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In-Silico design, synthesis and biological evaluation of N'-[(e)-(4-hydroxy-3-methoxyphenyl) methylidene] -2-methyl-1,3-benzoxazole-5-carbohydride

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ABSTRACT

Intention of this work was to enhance various biological properties of synthesized benzoxazole derivatives into significance scale in inexpensive methods with less toxicity. Synthesis was done by both conventional and microwave methods. In-Silico studies including docking, analysis of the drug-receptor diagram and ADME prediction were done before pharmacological evaluation. Quantitative Structure Activity Relationship (QSAR) studies were also performed using MLR method. From Structural Activity Relationship (SAR) studies, noticeable results were obtained when modifying 5th position of benzoxazole with Shiff's base having aldehyde bearing specific groups (OCH_3& -OH at the 3rd and 4th position respectively) and the presences of the CH_3 group at the 2nd position of the benzoxazole nucleus. So this work can be treated as a practical proof of theoretical statement that biological activity of benzoxazole can be enhanced by a small substitution (ie. up to 3 carbons) at 2nd position. Among the analogues synthesized BZ-24- (N'- [(e)- (4-hydroxy-3-methoxyphenyl) methylidene] -2-methyl-1,3-benzoxazole-5-carbohydride) was found to have significant anti-cancer, anti-tubercular, antimicrobial and anti-fungal effects.

INTRODUCTION

Benzoxazoles are derivatives of a fused heterocyclic ring system of benzene and oxazole ring at 3a-7a position. Benzoxazole has been studied for it wide array of biological properties [1-3]. It possesses a degree of structural similarity with purine nucleic bases such as guanine or adenine that can facilitate interactions with biopolymers such as DNA. In other groups of DNA-interactive molecules, these moieties (derivatives of this compound) are often encountered in compounds that present a variety of therapeutic activities, including anti-mutagen, anti-allergic, analgesic, anti inflammatory, anti-pyrectic, antioxidant, anti-aggregant, anti-parasitic, antibacterial, antiviral, anti-tumor properties. In many cases these compounds can coordinate metal ions through nitrogen or oxygen atoms of the ring system [4-5]. Benzoxazole having -NH=N- or/and ester-linkage with aldehyde bearing free OH, -OCH3, -NO2, -Cl groups scaffold inhibits HSP-90 protein which results in cytosolic vacuolization. And benzoxazole has ability to form complex with double stranded DNA which causes anti-cancer property. Ability to inhibit enzymes mycobacterium tuberculosis Enoyl-ACP reductase (Inh A, Rv1484) prevents mycolic acid production, which results in anti-tubercular activity.

Using SAR studies Ayopova et al. investigated the quantitative relationships between 2-(alkylthio) benzoxazole derivatives and their herbicide activity using Hansch's equations [6] and Evans et al. carried out QSAR studies on two anti-inflammatory analogues 4- benzoxazoleacetic acid and 7-benzoxazole methylacetic acid[7]. Manish Kumar Gautam et al. [8] established that Benzoxazole having hydrazide bridge (-NH=N-linkage) with aromatic rings shows anti-microbial activity. Different literature survey revealed that by attaching aromatic groups to benzoxazole moiety bearing free OH,-OCH_3,-NO_2,-Cl,-NH3, -N-$(CH_3)_2$ will increase biological activity[9-10]. Ramanpreet et al. established that fused heterocyclic ring system of benzene having substitution at the 2nd position with small groups is more pharmacologically active. Pantoprazole synthesis by Bernhard et al. [11] explained the biological importance of methoxy group. Shimamura et al. [12] in 1995 proposed different methods to reduce the basicity of ring nitrogen in the hetrocyclic nucleus. He also proved that substituting different groups in the

aromatic chain can reduce the enzyme irreversibility of compounds. Based on these results, an attempt was made to design, synthesis and study biological activities of benzoxazole derivatives having aldehyde with different groups at 3rd and/or 4th position. For the attachment of the aldehyde with the benzoxazole nucleus a Shiff's base was introduced at 5th position; with an expectation to enhance biological activity.

Study was conducted in In-Silico design, synthesize and biological evaluation. Advantage of such methodology was that unnecessary synthesis of several compounds in synthesis stage could be avoided since compound properties were pre-evaluated in software. After biological evaluation of the synthesized drug; QSAR studies were performed and derived mathematical equation representing correlation between biological activities and molecular descriptors.

MATERIALS AND METHODS

During In-Silico molecular studies various software were used. Initially ChemSketch version 10.1 was used for molecular design. Molinspiriation version v2011.04[13] was used to calculate the physicochemical descriptors like Lipophilicity/ Hydrophobicity, Electronic effect, Steric effect, Molecular volume, Molecular polar surface area(PSA), Number Of Rotatable Bonds(NORB), Lipinski's Rule Of Five[14]. Software Prediction of Activity Spectra for Substance (PASS)[15,16] was used to predict the biological activity. QikProp[17] utility of Schrödinger™[18] was used for prediction of pharmacokinetic properties like Absorption, Distribution, Metabolism, Elimination (ADME)[19]. Based on thus calculated physicochemical descriptors analogues were selected for docking with the specific protein for the selected activity from Protein Data Bank (PDB)[20] using software Schrödinger™ Ligprep ™[21]. Docking results of these derivatives were further analyzed in Discovery Studio [22] version 3.1 for finding the binding sites in amino acids at different positions and different bonds in drug-receptor complex were also analyzed in detail.

Synthesis

Synthetic pathways for preparation of the target compounds, listed in Table No. 1 are shown in Figure No. 1. Synthesis of compounds were performed by aromatic nitration in electrophilic substitution of methyl-p-hydroxy benzaldehyde to 4-hydroxy-3-nitro benzoic acid in the presence of acid mixture (H2SO4:HNO3 in 1:1ratio) at 0-5ºC. Then it was reduced in the presence of sodium dithionate to 3-amino-4-hydroxyl-benzoic acid methyl ester and it was cyclized to benzoxazole moiety by the action of acetic acid or formic acid in ethanol. The hydrazide derivatives were obtained from hydrazine hydride and it was reacted with 4-hydroxy-3-methoxy benzaldehyde in ethanol and acetic acid.

Preparation procedure

Reactions were monitored and purity of the products ware checked by thin layer chromatography (TLC). Silica gel 60 F254 chromato plates were used for TLC. All the melting points were measured with a capillary melting point apparatus (Buchi SMP 20 and Electrothermal 9100) and were uncorrected. Yields were calculated after recrystallization. The IR spectra were recorded on a Jasco FT/IR-420 spectrometer as KBr discs. The 1H NMR spectra were recorded employing a VARIAN Mercury 400 MHz FT spectrometer; chemical shifts (d) were in parts per million relative to TMS and coupling constants (J) were reported in hertz.

Synthesis of 4-Hydroxy-3-nitro-benzoic acid methyl ester (2): A mixture of 12.4ml of con. sulphuric acid and con. nitric acid (1:1) were added to p-hydroxy methyl benzoate (10g, 0.74mol) at a temperature 0-10^0 C with continuous stirring. Temperature of the reaction was maintained between 5 to 15°C for 1 hour and then poured it into crushed ice (70g). From that crude m-nitro and p-hydroxy methyl benzoate were filtered off. Washed product was then added to ice cold methanol and stirred and filtered to remove the trace of ortho-isomer and other impurities. Then the product was re-crystallized using methanol as solvent. The purity of the compound was established by single spot on TLC plate. Solvent system used: Ethylacetate: methanol (1:1).

Synthesis of 3-Amino-4-hydroxy-benzoic acid methyl ester (3): To the compound 2 (10gm) in boiling alcohol (100ml), sodium dithionate was added until it became almost colorless. This mixture was refluxed for 1.5 hours. The alcohol was reduced to one third of its volume by distillation and the residual liquid was triturated with ice cold water. The resulting colourless, shiny product was filtered, washed with cold water, dried and recrystallize using methanol as solvent. The purity of the compound was established by single spot on TLC plate. Solvent system used: Saturated methanol.

Synthesis of 2-subtituted benzoxazole-5-carboxylic acid methyl ester (IV) (General procedure for basic ring formation)***(4)***: Compound 3 (0.01mol) with an appropriate aliphatic acid (formic acid /acetic acid) in excess was refluxed for 2 hours with anhydrous zinc sulphate. The reaction mixture was cooled and poured in to crushed ice (100gm) with stirring. The light yellowish product thus separated was filtered under suction and washed with cold water. The products were re-crystallized by using methanol as a solvent. The purity of the compound was established by single spot on TLC plate. Solvent system used: Saturated methanol.

Two derivatives thus obtained from different aliphatic acids were Benzoxazole-5-carboxylic acid methyl ester (4a) and 2-methyl-benzoxazole-5-carboxylic acid methyl ester (4b).

Synthesis of 2-substituted benzoxazole-5-carboxylic acid hydrazide (5) [24,25]: A mixture of an appropriate 2-subtituted benzoxazole-5-carboxylic acid methyl ester (4) (0.001mol) in alcohol (25 ml) and hydrazine hydrate (99%, 0.015 mol) was refluxed for 4 hours. The alcohol was reduced to half of its volume and cooled. The product separated was filtered and washed with small portions of cold alcohol and then with cold water repeatedly and dried. The resultant product was re-crystallized using methanol as solvent. The purity of the compound was established by single spot on TLC plate. Solvent system used: Methanol.

Synthesis of N'-[(E)-(4-hydroxy-3-methoxyphenyl) methylidene] -2-methyl-1, 3-benzoxazole5-carbohydrazide (6) [26]: Both conventional and microwave methods were done. For synthesis conventional method was performed and microwave method was done for comparison.

Conventional Method: A mixture of compound-5 (0.01mol) in 50ml ethanol, 4-Hydroxy-3-methoxy-benzaldehyde (0.01mol) and a few drops of acetic acid was refluxed for 2.5 hours at 60^{0}C. The resulting mixture was poured into ice cold water and then it was filtered. Pure compound was obtained from DMF and followed a column chromatography. The purity was checked by single spot on TLC plate, consistency in melting point and R_f value. Solvent system used: methanol and ethyl acetate-1:1

Microwave Method [27]: A mixture of compound-5 (0.01mol), 4-Hydroxy-3 methoxy benzaldehyde (0.01mol) and

2-3 drops of glacial acetic acid in ethanol (20Ml) was irradiated (400W, 76-780C) for 15 min. The resulting compound was recrystallized from ethanol or acetone. The purity was checked by single spot on TLC plate. Solvent system used: ethanol and acetone-2:1

Pharmacological Screening

Anti-cancer Studies [28]: In vitro Cytotoxicity study was performed by MTT Assay method. Cell growth was determined by counting viable cells after staining with a vital dye. Here an yellow tetrazole (3-(4, 5-Dimethylthiazole -2-yl)-2,5-diphenyltetrazolium bromide) was reduced to purple formazan in the mitochondria of the living cells. Using spectrophotometer (500&600nm) the absorbance of the coloured solution was measured (Alley et al. 1988)[29].

Cells were seeded (5000 cells/well) in 96-well; flat-bottom titer plates along with different concentrations of analogues (0.000001-100 µg/mL) were used and incubated it for 48 hours at 37^0C in 5% CO_2 atmosphere. After completion of incubation the medium was removed and wells were washed with Phosphate Buffered Saline (PBS). 100 µl of the working MTT dye in Dulbecco's Modified Eagle Medium (DMEM) was added and incubated for another 2 hours. MTT lysis buffer (100 µL) was added and incubation continued for 4 hrs. The absorbance was measured at 570nm and Proliferation Rate (PR) was calculated using formulae

$$PR = \frac{\text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

$$\text{Calculated the \% viability as \% Viability} = \frac{\text{Mean Absorbance of sample}}{\text{Mean Absorbance of control}} \text{ X } 100$$

Cytotoxicity of the drug PST001 on the cells was calculated as cell growth Inhibition Rate (IR). IR = 100 PR.

Anti-tubercular Screening

Anti-tubercular study was performed by using Alamar blue assay method [30]. Alamar blue is a dye used for screening of anti-tubercular activity. Alamar blue (oxidized form) turns pink in colour upon reduction due to the presence of live Mycobacterium (because Mycobacterium is an aerobic organism). This principle was used to predict the presence or absence of growth of Mycobacterium tuberculosis for testing anti-tubercular agents.

Procedure[31]: The bacterial cultures were grown till mid-log phase in the Middle brook 7H9 broth for Mycobacterium tuberculosis H37Rv. Stock solutions of the test compounds were prepared at a concentration of 2 mg/ml. 50 µL of the mid-log phase culture was added to 150µL of the media taken in microtitre plates. To the wells of BZ24 (having concentration 100, 250 and 500 µg/ml) above prepared stock solution was added. The plates were then incubated at 37°C for 7 days. After incubation 20µL of Resazurin dye was added. Those compounds which prevented the change of colour of the dye from blue to pink were considered to be inhibitory.

Antibacterial Screening [32]

Disc plate method was used for screening antibacterial activity on the selected synthesized analogues of 1,3-benzoxazole derivatives. Gentamicin was used as standard for both gram positive and gram negative organisms. The organisms used were *Staphylococcus aureus* ATCC 25923 (Gram positive) and *Escherichia coli* ATCC 25922 (Gram negative).

Antifungal Screening [33]

Antifungal screening was done on selected novel 1,3-substituted benzoxazole 5-carbohydrazide analogue(BZ-24) by disc plate method using *Candida albicans* strain. Clotrimazole was used as standard drug for reference.

Acute toxicity screening [34]

Acute oral toxicity test was carried out according to the Organization for Economic Co-operation and Development (OECD) guidelines for testing of chemicals number 423 December 2001. Female Wistar rats were used in this procedure. They were divided randomly into six groups of three rats each. First group served as the control and was given 0.01%CMC orally at a dose of 10ml/Kg body weight. The selected analogue was taken at a dose 5, 50, 300, 1500 and 2000 mg/Kg body weight were given orally as a suspension in CMC(0.1%) to the remaining groups. All the animals were observed continuously for two hours, then intermittently for another four hours and also at the end of 24 hours. The number of animal deaths was noted after 24 hours.

Anti-inflammatory Studies [35, 23]

In vitro anti-inflammatory activity was evaluated by Carrageenan induced Rat Paw Edema Method with better Glide™ scored analogue. Diclofenac sodium (10 mg/kg) was used as standard. The experimental procedures were carried out in strict compliance with Institutional Animal Ethical Committee regulations-Ref No:03/2012/MCT. Albino rats of both sexes were weighted and divided into four groups of six each. The right paw of each animal was taken as the reference, non-inflamed paw for comparison and left paw for injecting carrageenan. Synthesized analogue *BZ24*-(2-[(2E)-2-(4-hydroxy-3-methoxybenzylidene) hydrazinyl]-1-(2-methyl-1,3-benzoxazol-5-yl) ethanone) in a dose of 200 mg/kg and 250 mg/kg were given orally for groups A1 and A2 respectively. Dose for the anti-inflammatory activity was determined after the acute toxicity studies up to 1734.20 ml/kg of body weight of the synthesized drug was found safe. Diclofinac (10 mg/kg) was given to the fourth group and 0.1% CMC to the third group as control. After one hour of the drug administration 0.1 ml of 1% w/v carrageenan was injected into the planar region of the left hind paw of all the animals. Using a Vernier Caliper the thickness of both hind paws of the animals were measured at tibiotarsal junction and calculated the average, after three hours of carrageenan administration. The paw thickness was again measured and noted the difference in paw thickness.

The percentage inhibition of edema was calculated as ***Percent inhibition = 1 VT/VC × 100.*** Where, VC and VT represented the mean increase in paw volume in control and treated groups respectively.

QSAR Studies [36]

QSAR approach attempts to identify and quantify physicochemical properties of a drug and to determine whether any of these properties has an effect on the drug's biological

activities. Thus QSAR techniques develop correlations between biological activity and physicochemical properties. It quantifies physicochemical properties in terms of the general concepts of electronic, steric and hydrophobic effects. QSAR data is applied for the determination of stability, distribution, toxicity prediction and lead optimization of drug candidates from structure-activity data.

QSAR tool of Schrodinger™ software Strike™ was used for performing QSAR studies. Strike™ workflow for QSAR model generation/validation generally consists of following steps; Data preparation, Model generation and validation, Model application. Data preparation was done using Maestro™ and QuikProp™ utilities, whereas Model generation and validation and Model application were done using Strike™. Four benzoxazole analogues (BZ10, BZ20, BZ23 and BZ24) were fed into QuikPrep™ to calculate various descriptors. And these calculated descriptors were used in Strike™ to model QSAR.

RESULTS AND DISCUSSION

Molecular Modeling: Using ChemSketch six analogues were drawn. The basic structure of those analogues is shown in Figure No.2. Then SMILE notation [32] and CLogP values of those analogues were calculated using EPI SuiteKnnowit software. Results are given Table No.1.

In-Silico design: Descriptors Molar Refractivity, Molecular Volume, Parachor, Polarizability were calculated using EPI Suite, results are given in Table No.2

Molecular properties and biological activities were calculated using Molinspiration software; results are given in Table No. 3.

Drug likeness was also evaluated using Molinspiration; results are in Table No.4.

Then biological active analogues were predicted by using PASS software, those results are given in Table No.5. Using QikProp, all proposed analogues were efficiently evaluated for pharmaceutically relevant properties, results of ADME of each analogue are given in Table No. 6.

Molecular Docking- Glide Scores: All the proposed derivatives were subjected to flexible docking using Schrodinger Glide™ (Extra Precision). The tabulated results are shown in Tables No.7-11. Corresponding Receptor-Ligand binding diagrams are shown in Figure No.3.

Analysis of the drug-receptor complex

Using discovery studio the receptorligand complex was analysed to identify docked amino acids. It also gave idea of type and length of bonds. (Fig: No. 4)

Synthesis

As per the scheme analogue synthesis was done using methyl

Table No. 1: Molecular descriptors of proposed analogues

Compound	Substituent		Molecular formula	Molecular weight	Melting point (^{0}C)	R_f
	R	Ar		(g)		
BZ10	H	4-hydroxy,3-methoxy benzaldehyde	$C_{16}H_{13}N_3O_4$	311.29	-	-
BZ20	CH_3	p-hydroxy benzaldehyde	$C_{16}H_{13}N_3O_3$	295.29	-	-
BZ23	CH_3	4-methoxy benzaldehyde	$C_{17}H_{15}N_3O_3$	309.31	-	-
BZ24	CH_3	4-hydroxy,3-methoxy benzaldehyde	$C_{18}H_{17}N_3O_4$	325.32	228-230	0.73

Table No. 2: Molecular descriptors of proposed analogues using EPI Suite

Compound	Molecular formula	Molar Refractivity (cm3)	Molecular Volume (cm3)	Parachor (cm3)	Polarizability ((Cm3) -24)	C Log P
BZ10	$C_{16}H_{13}N_3O_4$	82.09 ± 0.5	225.4 ± 7.0	609.1 ± 8.0	32.54 ± 0.5	2.11
BZ20	$C_{16}H_{13}N_3O_3$	80.70 ± 0.5	218.9 ± 7.0	589.9 ± 8.0	31.99 ± 0.5	2.57
BZ23	$C_{17}H_{15}N_3O_3$	85.66 ± 0.5	243.3 ± 7.0	634.5 ± 8.0	33.96 ± 0.5	3.13
BZ24	$C_{18}H_{17}N_3O_4$	86.51 ± 0.5	240.6 ± 7.0	640.2 ± 8.0	34.29 ± 0.5	2.65

Table 3: Violation of Lipinski's rule and comparison with standard drugs

Compound	CLog P	Molecular Weight	noN	nOHNH	Nrotb	N violation
Isoniazid (Anti-tubercular)	-0.81	137.14	4	3	1	0
Gentamicine(Anti-bacterial)	-4.213	477.603	12	11	7	2
Clotrimazole (Anti-fungal)	5.466	344.845	2	0	4	1
BZ10	2.11	311.30	7	2	4	0
BZ20	2.57	295.30	6	2	3	0
BZ23	3.13	309.33	6	1	4	0
BZ24	2.65	325.32	7	2	4	0

Table 4: Drug Likeness Analysis of Standard drugs with proposed analogues

Compound	GPCR Ligand	Ion Channel Modulator	Kinase Inhibitor	Nuclear receptor Ligand
Isoniazid (Anti-tubercular)	-3.89	-3.74	-3.50	-3.84
Gentamicin(Anti-bacterial)	0.34	0.19	0.18	-0.06
Clotrimazole(Anti-fungal)	0.17	0.03	0.14	-0.21
BZ-10	-0.20	-0.63	-0.33	-0.29
BZ-20	-0.39	-0.74	-0.38	-0.52
BZ-23	-0.45	-0.83	-0.54	-0.65
BZ-24	-0.84	-0.77	-0.68	-0.56

Table 5: Pass of Novel Proposed Analogue

Compound	Effect	Pa	Pi	Compound	Effect	Pa	Pi
BZ10	Anti-cancer	0.591	0.031	BZ23	Anti-cancer	0.530	0.033
	Anti-tuberculosis	0.448	0.050		Anti-tuberculosis	0.448	0.007
	Anti-bacterial	0.321	0.033		Anti-bacterial	0.667	0.003
	Anti-fungal	0.328	0.043		Anti-fungal	0.468	0.012
BZ20	Anti-cancer	0.561	0.054	BZ24	Anti-cancer	0.894	0.002
	Anti-tuberculosis	0.446	0.014		Anti-tuberculosis	0.828	0.008
	Anti-bacterial	0.423	0.021		Anti-bacterial	0.731	0.016
	Anti-fungal	0.327	0.039		Anti-fungal	0.653	0.016

Table 6: Prediction of ADME profile of selected BENZOXAZOLE analogues

Compd.	HOral Abs High/medium/low	% HOral Abs (>80% is high)	QP logKhsa (-1.5 - 1.5)	QP PCaco (<25 poor, >500 great)	QP logBB (-3.0 / 1.2)	QP logKp (Kp in cm/hr)	QP log HERG(concern below -5)	#metab (1 – 8)	QPP MDCK (<25 poor, >500 great)
BZ10	H	88	-0.167	503	-1.099	-2.324	-5.716	3	235
BZ20	H	87	-0.019	405	-1.194	-2.630	-5.963	3	186
BZ23	H	100	0.138	1336	-0.662	-2.670	-5.987	3	676
BZ24	H	91	-1.022	739	-0.521	-2.406	-4.021	2	766
	H-high / M-medium / L-low (=80 is high)								

Table 7: Glide Scores for docking with 2UWD (Inhibition of the HSP90 molecular chaperone *in vitro* and *in vivo* by novel, synthetic, potent resorcinylicpyrazole, isoxazole amide analogues)

Ligand	GScore	Lipophilic EvdW	PhobEn	PhobEnHB	PhobEnPairHB	HBond	Electro	Sitemap	PiStack	ClBr	LowMW	Penalties	HBPenal	PhobicPenal	RotPenal
BZ24	-12.36	-9.5	0	0	0	-1.57	-0.5	-0.22	0	0	-1.46	0	0	0.62	0.20
BZ23	-8.36	-4.4	-2.1	0	0	-0.56	-0.33	-0.8	0	0	-0.45	0.04	0	0	0.24
BZ10	-7.52	-5.24	0	0	0	-1.74	-0.39	-0.3	0	0	-0.37	0	0	0.17	0.35
BZ20	-5.48	-3.23	0	0	0	-1.36	-0.36	-0.57	0	0	-0.37	0	0	0.06	0.35

Anti-Cancer

Table 8: Docking score of the synthesized derivative with 3Q3S (Ether from Mycobacterium tuberculosis in complex with compound BDM56)

Ligand	GScore	Lipophilic EvdW	PhobEn	PhobEnHB	PhobEnPairHB	HBond	Electro	Sitemap	PiStack	ClBr	LowMW	Penalties	HBPenal	PhobicPenal	RotPenal
BZ24	-10.32	-9.1	0	0	0	-1.38	-0.5	-0.22	0	0	-0.42	0	0	0.92	0.38
BZ23	-9.61	-7.09	0	0	0	-1.54	-0.43	-0.66	0	0	-0.48	0	0	0.17	0.42
BZ10	-9.56	-7.46	0	0	0	-1.55	-0.71	-0.21	0	0	-0.5	0	0	0.51	0.36
BZ20	-7.52	-5.24	0	0	0	-1.74	-0.39	-0.3	0	0	-0.37	0	0	0.17	0.35

Anti-Tuberculosis

paraben as the starting compound. Compounds were synthesized as per the result obtained from the molecular modeling and In-Silico studies. Those compounds having high docking score (Glide score) and obeys Lipinski's rule of five were synthesized. So BZ-24 (2:N'-[(E)-(4-hydroxy-3-methoxyphenyl) methylidene]-2-methyl-1,3-benzoxazole 5 carbohydrazide) was synthesized; also literature survey supported it. For synthesize conventional methods were used. Also microwave assist method was performed for comparisons; results are given in Table No. 12

Structure Conformation

The synthesized analogues were characterized by analytical spectral methods. The purity of these compounds was asserted by consistency in melting point and Rf values, those are shown in Table No.1. The spectral conformation results of those analogues are shown in the Table No:13-15. 13C NMR spectral data is shown in Figure No.5

Anti-cancer Studies

We have investigated cytotoxic activity of the compounds in various cancer cell lines such as HeLa (Cervical cancer cell line), A375 (Skin cancer cell line), HCT116 (Colon cancer cell line), MCF7 (Breast cancer cell line) and FA549 (Lung Cancer).

Anti-cancer results revealed that fine-tuned 1,3-benzoxazole-5-carbohydrazide derivative can be a drug for colon cancer because of

Table 9: Glide scores for docking with 2BRD (crystal structure of bacteriorhodopsin in purple membrane)

GScore	Lipophilic EvdW	PhobEn	PhobEnHB	PhobEnPairHB	HBond	Electro	Sitemap	PiStack	ClBr	LowMW	Penalties	HBPenal	PhobicPenal	RotPenal
-9.85	-7.75	0	0	0	-1.80	-0.5	-0.32	0	0	-0.42	0	0	0.71	0.23
-9.73	-6.51	-1.9	0	0	-0.6	-0.47	-0.45	0	0	-0.25	0.23	0	0	0.24
-9.56	-7.46	0	0	0	-1.55	-0.71	-0.21	0	0	-0.5	0	0	0.51	0.36
-7.52	-5.24	0	0	0	-1.74	-0.39	-0.3	0	0	-0.37	0	0	0.17	0.35

Anti-Bacterial

Table 10: Glide score for docking with BZ 24Dock with HS-90 (Anti-fungal).

GScore	Lipophilic EvdW	PhobEn	PhobEnHB	PhobEnPairHB	HBond	Electro	Sitemap	PiStack	ClBr	LowMW	Penalties	HBPenal	PhobicPenal	RotPenal
-9.85	-7.75	0	0	0	-1.80	-0.5	-0.32	0	0	-0.42	0	0	0.71	0.23
-9.73	-6.51	-1.9	0	0	-0.6	-0.47	-0.45	0	0	-0.25	0.23	0	0	0.24
-6.88	-5.07	-1.92	0	0	0	-0.06	-0.69	0	0	-0.5	1.13	0	0	0.22
-6.01	-4.98	-1.75	0	0	-0.22	-0.26	-0.69	0	0	-0.3	2.01	0	0	0.19

Anti-Fungal

Table 11: Glide Scores for Docking with 6COX (Cyclooxygenase-2 (prostaglandin synthase-2) completed with a selective inhibitor, sc-558 in i222 space group)

GScore	Lipophilic EvdW	PhobEn	PhobEnHB	PhobEnPairHB	HBond	Electro	Sitemap	PiStack	ClBr	LowMW	Penalties	HBPenal	Phobic Penal	RotPenal
-10.37	-6.61	-2.21	0	0	-0.7	-0.19	-0.6	0	0	-0.3	0.06	0	0	0.19
-9.12	-6.28	-2.7	0	0	-1.01	-0.58	-0.66	0	0	-0.5	2.44	0	0	0.18
-8.27	-6.15	-2.7	0	0	-0.62	-0.56	-0.42	0	0	-0.47	2.4	0	0	0.25
-7.83	-6.26	-2.7	0	0	-0.14	-0.39	-0.69	0	0	-0.45	2.56	0	0	0.24

Anti-Inflammatory

- Restoration of normal apoptosis in APC-deficient cell and inhibition of angiogenesis and neovascularization.
- Inhibiting transcriptional activation by the nuclear peroxisome proliferator activated receptor **δ** (nuclear hormone receptor regulated by APC-gene).
- Ability to generate new radicles
- Ability to react with inorganic molecules

Colon cancer produced large amount of prostaglandin-E2, causes inflammation. This can be prevented by 1,3-benzoxazole-5-carbohydrazide derivatives due to COX-2 inhibition (anti-inflammatory property). Results of studies are given in Figure No.6 and Table No. 17.

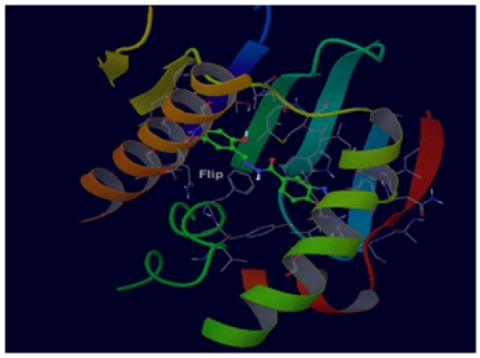
Anti-Tubercular Activity

Synthesized analogues that exhibited good glide score on docking were selected for anti-tubercular activity. *Mycobacterium tuberculosis* H37Rv maintained in Lowenstein Jensen medium was used as the test organism for anti-mycobacterial screening studies. Anti-mycobacterial screening of selected 1,3-Benzoxazole-5-carbohydrazide analogues results are shown in Figure No. 7, Table No. 17.

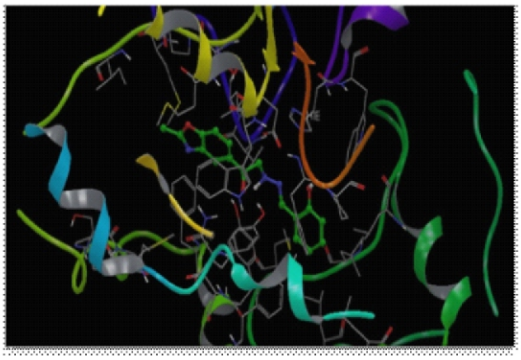
Anti-bacterial Activity

Gentamicin was used as standard for both gram positive (*Staphylococcus aureus* ATCC 25923) and gram negative organism (*Escherichia coli* ATCC 25922). The values of antibacterial activity of the BZ24 against the tested Gram-

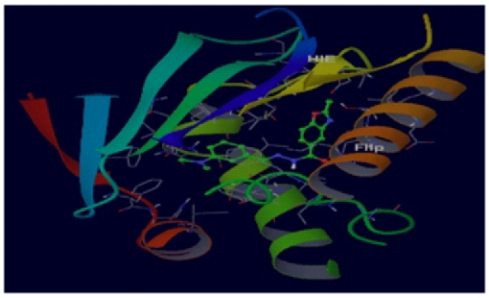
Figure No. 3: Receptor-Ligand binding diagram for Schrödinger software.



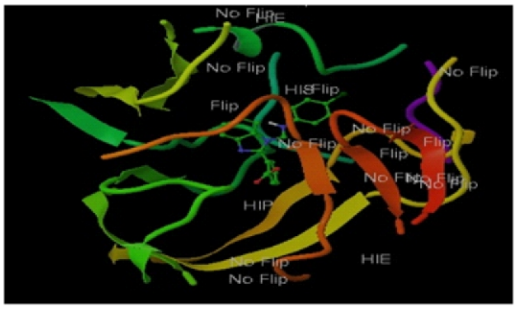
BZ24 Dock with 2UWD(Anti-cancer)



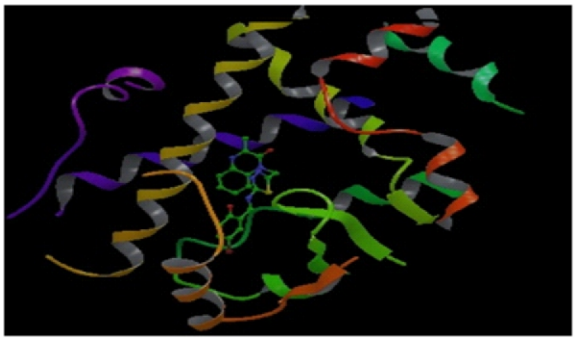
BZ 24Dock with 2BRD (Anti-tubercular)



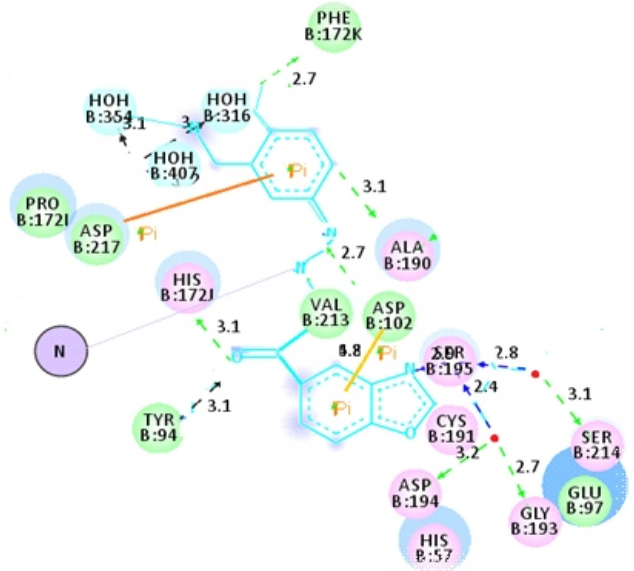
BZ 24Dock with 2BRD (Anti-bacterial)



BZ 24Dock with HS-90 (Anti-fungal)



BZ 24Dock with 6COX(Anti-inflammatory)



Analysis of docking of BZ24 with 3E16

- pi-bond between the aromatic ring due to weak Vander Walls interaction.
- pi-bond between the aromatic ring of the nucleus at a distance 6.8
- pi-bond between the aromatic group at the side chain with a distance of 8.9
- C=O makes a hydrogen bond with HIS B:1721
- N forms an ionic bond with the cavity
- N= forms hydrogen bond and ionic bond with ASP B:102 and ALAB:190 at a distance 2.7 &3.1 respectively.
- Methoxy group produce a HOH B:354 &HOH B:316(hydrogen bond by hydrolysis) at a distance 3.1
- The carbon atom on the aromatic side chain form a bond of 3.1 from ALA B: 190

OH at the para position forms a bond with PHE B:172K having 2.7 bond length.

Figure No.4: Analysis of the receptor-ligand diagram using discovery studio

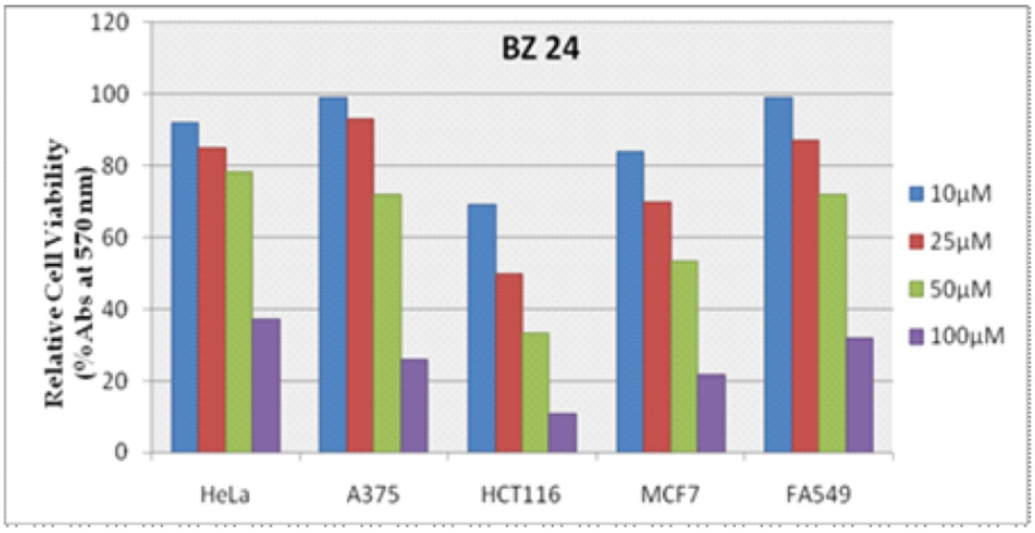


Figure No. 6: MTT Assay- Relative cell viability of BZ24 in various cancer cell lines

Table No.17: Sensitive Cells and their IC50, DAU=daunorubicin

Compound	IC50 (µM)				
	HeLa	A375	HCT116	MCF7	FA549
BZ24	172.57	109.40	56.44*	254.32	225.15
DAU	94.80	70.10	44.42	102.73	70.84

* The most sensitive cells and their Ic50

Table No.17: Alamar Blue Assay Method REMA

Sl No:	Compound code	Concentration (µg/ml)		
		10	100	250
1	BZ24	P	B	B++

P=Pink(Resistant) , B=Blue (Sensitive), B++=Blue (Very Sensitive)

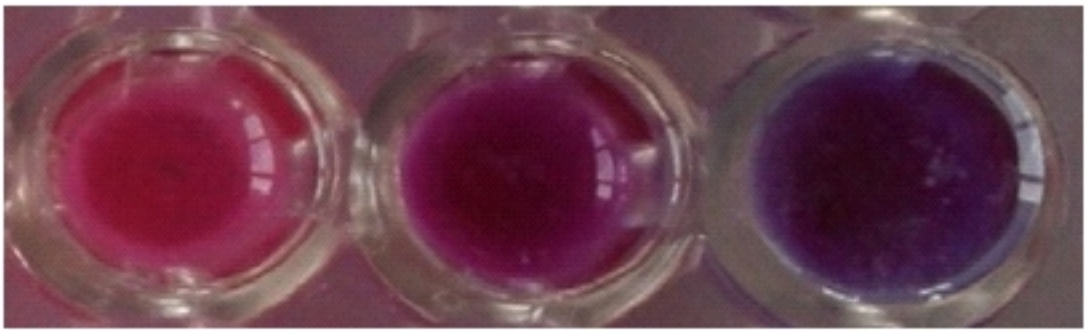


Figure No. 7 : Alamar Blue Assay for BZ-24

Table No.18: Anti-bacterial activity of selected 1,3-Benzoxazole-5-carbohydrazide analogues

Compound	Diameter of Zone Of Inhibition in mm									
	Gram positive organism					Gram negative organism				
	Staphyloccusaureus					Escherichia coli				
	A	B (100µg)	C (200µg)	D (300µg)	E	A	B (100µg)	C (200µg)	D (300µg)	E
BZ24	-	14	17	21	20	-	15	17	20	19

Table No.19: Antifungal Activity (Candida albicans)

Sample	ZONE OF INHIBITION (mm)		
	100 (µg)	250 (µg)	500 (µg)
Control	-	-	-
Standard (Clotrimazole)	15	-	-
BZ24	-	13	15

Table No.20: Effect of Analogues on Carrageenan induced paw edema

Group	Dose mg/kg	Log10 of dose	Dead/total	Dead %	Corrected %	Probit
1	5	0.70	0/3	0	-	
2	50	1.70	0/3	0	-	
3	300	2.48	0/3	0	-	
4	1500	3.18	1/3	33.33	-	
5	2000	3.30	2/3	66.67	50.01	5.03

Table No.21: Effect of Analogues on Carrageenan induced paw edema

Sl No: Compound	Dose (Oral) mg/kg	Paw thickness in mm (mean)				Mean change in paw edema (mm)* SD	% Inhibibtion of edema(1 – Mean/2.25) * 100
		1hr		3hr			
		R	L	R	L		
1(A1)	BZ 24-200	3.45	4.23	3.45	4.12	0.67 ±0.02	70.22
2 (A2)	BZ 24-250	3.82	4.25	3.82	4.10	0.28±0.03	87.56
3 (B)	Vehicle -1ml (0.1% CMC)	3.67	4.45	3.67	5.92	2.25±0.05	-
4 (D)	Diclofenac10	3.66	4.25	3.66	4.17	0.35±0.03	84.44

negative and Gram positive bacteria are summarized in Table No.18. Results revealed that the investigated compounds expressed inhibitory activity against *Escherichia coli*. and *S. aurease*. Compound having high log 1/cMIC (or low MIC) showed best anti-bacterial activity.

Antifungal Activity

Results of antifungal studies of BZ24 against *Candida albicans* are summarized in Table No.20. As indicated, the compound showed noteworthy antifungal activities against the tested yeast because it had high log1/cMIC (or low MIC).

Acute Toxicity Study

Derivative BZ24 (N'-[(E)-(4-hydroxy-3-methoxyphenyl) methylidene]-2-methyl-1,3-benzoxazole - 5-carbohydrazide) was selected according to the Glide score for the study of Acute Toxicity at safety dose range. According to the OECD guide line-423, the doses taken were 5, 50, 300, 1500 and 2000 mg/ kg bodyweight of the animal. Table No. 21 gives the result of acute toxicity study of the derivativeBZ24 (N'-[(E)-(4-hydroxy-3-methoxyphenyl) methylidene]-2-methyl-1,3 benzoxazole - 5-carbohydrazide) in Wistar albino rats by Fixed Dose Method. Route of administration was Oral as well as number of animals in a group was 3.

Estimate LD50: Conventional Method

In conventional method a correction factor was applied based on 0% mortality group. Then percent mortality values were converted to probit values by reading the corresponding probit units from the probit table. Then LD50 value was expected to

Figure No.8: Log10 (Dose) Vs Death % plot Acute Toxicity study details

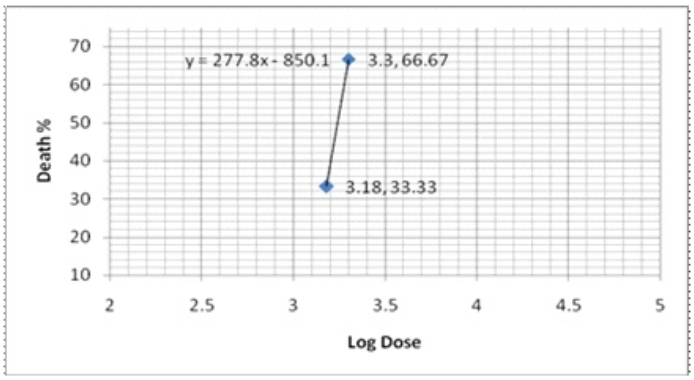
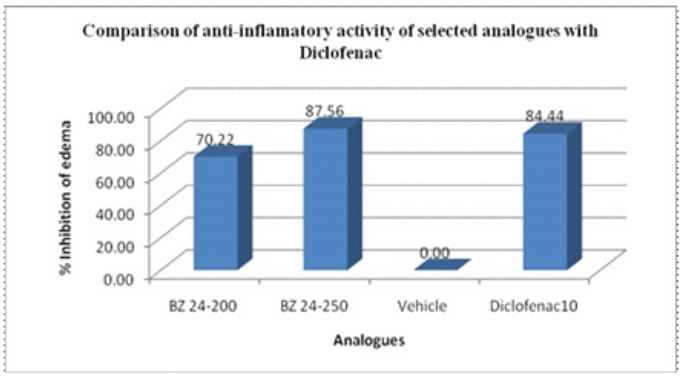


Figure No.9: Comparison of Anti-Inflamatory activity of selected derivatives with Diclofenac



Correlation Matrix for input variables

	mol MW	SASA
mol MW	1.0000	
SASA	0.5686	1.0000

MLR Regression Statistics

S.D.	R-Squared	F(2.0,1.0)	P
0.2565	0.4345	0.4	7.520e-001

MLR Regression Coefficients and T-Values

Variable	Coefficient	Std. Err.	T
Intercept	8.3589e+000	4.6010e+000	1.8167
mol MW	1.2285e-002	1.4655e-002	0.8383
SASA	-2.3876e-003	8.9785e-003	0.2659

Check independent variables

r^2	S.D.	p
0.6671	0.1728	0.000e+000

Predicted Results

	QPlogPC16	Strike Prediction	QPlogPC16
BZ-10	10.6133		10.7390
BZ-24	10.8382		10.7720
BZ-20	10.7268		10.5490
BZ-23	10.9097		11.0280

RMS	0.1283
Q^2	0.4345

calculate from plotting probit values against log doses. Since number of probit values were not enough to estimate LC50 value, percentage of death and log10 of dose were used to estimate LC50. Using linear regression analysis the relationship of log dosage and percentage of death was obtained as **Percentage of death = 277.8 * (log_{10} (dose)) - 850.1**

Based on the equation, LD50 value of the derivative BZ24-(N'-[(E)-(4-hydroxy-3-methoxyphenyl) methylidene] -2-methyl-1, 3-benzoxazole - 5-carbohydrazide) was obtained as 1734.20 mg/kg of body weight.

Anti-inflammatory

In vitro anti-inflammatory activity was evaluated by Carrageenan induced Rat Paw Edema Method with better Glide scored analogue. Diclofenac sodium was used as standard. Analogue BZ-24: 2-[(2E)-2-(4-hydroxy-3-methoxybenzylidene) hydrazinyl]-1- (2-methyl-1,3-benzoxazol-5-yl) ethanone) was found to possess anti-inflammatory activity in comparison with the standard. The results were statistically significant in comparison to the standard.

QSAR Studies

In anti-bacterial studies, QSAR modeling experiment was done using descriptors "mol MW", "SASA", "QPlogPC16". And activity was selected as CIQPlogS. Then QSAR modeling was started using MLR method, which produced Correlation Matrix and Regression Coefficients; QSAR results are given in below.

The generated equation is depicted in Figure 10. Result reveals BZ24 is the analogue which is very close to the straight line.

CONCLUSION

From the results discussed above, it can be concluded that the

Figure No.10: Graph of QSAR equation.

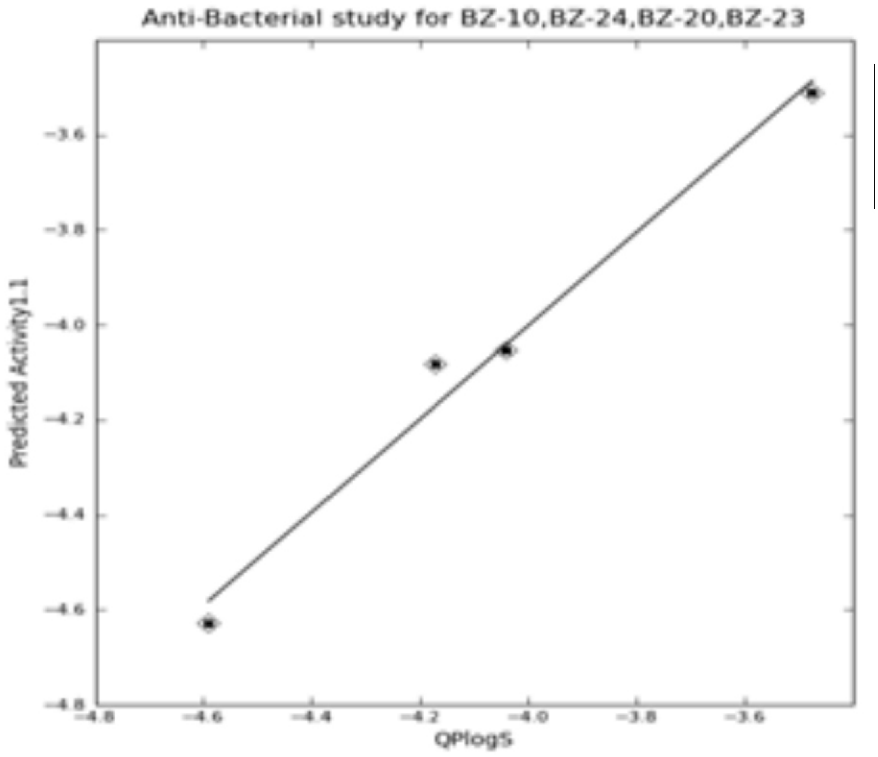


Figure No.5: ^{13}CNMR of BZ24- N'-[(E)-(4-hydroxy-3-methoxyphenyl) methylidene]-2-methyl-1,3-benzoxazole-5-carbohydrazide

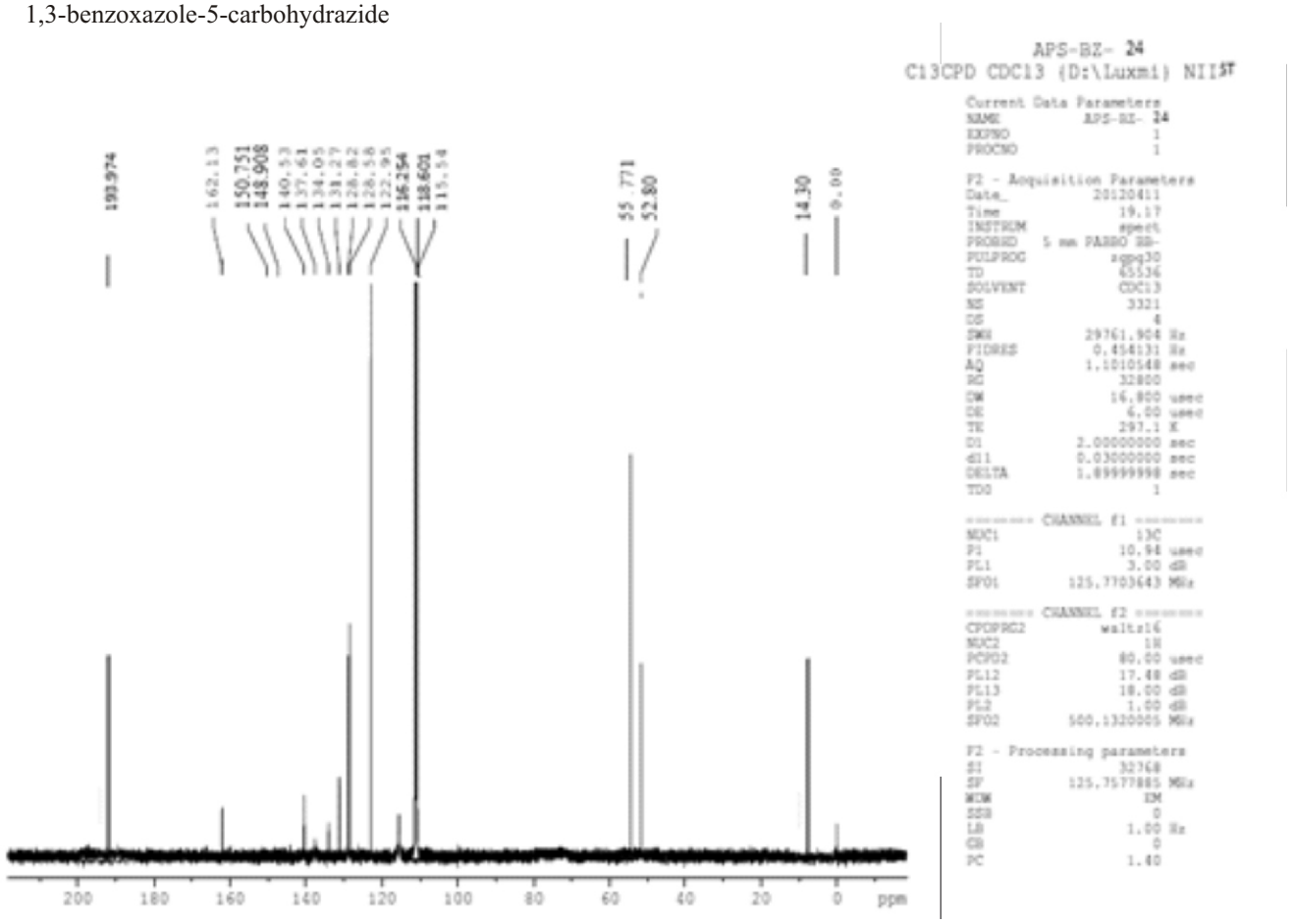


Table No.14: 13CNMR of BZ24- N'-[(E)-(4-hydroxy-3-methoxyphenyl) methylidene]-2-methyl- 1, 3-benzoxazole-5-carbohydrazide

Atom	*Shift*	*Atom*	*Shift*
CH3	14.304	C	131.27
CH	52.802	C	134.05
CH3	55.771	C	137.61
CH	115.54	CH	140.53
CH	118.601	C	148.908
CH	116.254	C	150.751
CH	122.95	C	162.09
CH	128.58	C	162.13
CH	128.82	C	193.97

Table No.13: IR spectrum obtained for BZ-24 synthesized compound

Compound	**IR (KBr υ cm-1)**
I-Methyl paraben	OH 3306.36,C-O-H 1433.82, C=O 1740, CH3 deformation 1360.36(bend)
II- 4-Hydroxy-3-nitro-benzoic acid methyl ester	O-H (stretch) 3360 (str), N=O1580.23, N-O 985.45,C=O(ester)1755.53,C-O(2ndband)123.85, α-CH3(bend)1382.53
III-3-Amino-4-hydroxy-benzoic acid methyl ester	NH2=3310.21 , Ar-H (str)=2962.13,C-O-H 1433.82 ,OH (bend,out plane) 655-730 (str) ,O-C 100-1300, C-O-H (bend)- 1395-1440, C-C-C (bend) 1100C-N 1100
IVa- Benzoxazole-5-carboxylic acid methyl ester	NH (str) 3312.14, C=C (in ring) 1607.38, C-C (str) 1917.86 , C=O 1740 (str) ,O-C 100-1300 , C-O-H (bend)- 1395-1440C-C-C (bend) 1100, C-N 1100,C=N (str) 1588.09 -C-O-C 1117.55,-C= C(bend)
IVb-2-methyl-benzoxazole-5 carboxylic acid methyl ester	NH (str) 3312.14, C=C (in ring) 1607.38, C-C (str) 1917.86 ,C=O 1740 (str) ,O-C 100-1300 ,C-O-H (bend)- 1395-1440, C-C-C (bend) 1100 C-N 1100,C=N (str) 1588.09 , -C-O-C 1117.55 ,-C= C(bend) αCH3 1355.35
Va- Benzoxazole-5-carboxylic acid hydrazide	C=O 1740 (str) ,O-C 100-1300,C-O-H (bend)- 1395-1440, C-C-C (bend) 1100, C-N 1100,C=N (str) 1588.09, C-O-C 1117.55,-C= C(bend) ,NH-NH2 3317.93
Vb- 2-methyl-benzoxazole-5-carboxylic acid hydrazide	C=O 1740 (str) ,O-C 100-1300,C-O-H (bend)- 1395-1440, C-C-C (bend) 1100, C-N 1100,C=N (str) 1588.09 ,C-O-C 1117.55,-C= C(bend) ,NH-NH2 3317.93,αCH3 1355.53

investigated 1,3-benzoxazole-5-carbohydrazide derivatives showed anti-cancer activity against cell lines such as HeLa (Cervical cancer cell line), A375 (Skin cancer cell line), HCT116 (Colon cancer cell line), MCF7 (Breast cancer cell line) and FA549 (Lung Cancer). Out of the synthesized analogues, BZ-24 (N'-[(e)-(4-hydroxy-3-methoxyphenyl) methylidene] -2-methyl-1,3-benzoxazole-5-carbohydride) is more potent against colon cancer due to its action on prostaglandins. Also it showed in-vitro inhibitory activity against Mycobacterium tuberculosis H37Rv strain, Gram-negative bacteria Escherichia coli, Gram positive bacteria S.aurease and Candida albicans(diploid-fungus). QSAR analyses were also employed to study the quantitative effects of the lipophilicity of the benzoxazole derivative on their antibacterial activity. Since this work proposes a low budget synthetic method for 1,3-benzoxazole-5-carbohydrazide derivatives, further studies may lead to a cost-effective anti-cancer drug production in future.

Table No. 15: H1NMR for BZ-24

Compound	*1H NMR (400 MHz,MeOD) δ ppm*
BZ24	7.52(CH),9.49(OH),3.35(CH3-triplet), 8.945(CH) , 6.96(CH) , 7.53(CH) ,7.55(CH) , 7.557(CH) , 7.901(CH) , 5.771(NH) , 4.38(CH2doublet),2.513(CH3triplet)

Table No. 12: Comparison of Conventional method and Microwave method used in synthesis.

Compound	Microwave		Conventional	
	Time (min)	Yield (%)	Time (hr)	Yield (%)
BZ 24	15	77%	2.5	66%

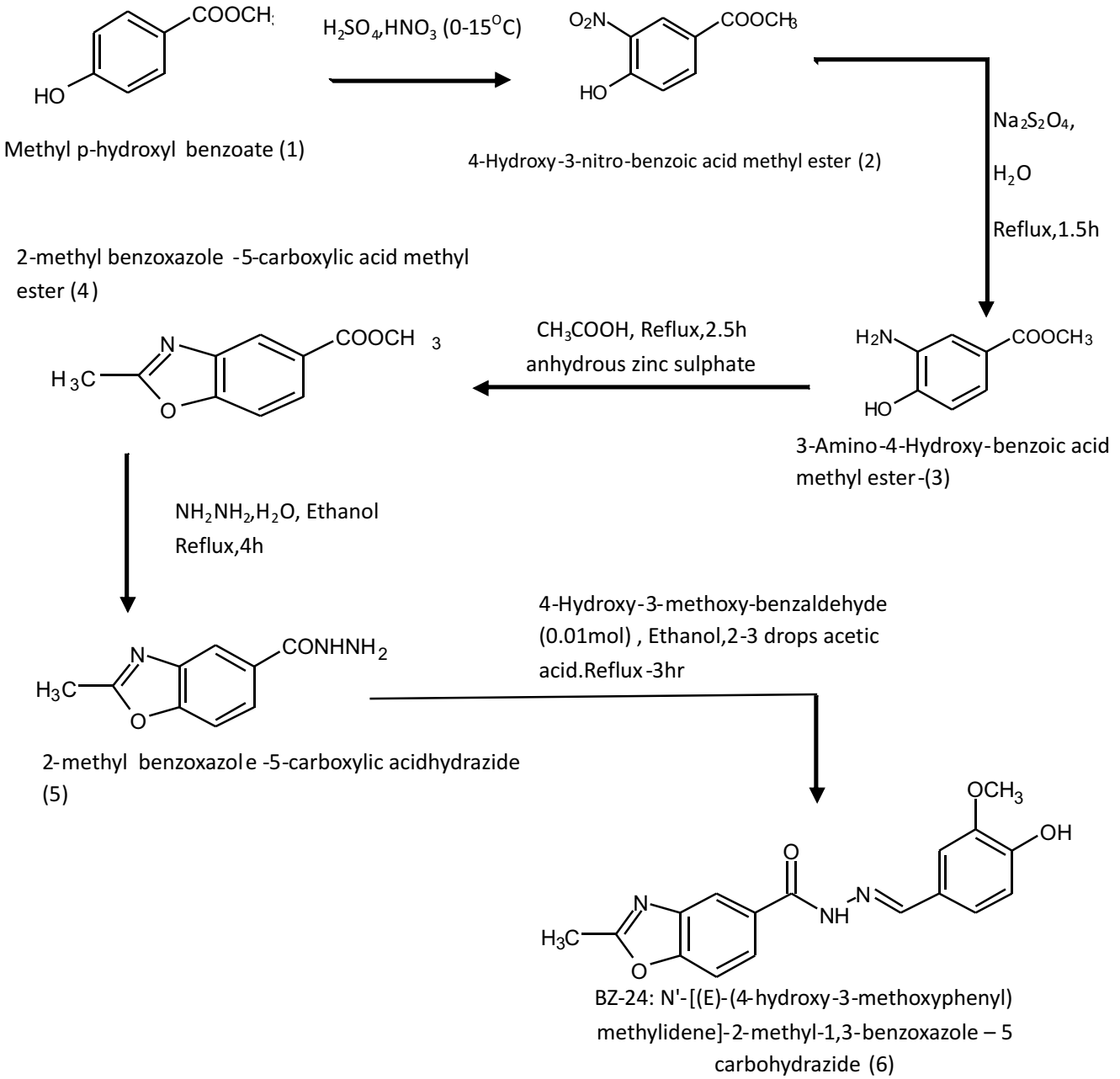


Figure-1: Synthesis of the target compound BZ-24

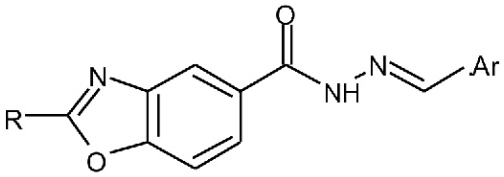


Figure No.2: Molecule designed by using ACD Lab ChemSketch

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