SAR) and identifying drug candidates at molecular level (IUPAC 1974). After many centuries in which the search for therapeutic substances was based on the exploitation of natural resources, the advent of organic chemistry radically changed the perspective of drug discovery towards purification and synthesis of bioactive principles, suggesting that new active drugs could be easily obtained by synthesis. As a consequence, at present, the synthesis of active principles is largely guided by the study of the interactions between active substances and their molecular targets that have been disclosed by progress in genetics and molecular biology[1].

Since prehistoric times, humans have used natural products, such as plants, animals, microorganisms, and marine organisms, in medicines to alleviate and treat diseases. According to fossil records, the human use of plants as medicines may be traced back at least 60,000 years. This was evident with the earliest records depicted on clay tablets in cuneiform from Mesopotamia 2600 B.C. which document the use of oilsfrom *Cupressussempervirens* and *Commiphora* sp ecies to treat coughs, colds and inflammation [2]. The Ebers Papyrus, 2900 B.C. an Egyptian pharmaceutical record which documents over 700 plant-based drugs, the Chinese Materia Medica 1100 B.C., Shennong Herbal are other documented records on the uses of natural products, and together provokes urge towards the search for naturally hidden raw medicinal treasures. Continuity on the findings reveals the medicinal property of natural product to be accompanied with its toxic effects by the Greeks, thus, the word "Pharmakon" used to indicate both remedy and poison [3].

With these, highest impact of organic compounds in the field of medicine has been brought to attention by the scientist Berzelius (1807) through his statement that organic chemicals found in nature contain a special vital force for the natural synthesis which can't be accomplished in laboratory. Significance hampered when the extraction of pure substance from plants ensured consistent quality with major toxicities. Therefore, the search for less toxic medicinal products resulted in the introduction of synthetic substances. Later in century, Frederich Wohler (1828) discovered that urea, a natural component of urine, could be synthesized in laboratory by heating ammonium cyanate and proved that the vital force in nature also paved the way for many researchers to the field of synthesis, focusing biologically active organic compound. The adopted approach was to synthesize drug structures based on the pharmacologically active compounds referred to as "leads".

Paul Ehrlich and Sacahiro Hata contribute to the first rational synthetic development of Arsphenamine for syphilis in 1910 and recognition of Ehrlich on the importance of both medicinal and toxic property of a drug towards the microorganism than the host cell, contribute to the concept of chemotherapeutic index. Fortunately, during the late 1990s synthetic chemists realized that combinatorial libraries that consist of hundreds to thousands of new compounds lacked the complexity of the intricate natural products synthesized by nature. Therefore, the concept of diversity-oriented synthesis (DOS) was adopted in which synthetic chemists would synthesize compounds that resembled natural products or that are based on natural product topologies [4].

Cancer is the most leading cause of mortality, characterized as a chronic disorder involved in various cell signaling pathways and disorganized cell functions like irregular cell proliferation with disturbed apoptosis. Worldwide reports on cancer supported that among all the types of cancers breast cancer, blood cancer, liver cancer, lung cancer, brain cancer, colon cancer, prostate cancer, cervical cancer and ovarian cancer plays a vital role in the mortality. Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired damage to genes that directly regulate their cell cycles [5]. During the G, phase, cells respond to extracellular signals by either advancing toward another division or withdrawing from the cycle into a resting state. Unlike transit through the S, G2, and M phases, G progression normally relies on stimulation by mitogens and can be blocked by antiproliferative cytokines. Cancer cells devoid of these controls and tend to remain in cycle. The decision to divide occurs as cells pass a restriction point , after which they become refractory to extracellular growth regulatory signals and instead commit to the autonomous program that continue through to division [5].

Clinically chemotherapeutic agents showed beneficial effects in cancer treatment. These chemical compounds exhibited fatal adverse effects like bone marrow depression and some drugs produces alopecia [6]. Currently there is a huge scientific and commercial interest in the discovery of potent, safe and selective anticancer drugs, till today development of anticancer agents without any adverse effects and with lowest possible cost is a potential research area

for pharmaceutical companies as well as nonprofit government and non-government organizations, like the National Cancer Institute (NCI) in the United States, the European Organization for Research and Treatment of Cancer (EORTC), and the British Cancer Research Campaign (CRC) [7]. Identification of cytotoxic compounds led the development of anticancer therapeutics for several decades. Advances in cancer treatment, however, continued to be limited by the identification of unique biochemical aspects of malignancies that could be exploited to selectively target tumor cells. For the last three decades, the "Oncogene Revolution" prompted investigators to concentrate on the development of agents against oncogenes, with the goal of blocking cell growth and metastasis. It has now become clear that the cancer cell genome is too varied and the number of oncogenes too numerous for this strategy to work effectively for most tumours. Thus, the most reliable target for drug efficacy in all type of cancers will be the molecular level cell cycle targets, with more significant multiple mechanism of action [8].

The compounds selected for evaluation as potential anticancer agents could be of natural or synthetic origin. Compounds of natural origin have often provided new leads in the novelty of structures with anticancer activity. Vincristine derived from the periwinkle plant Vinca rosea, etoposide is derived from the mandrake plant Podophyllum peltatum, and taxol, which is derived from the pacific yew, Taxus brevifolia. Similarly, doxorubicin and bleomycin are fermentation products of the bacteria Streptomyces; L-asparaginase is derived from the broths of Escherichia coli or Erwinia carotovora; rhizoxin is derived from the fungus Rhizopus chinensis; cytarabine was discovered from the marine sponge Cryptotethya crypta; and bryostatin from the sea moss Bugula neritina. Analogs of natural compounds have often been synthesized to improve their efficacy or toxicity profiles [9]. For example, carboplatin was developed as an analog of cisplatin with reduced renal toxicity, doxorubicin is an analog of daunomycin that reduces its cardiotoxicity, and topotecan is an analog of camptothecin with better toxicity profile. The synthetic compounds could be the analogs of known compounds or novel structures. The process of identifying and selecting these candidates has undergone a sea change in the recent decades with the development of solid-state and combinatorial chemistry and computer modeling of drug–receptor interactions. Discovery of new anticancer agents by laboratory synthesis has evolved from analog evaluation and improvement of new leads to rational design based on drug–receptor or drug–enzyme interactions [7].

It is almost 50 years since antimetabolites were first found to have clinical antitumour activity, with Farber's discovery that aminopterin could cause remission in acute leukaemia. An antimetabolite is defined as a drug that interferes with the normal metabolic processes within cells. Knowledge of these processes at a cellular level has increased, leading to the identification of a number of potential new targets. The metabolic processes of the cell are complex and involve many enzymes. Two important pathways exist, which give rise to the synthesis of purines and pyrimidines. Inhibitors of vital enzymes in these pathways are being studied, including dihydrofolate reductase (DHFR), thymidylate synthase (TS), and glycinamide ribonucleotide formyltransferase (GARFI). Methotrexate, one of the earliest antimetabolites discovered, has been in use in cancer chemotherapy for over 30 years [10]. It is an inhibitor of DHFR, which occupies a central position in the metabolic pathway. DHFR is responsible for the conversion of dihydrofolate to tetrahydrofolate and ultimately to 10-formyl tetrahydrofolate. The last compound provides the formyl group for glycinamide ribonucleotide formyltransferase (GARFT) and aminoimidazole carboxamide ribonucleotide formyl transferase (AICARFT).

Resistance to methotrexate arises by a variety of mechanisms, including impaired transport via the reduced folate carrier and multi drug resistance pathway [11]. Clearly, resistance to antimetabolites remains a formidable obstacle, but increasing opportunities for translational research which inspired the search for other novel antimetabolite, offering prospect of improvements in this key area in the future [12].

Chalcones the compound bearing 1,3-diaryl-2-propen-1-one system are found in two moieties together as a single molecule of bis-chalcones. The compounds with chalcone skeleton have been reported to possess broad spectrum of biological activities such as anti-inflammatory [13], anti-proliferative [14], anti-malarial [14], anti-pyretic[15], antibacterial [16], antifungal[17], anti-ulcerative [18], antiviral [19] and hepatoprotective [20]. These activities are largely attributed due to the presence of two chalcone moieties in a single molecule. The chalcones gained recognition as cytotoxic agents in 1990, and have since been presented in an increasing number of scientific papers and patents, including reports on the anticancer properties of these agents [21].

Worldwide, one in seven deaths is due to cancer; cancer causes more deaths than AIDS, tuberculosis, and malaria. By 2030, the global burden is expected to grow to 21.7 million. Despite the availability of

many anticancer agents to treat different types of cancer efficiently, the toxicity profile remains largely and difficult to modify. Therefore, the demand for novel molecules to treat cancer efficiently with high safety profile is increasing. Hence, we proposed worthwhile to synthesize some novel bischalcones derivatives and studied their cytotoxicity properties using brine shrimp (Artemia Salina) lethality bioassay. Due to their abundance in plants and ease of synthesis, this class of compounds has provoked great interest for possible therapeutic uses. In the design of new drugs, the development of a single molecule through combining two chalcone pharmacophore moieties may lead to compounds with interesting enhanced biological profiles. Numerous reviews compiling the results of investigations on the anticancer potential of chalcones based structures in research articles are already present. In recent years, chemotherapy with agents possessing different mechanisms of action such of bis-chalcones places them in forefront as potential future drug candidates.

MATERIALS AND METHODS:

Materials

Instrumentation

Melting points will be taken in open capillary tubes. Purity of the compounds will be checked on silica gel G TLC plates of 2 mm thickness using n-hexane and ethyl acetate as solvent system. The visualization of spot was carried out in an UV chamber. The spectra, IR, NMR and Mass have been recorded by sending the pure sample to Universiti Malaya.

Reagents and chemicals

Substituted aldehydes and ketones, urea, Brine Shrimp eggs, n-hexane, ethyl acetate, acetone, chloroform, methanol, ethanol, iodine, sodium hydroxide, sulphuric acid, Silica gel (column and TLC) and other regular laboratory chemical of AR grade were procured from the local chemical suppliers via purchase indent from Laboratory Department, Asia Metropolitan University.

Computational software requirements

Computer aided drug discovery softwares along with graphical user interface (GUI) were utilized for molecular modelling, energy minimization, molecular docking and virtual screening protocols.

Table 1: List of software applications used in present study

S.No	Activity (Performed)	Software (Used)	License type (Obtained)	Source
1)	Molecular modelling (2D-Drawing)	Accelrys Draw	Academic License GPU (General Public User) License	Open Source
2)	Molecular modelling (2D-3D Conversion)	Open Babel	Academic License GPU (General Public User) License	Open Source
3)	Molecular modelling (Molecular Mechanics & Energy Minimization)	Argus Lab v 4.0	Academic License GPU (General Public User) License	Open Source
4)	Molecular Docking & Virtual screening	iGemdock v 2.1	Academic License GPU (General Public User) License	Open Source

Table 2: Citations for software applications used in the present study

S.No	Software	Citation
[1]	**Accelrys Draw**	Draw, A. (2011). Acceryls Software Inc. San Diego.
[2]	**Open Babel**	OLBoyle, N.M., Banck, M., James, C.A., Morley, C., Vandermeersch, T., & Hutchison, G. R. (2011). Open Babel: An open chemical toolbox. J Cheminf, 3, 33.
[3]	**Argus Lab**	Thompson, M. A. (2004). Argus Lab 4.0. 1. Planaria Software LLC, Seattle, WA.
[4]	**iGemdock v 2.1**	Hsu, K.C., Chen, Y.F., Lin, S. R., & Yang, J. M. (2011). iGEMDOCK: a graphical environment of enhancing GEMDOCK using pharmacological interactions ant post screening analysis. BMC bioinformatics, 12(1), 1.
[5]	**Protein Data Bank (PDB)**	Bank, P. D. (1971). Protein Data Bank. Nature New Biol, 233, 223.

Computational hardware requirements

The minimum central hardware system configuration include Intel (R) Core (TM) 2Duo Central Processing Unit (CPU), 2.5 GHz, 1 TB hard disk, 2 KV Power Backup, WinXP was used for running all the selected computer aided drug discoveri softwares. All softwares were well compatible with the selected system configuration.

X-ray crystallographic structure of

X-ray crystallographic data of DHFR Ligand Binding Domain (LBD) was obtained from Brookhaven Protein Data Bank (http://www.rcsb.org/pdp). The protein data bank code (PCB ID: 4KD7) deposited by Lamb, et.al., 2013[22].

Methods

General procedure for the synthesis of bis-chalcone derivatives

As shown in the scheme 1, we evaluated the pre-existing methods for the proposed synthesis and further studies was done to optimize the reaction conditions for further improvement of the product purity & yield.

Scheme 1: Synthesis of Bischalcones

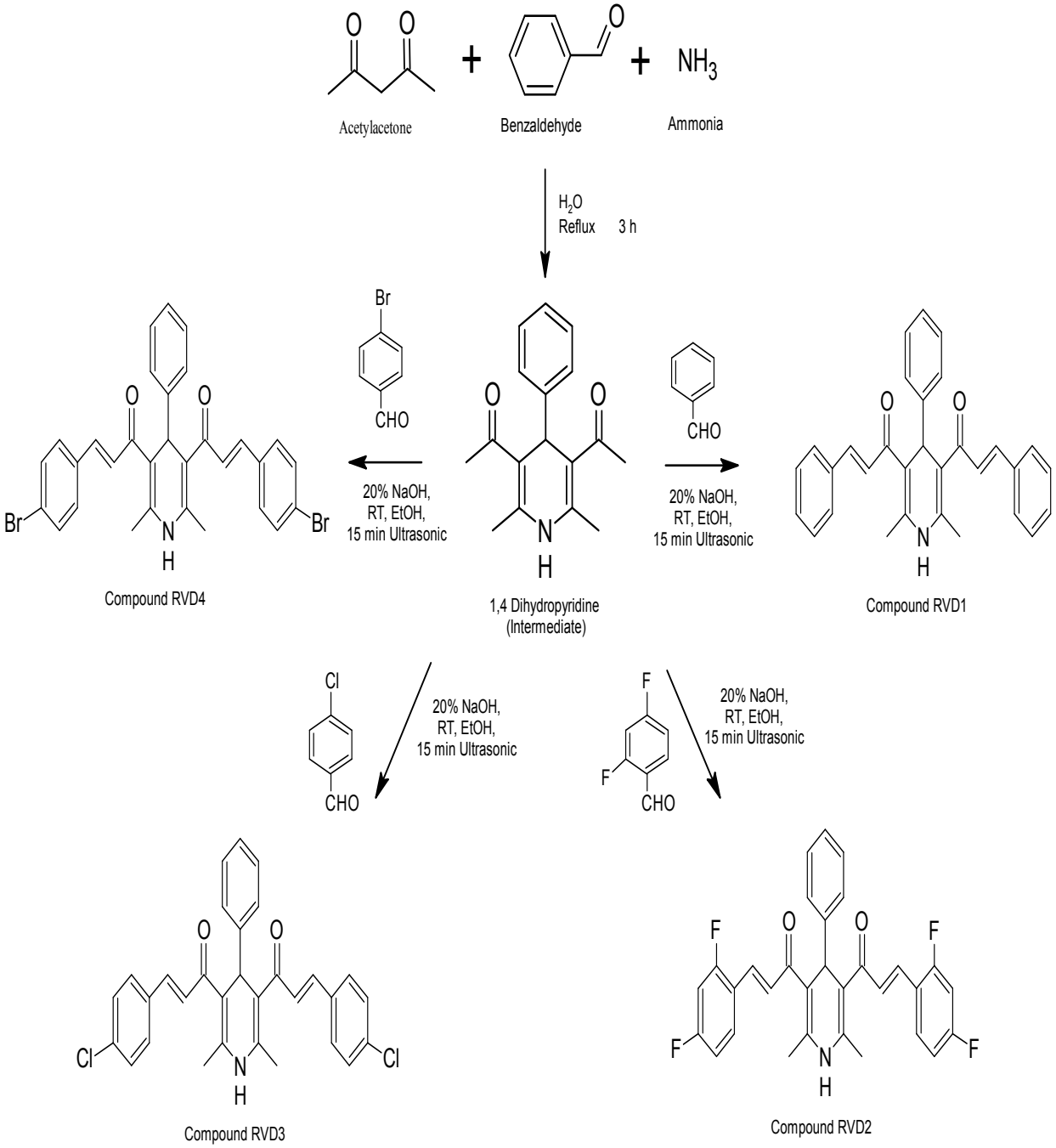
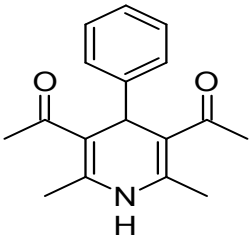
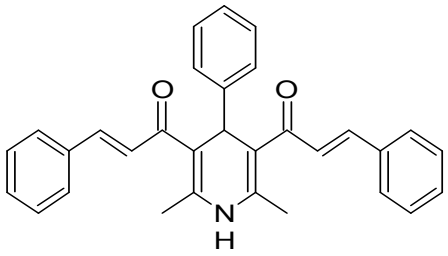


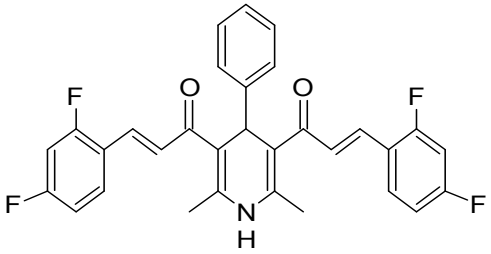
Table 3: Chemical structure of the titled molecules



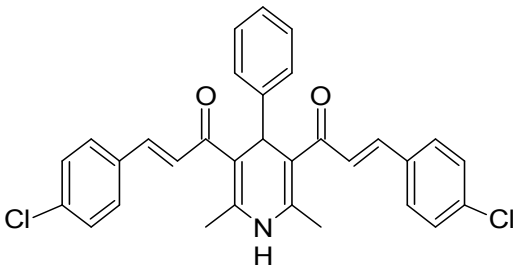
Intermediate



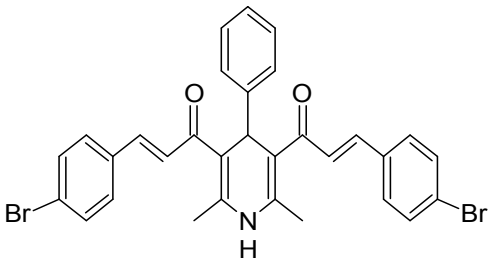
Compound **RVD1**



Compound **RVD2**



Compound **RVD3**



Compound **RVD4**

The reaction seen in scheme 1 was carried out by adding substituted benzaldehydes to the intermediate in 2:1 mole concentration in q.s amount of ethanol. To this reaction mixture, 15mL of 20% NaOH solution was added in drop wise and the mixture was irradicated by an ultrasonic generator in water bath at 30-35 ºC for 15 minutes. The reaction mixture turns yellow. After completion of the reaction, the mixture was poured in crushed ice, acidified with 1:1 dilute hydrochloric acid, yellow coloured solids separate out, which later was isolated by filtration using Buchner funnel under vacuum filtration setup, dried and purified by recrystallization with ethanol. The solids of bischalcones RVD1-RVD4 were collected and used for doing physical characterization and bioassay test. The physical properties are depicted in separate table as given under Chapter 5 for an every individual compound.

Identification of bis-chalcones (RVD1-RVD4)

The formation of bischalcones was identified and analysed by Co-TLC technique with starting materials. The visualization of the spots was carried out under UV light and in Iodine chamber.

Characterization of bis-chalcones (RVD1-RVD4)

The chemical structures of the synthetic bis-chalcones was established based on their Physical, chemical and spectral analytical data.

Melting points was determined in open capillary tube, and expressed in degree Celsius.

The synthetic was characterized by UV, IR, NMR & Mass spectral methods.
NMR and IR were obtained by sending the samples to Universiti Malaya.

Biological evaluation of bis-chalcones
It has been demonstrated that a positive relationship exists between brine shrimp lethality and human carcinoma by Kesavan SS et al. Principle of this method is based on the ability of synthesized bis-chalcones to kill laboratory cultured Artemia nauplii brine shrimp. This brine shrimp lethality assay was carried out according to Mellany et al., with minor modifcations. About 1 g of Artemia salina (Linnaeus) cysts was aerated in 1 L capacity cone shaped container containing seawater (Sea water salt 38g/L and pH of 8.2 maintained). Air pump was fitted to the water to ensure complete aeration of the cysts. After 30 hours of incubation at room temperature (25-29°C), under continuous illumination of incandescent lamp newly hatched free-swimming brown-coloured nauplii were harvested from the bottom. As the cyst capsules floated on the surface, this collection method ensured pure harvest of nauplii. The freshly hatched free-swimming nauplii were used for the bioassay. Ten nauplii were placed in each vial containing 5mL of drug solution of various concentrations. The setup was allowed to remain for 24 h, under constant illumination of lamp. Three replicates were prepared for each dose level and conducted along with control (vehicle treated). Number of survived nauplii was counted with a hand lens after 24 hours. LC50 values (µg/mL) were determined by comparing mean surviving larvae of test and control tubes. The results of cytotoxicity study are given under results section.

General procedure for the ligand preparation
The chemical structure of the selected ligand **RVD3** was initially modelled as 2D chemical structures using Accelrys Draw software and transformed into 3D chemical structure using Open Babel software and subjected for energy minimization using ArgusLab v 4.0 software. The minimization was executed until the root mean square gradient value reached a value smaller than 0.0001 kcal/mol. Such energy minimized structures were considered for molecular docking studies using iGemdock v 2.1 software. The corresponding docking engine compatible 'MDL MOL' file format has been adapted to ligand by using integral option (save as /MDL MOL).

General procedure for the protein target selection, preparation and validation
The selection of DHFR Ligand Binding Domain (LBD) for molecular docking studies was carried out based upon several factors such as determination of structure by X-Ray diffraction spectroscopy with resolution between > 2.5 Aº, and it should contain a co-crystallized ligand. The selected protein should not have any protein breaks in their 3D structure. On the other hand, we further consider Ramachandran plot statistics as the important filter for protein selection with none of the residues present in disallowed region. Finally, the resultant protein target was prepared for molecular docking simulation in such a way that all heteroatoms (eg; non-receptor atoms such as water, ions, etc.) were removed.

General procedure for the software validation
iGEMDOCK v 2.1 software validation was performed by using X-ray structure **(4KD7)** deposited with co-crystallized ligand was obtained from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb). The Root Mean Square Deviation (RMSD) between the X-ray co-crystallized ligand and docked confirmation was 1.84 Aº indicated that the parameters for docking simulation was good in reproducing X-ray crystal structure.

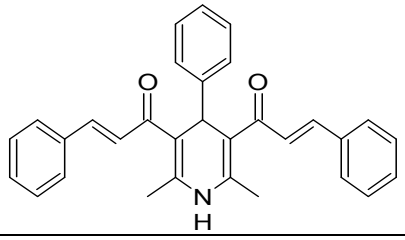
General procedure for the Molecular Docking
Molecular docking technique was employed to dock the bioactive bischalcone **RVD3** against **4KD7** using iGEMDOCK to locate the interaction between **RVD3** and **4KD7**. iGEMDOCK requires the receptor and ligand coordinates in either MOL2 or PDB format. Non polar hydrogen atoms were removed from the receptor file and their partial charges were added to the corresponding carbon atoms. Molecular docking was performed using standard protein-ligand docking protocol. The binding site was defined by crystallographic ligand of **4KD7**. Default setting was used for all the calculations and docking run was performed.

Satistical analysis
The resulting data will be converted using SPSS 20 as probit analysis method for determination of lethal concentration 50% (LC_{50}) values of the tested compounds. Data was expressed as mean ±SEM.

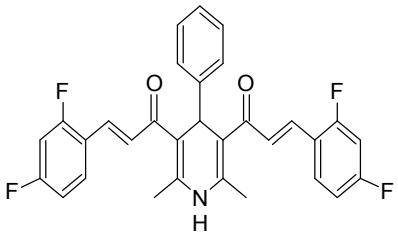
RESULTS

Results of chemical synthesis

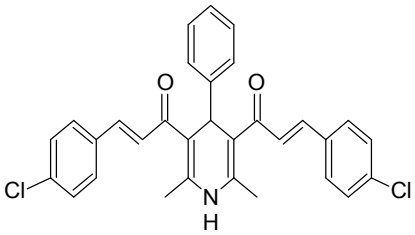
Physical characterization of bischalcone RVD1

Bischalcone (RVD1)	
	
(a) Physical state	Solid
(b) Colour	Dark yellow
(c) Nomenclature	1,1-(4-phenyl-1,4-dihydropyridine-3,5-diyl)bis(3-phenyl-prop-2-en-1-one)
(d) Molecular weight	417.51 g/mol
(e) Molecular formula	$C_{29}H_{23}NO_2$
(f) Melting point	186
(g) Yield (%)	87
(h) Recrystallization solvent	Ethanol
(i) Thin Layer Chromatography (TLC) ❖ **Mobile phase concentration** ❖ **R_f value** ❖ **UV-254nm observation**	Thin Layer Chromatography • 25% Ethylacetate/Hexane • 3.7 • Green Fluorescence
(j) UV spectrum data (λ_{max})	311
(k) IR spectrum data (cm^{-1})	3319.32 (ar NH), 1621.80 (C=O), 1014.34 (C=C–H)

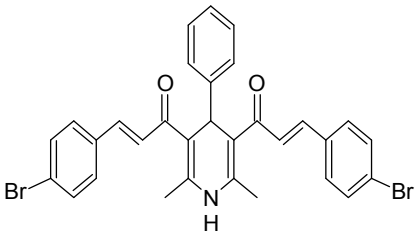
Physical characterization of bischalcone RVD2

Bischalcone (RVD2)	
	
(a) Physical state	Solid
(b) Colour	Yellow
(c) Nomenclature	1,1-(4-phenyl-1,4-dihydropyridine-3,5-diyl)bis(3-(2,4-diflorophenyl)prop-2-en-1-one)
(d) Molecular weight	489.46 g/mol
(e) Molecular formula	$C_{29}H_{19}F_4NO_2$
(f) Melting point	183
(g) Yield (%)	85
(h) Recrystallization solvent	Ethanol
(i) Thin Layer Chromatography (TLC) ❖ **Mobile phase concentration** ❖ **R_f value** ❖ **UV-254nm observation**	Thin Layer Chromatography • 25% Ethylacetate/Hexane • 4.3 • Green Fluorescence
(j) UV spectrum data (λ_{max})	265
(k) IR spectrum data (cm^{-1})	3295.82 (ar NH), 1607.60 (C=O), 1013.35 (C=C–H), 1272.76 (– F)

Physical characterization of bischalcone RVD3

Bischalcone (RVD3)	
	
(a) Physical state	Solid
(b) Colour	Dark yellow
(c) Nomenclature	1,1-(4-phenyl-1,4-dihydropyridine-3,5-diyl)bis(3-(4-chlorophenyl)prop-2-en-1-one)
(d) Molecular weight	486.39 g/mol
(e) Molecular formula	$C_{29}H_{21}Cl_2NO_2$
(f) Melting point	143
(g) Yield (%)	87
(h) Recrystallization solvent	Ethanol
(i) Thin Layer Chromatography (TLC) ❖ **Mobile phase concentration** ❖ **R_f value** ❖ **UV-254nm observation**	Thin Layer Chromatography • 25% Ethylacetate/Hexane • 3.2 • Green Fluorescence
(j) UV spectrum data (λ_{max})	225
(k) IR spectrum data (cm^{-1})	3279.13 (ar NH), 1616.54 (C=O), 1011.91 (C=C–H), 817.76 (– Cl)

Physical characterization of bischalcone RVD4

Bischalcone (RVD4)	
	
(a) Physical state	Solid
(b) Colour	Yellow
(c) Nomenclature	1,1-(4-phenyl-1,4-dihydropyridine-3,5-diyl)bis(3-(4-bromophenyl)prop-2-en-1-one)
(d) Molecular weight	575.29 g/mol
(e) Molecular formula	$C_{29}H_{21}Br_2NO_2$
(f) Melting point	145
(g) Yield (%)	83
(h) Recrystallization solvent	Ethanol
(i) Thin Layer Chromatography (TLC) ❖ **Mobile phase concentration** ❖ **R_f value** ❖ **UV-254nm observation**	Thin Layer Chromatography • 25% Ethylacetate/Hexane • 2.8 • Green Fluorescence
(j) UV spectrum data (λ_{max})	367
(k) IR spectrum data (cm^{-1})	3292.38 (ar NH), 1584.96 (C=O), 1007.67 (C=C–H), 699.42 (– Br)

Results of biological evaluation
Cytotoxicity data of bischalcones RVD1-RVD4.

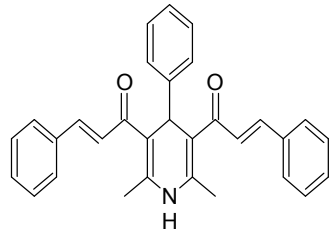
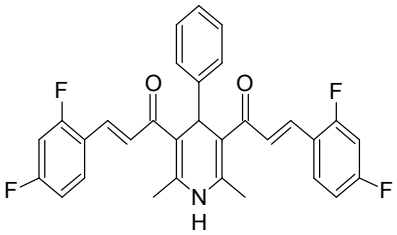
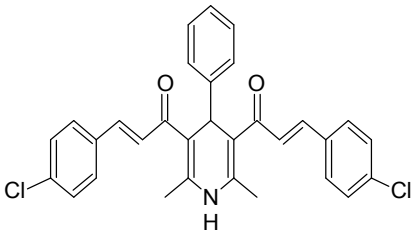
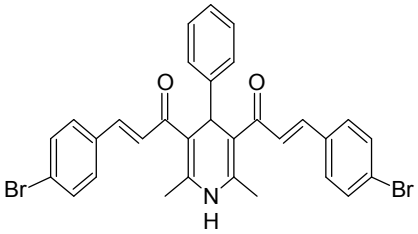
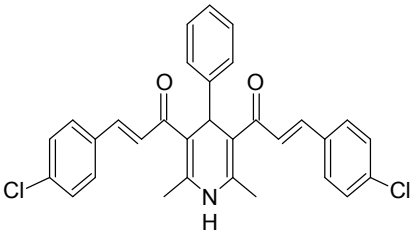
Compound	Artemia salina lethality (Brine shrimp) Lethal Dose Concentration ($LC_{50,}$ µg/mL)*
 **RVD1**	30.19µg/mL ± 0.11
 **RVD2**	28.75 µg/mL± 0.12
 **RVD3**	13.18 µg/mL± 0.12
 **RVD4**	13.80 µg/mL± 0.11

Table 3:Molecular docking information of bischalcone RVD3 against 4KD7

Compound	**iGemdock score kcal/mol**	**No. of Hydrogen bonds/ Interacting Amino acid residues**
 **RVD3**	-131.25	3/ Ser 119 (1), Thr 56 (1), Gly 117 (1)

Binding orientation and H-bond interactions (Green) of **RVD3** (Yellow) within the active binding site region of **4KD7** (Residues)

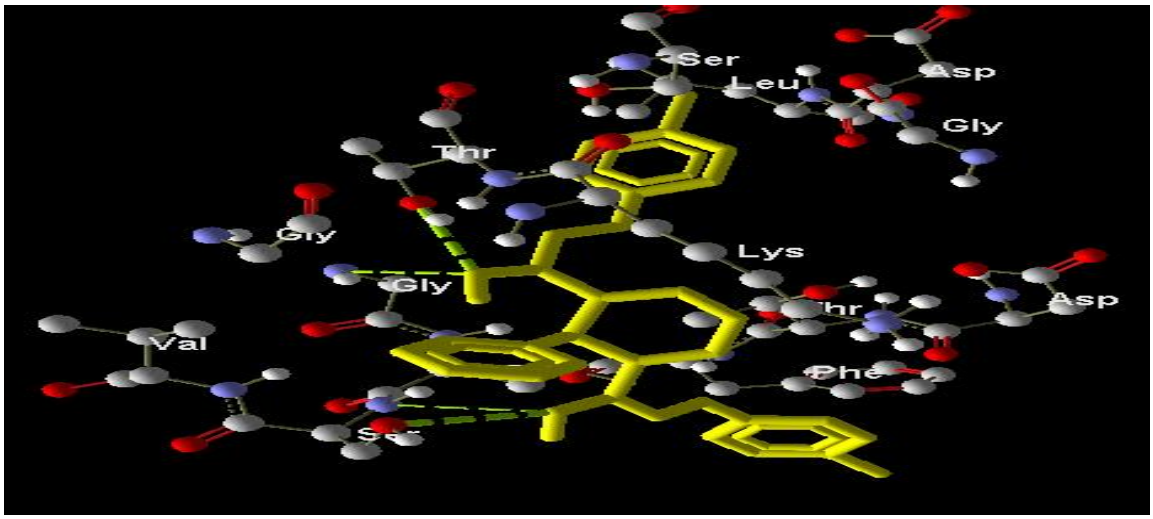
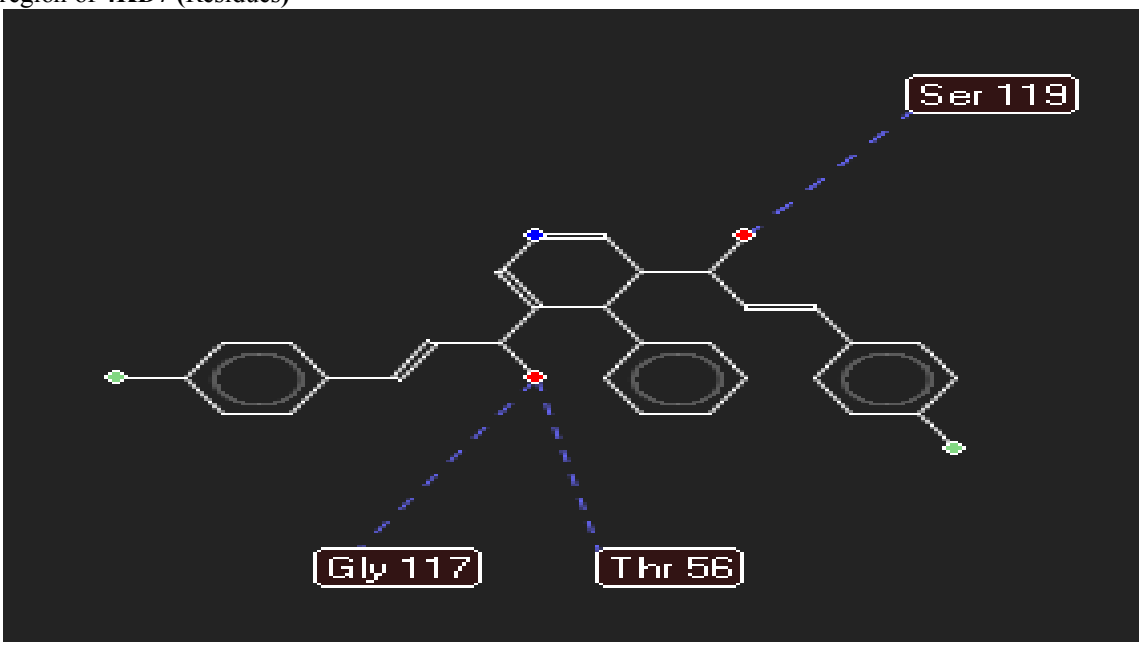


Fig 1: 2D H-bond interactions (Blue) interactions diagram of **RVD3** (white) within the active binding site region of **4KD7 (**Residues**)**



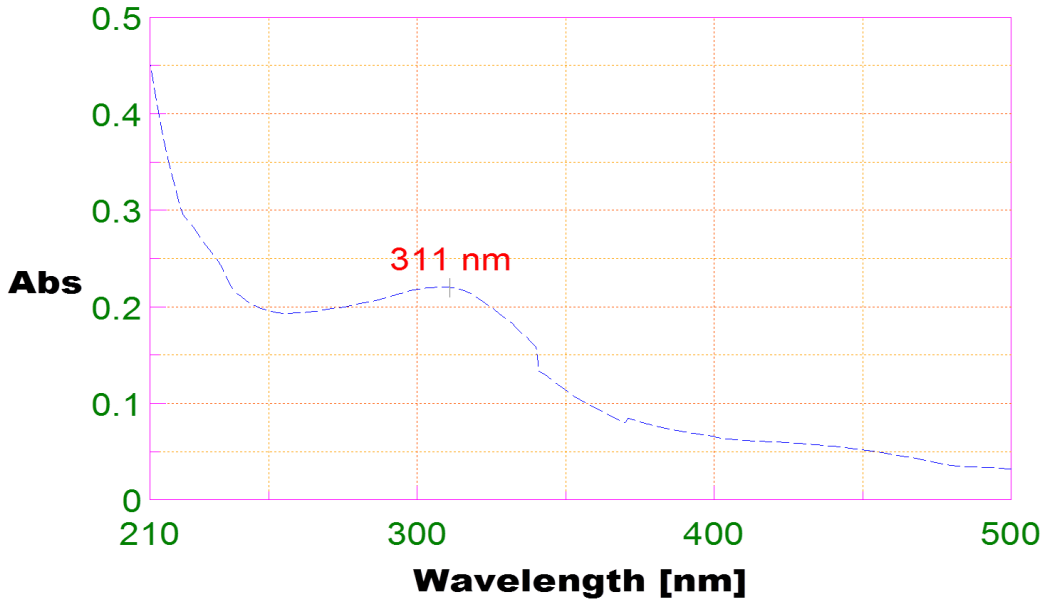
DISCUSSION:

Synthesis

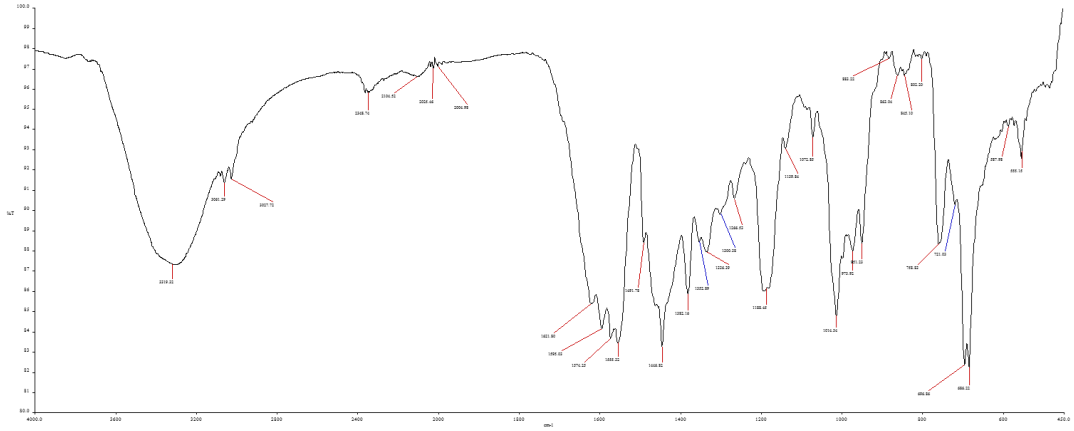
The compounds synthesized in the present study kept with the expected structures by its spectral data as shown in Tables **5.1-5.4** under chapter 5. Following are the representative UV and IR spectra of the compounds **RVD1-RVD4** respectively. The results of the spectral data of the compounds **RVD1-RVD4** were in close agreement with expected chemical structures of the bischalcones and the structures of all compounds were confirmed by following spectral data.

The results of the UV spectras for compound **RVD1, RVD2, RVD3** and **RVD4** is, 311 nm, 265 nm, 225nm, and 307 nm respectively. Originally coloured compounds such as the bischalcones derivatives should give UV spectra in the range from 200nm – 400nm. The observed colour of the bischalcones is caused the presence of the chromophoric unit –CO-CH=CH-. Besides, the existence of auxophore in the synthesized compounds responsible for the shift in the UV spectra values. It is shown that the UV spectra values of bischalcone derivatives are 260nm, 310nm and 386nm [23], 200nm to 400nm [24].

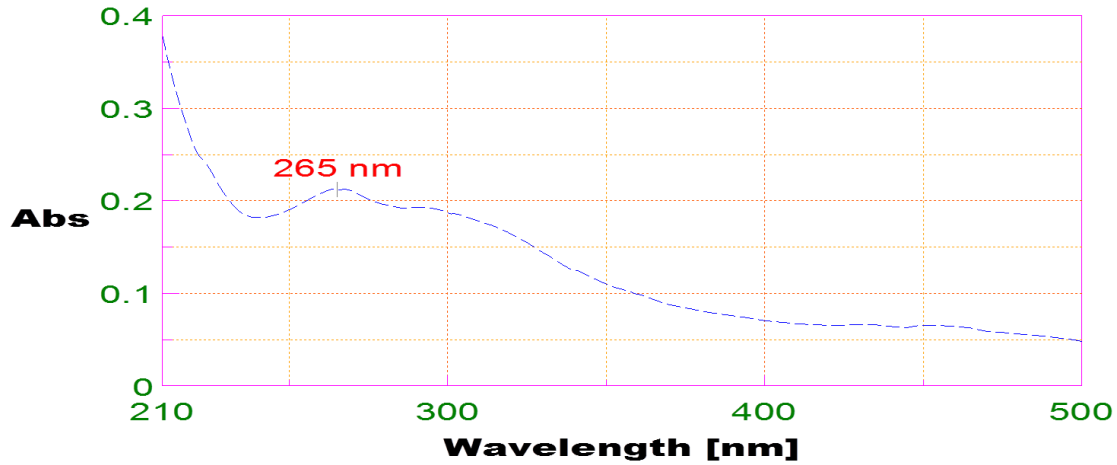
UV spectrum of RVD1



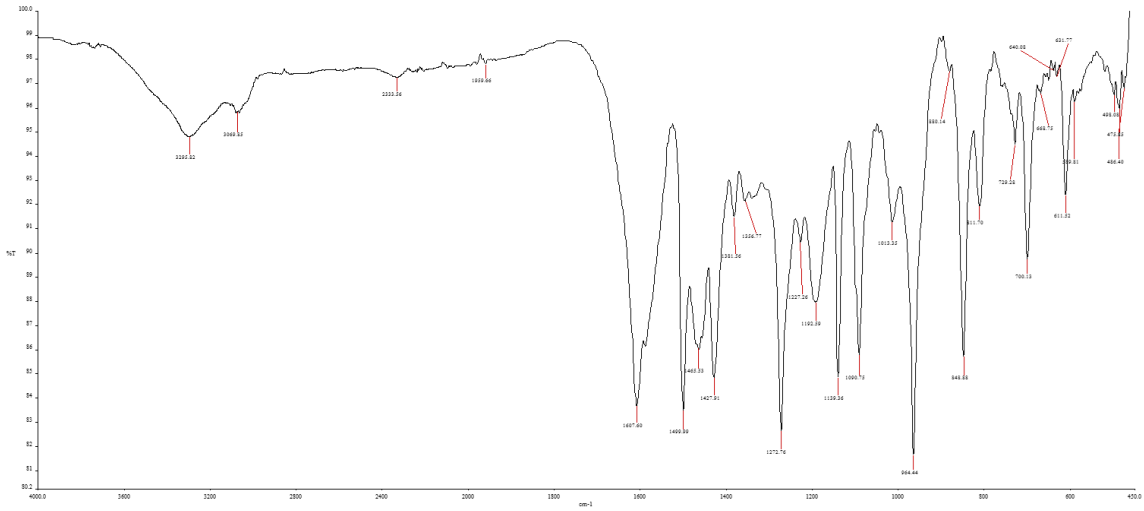
IR spectrum of RVD1



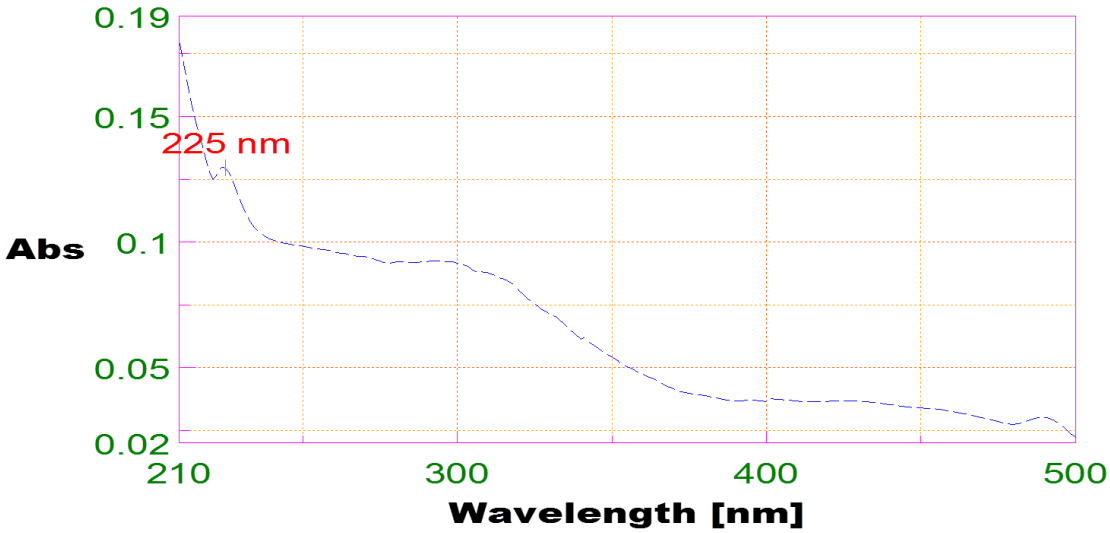
UV spectrum of RVD2



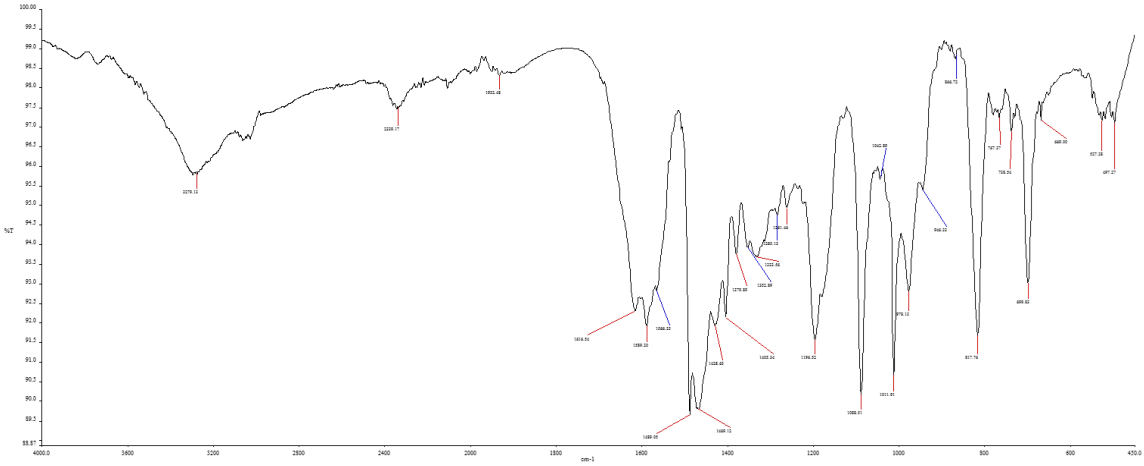
IR spectrum of RVD2



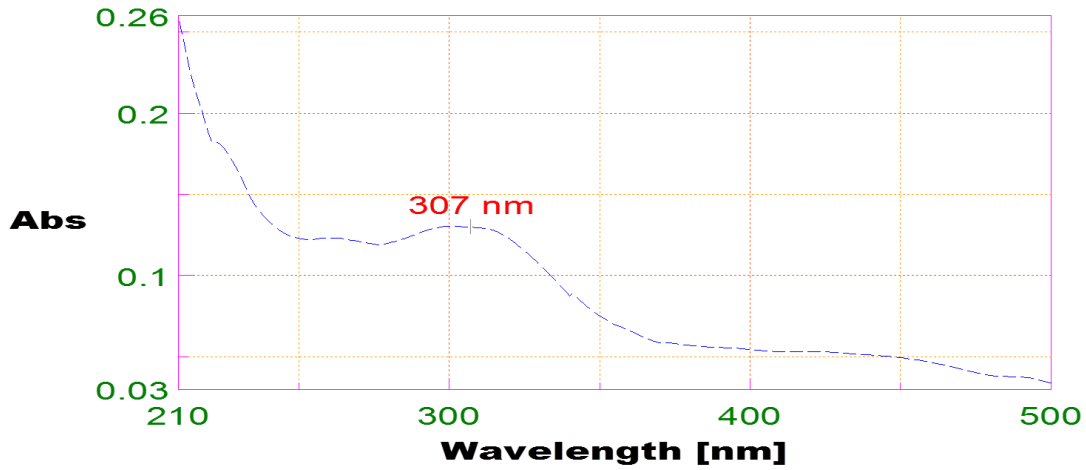
UV spectrum of RVD3



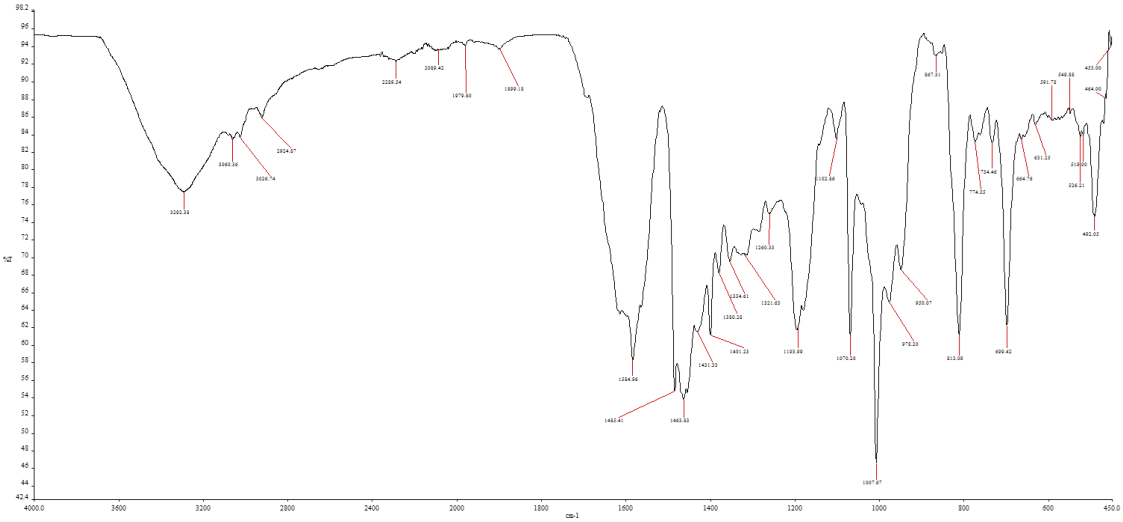
IR spectrum of RVD3



UV spectrum of RVD4



IR spectrum of RVD4



Biological evaluation

Cancer consists of a new growth of cells in which the cells exhibit high growth rate, loss of differentiation, loss of cell apoptosis control, ability for angiogenic modulation, and metastatic capacity. Inducing apoptosis spontaneously to cancer cells, often markedly retarding their growth, seen in tumors responding to irradiation, cytotoxic chemotherapy, and hormone ablations [25].

It is possible to detect and monitor the cytotoxicity of a bioactive compound, using the brine shrimp lethality bio-assay than more tedious and expensive in vitro and in vivo antitumor assays. The brine shrimp assay has advantages of being rapid, inexpensive, and simple method. The brine shrimp test was used as prescreen for a panel of six human solid tumor cell lines at the Cell Culture Laboratory of the Purdue Cancer Center suggest the resemblance of the brine shrimp as solid tumor. Therefore, this is a internationally accepted bioassay for screening of antitumor compounds[26].

Previously reported brine shrimp results provide a circumstantial evidence that bioactive compound with LC50 values below 20 µg/ml have a likelihood of yielding potent anticancer compounds[27]. Such results demonstrated by Bridelia cathartica[28,29] Croton macrostachys [28,30], Maytenus putterlickioides [28]; Ozoroa insignis[28], Psorospermum febrifugum[27,30], Phyllanthus engleri[27,33] and Ximenia Americana[28,32]. Proceeding the studies, englerin A proved to be a selective anti-cancer compound against kidney cancer cells [31], which provides further corroborative evidence on the potential of the brine shrimp test to predict the cytotoxicity potency of anti-cancer compounds.

It is therefore possible that in this study, the exerted cytotoxic effect by the synthesized novel compounds **RVD1-RVD4** creates pavement to yield synthetic compounds that is active against cancer cell lines. The LD50 values of lesser than 20µg/mL such as 13.18 µg/mL ± 0.12 and 13.8 ± 0.11 demostrated by compound **RVD3** and **RVD4** respectively, clearly

defined its high potency towards solid tumors. Whereas, compound **RVD1** and **RVD2** devoid of the expected potency because the demonstrated LD50 values was beyond 20µg/ml which is 30.19µg/mL ± 0.11 and 28.75± respectively. The results demonstrated were predictable earlier basing on the selection of the starting material in the synthesis of the target structures. This was further confirmed with obtained spectral datas in this present study. Close insight into the basic chemical structures of the synthesized molecules clearly display two chalcone moieties bridged together by dihydropyridine structure forming the bischalcone pharmacophore.

As discussed earlier, the single moiety parent chalcone proved high potency as anticancer agent through multiple mechanisms. Thus, the presence of additional chalcone moiety in a single chemical structure in this study may have highest impact more likely twice as potent as the parent chalcone. In addition, dihydropyridine with bulky substituent at position 3 and 5 proved to have equipotent effect as the antimetabolite Methotrexate and also with remarkable novelty through its multi drug resistance inhibitor activity [34]. All the synthesized compounds in this study contains chalcone moiety at position 3 and 5 of dihydropyridine structure where compound **RVD3** and **RVD4** being comparatively potent. Difference in the potency of compounds **RVD1-RVD4** reveals the influence of the halogen substitution on the phenyl ring of the chalcone moieties, thus the contribution of the halogens in retaining the potency.

Computatinal evaluation

In the present investigation, we have selected inhibition of new DNA as the potential hypothetical mechanism for the observed cytotoxicity potential of bioactive bischalcone **RVD3**. Hence, docking simulation has been performed to ddetermine possible protein-ligand interactions within the active binding site region of the **4KD7** (selected anticancer metabolite target protein DHFR). In the recent past, the interactions between the selective synthetic DHFR ligands revealed the importance of hydrogen bonding with the amino acids **Asn 64, Phe 31** and **Phe 34** are important for increased affinity to human DHFR[22] (Lamb et.al., 2013). The compound **RVD3** revealed three hydrogen bond interactions with amino acids; **Ser 119 (1H), Thr 56 (1H), Gly 117 (1H)**. Since the highly potent bioactive bischalcone **RVD3** did not display the interactions with previously reported amino acids that serve as the most vital towards the inhibition of DHFR, we hypothesized that the cytotoxicity potential of **RVD3** might not be due to DHFR inhibition. Therefore further target based studies should be performed for understanding the complete mechanism of action of the synthesized bischalcones derivatives in the present study.

CONCLUSION:

In conclusion, we could synthesize and characterize some novel bischalcones **RVD1-RVD4**. These compounds were screened for in vitro cytotoxicity potential by using Brine Shrimp (*Artemia salina*) lethality bioassay and the results revealed the positive and significant contribution of **RVD3** consisting chloro substitution at position 4 of both ring A and B of chalcone moieties which is covalently attached to the dihydropyridine nucleus, towards the observed cytotoxicity (*Artemia salina* lethality) at LD_{50} value of **13.18µg/mL ± 0.12**. Likewise, the compound **RVD4** also exhibited significant activity comparatively with RVD3 at LD_{50} value of 13.80µg/mL ± 0.11. The observed remarkable activity of RVD3 and RVD4 may be due to the pharmacophores such as α,β-unsaturation of the bischalcone moiety, halogen substituents which forms part of the basic skeleton of both the molecules. Subsequently, molecular docking studies revealed that the possible underlying mechanism might not be due to the inhibition of DHFR. Therefore, further target based studies should be the future research target to understand inherent mechanism of cytotoxicity of the synthesized bischalcones **RVD1-RVD3**.

In the current drug design and development in addition to designing ligands against any target, necessary concern has to be taken in addressing the importance of selectivity and specificity against target and also includes the pharmacokinetics profile such as absorption, distribution, metabolism and elimination. Together with the biopharmaceutical considerations, the behavior of compounds in the real environment can be clearly justified.

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