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Microwave-Assisted Synthesis, Characterization, Computational studies and *in-vitro* anthelmintic, antioxidant and anticoagulant activities of some Novel Benzimidazoles

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Abstract

Benzimidazole derivatives have played an essential role in the field of medicinal chemistry since decades. They are reported to possess a broad spectrum of activities of immense therapeutic potential. In the present study an attempt has been made to synthesize various benzimidazole derivatives through Microwave assisted reaction. In order to predict the probability of the active compounds for being therapeutically active, molecular docking, ADMET and adverse effect studies were performed. The compounds had appropriate ADMET values which suggests that these derivatives are likely to have drug-like property with a good safety profile. The synthesized compounds were characterized by IR, ^{1}H and ^{13}C NMR spectroscopy, LC-MS and Elemental analysis and screened for *in-vitro* anthelmintic, antioxidant and anticoagulant activities. The experimental observation enables the newly generated Benzimidazole derivatives to be attractive candidates as anthelmintic, antioxidant and anticoagulant agents

Key words: Benzimidazole, Antioxidant, Anthelmintic, Docking, ADMET, Spectral study

INTRODUCTION

Benzimidazole behaves as a good drug molecule due to the presence of nitrogen atoms in its core moiety and this type of complexes are found in a variety of biological molecules including, vitamin B_{12} and its derivatives, ion-heme systems and several metalloproteins. Benzimidazoles have become the most studying heterocyclic motif by synthetic organic chemists and biologists

owing to its continuously increasing demand[1]. A number of methods have been developed for the synthesis of compounds containing benzimidazole moiety and the most popular synthetic method is the dehydration of 1,2-diaminobenzenes with carboxylic acids or its derivatives such as anhydrides, lactones, ortho esters and nitriles under vigorous dehydrating reaction conditions in presence of strong acids such as polyphosphoric acid, hydrochloric acid, boric acid, or p-toluene sulphonic acid[2-4]. However, both the yield and purity of this reaction can be improved by using milder reagents such as lewis acids, inorganic clays, or mineral acids[5-7]. Another important method for synthesis is the condensation of 1,2-diaminobenzenes with aldehydes in the presence of an oxidative reagent such as benzoquinone, sodium metabisulfite, mercuric oxide, ytterbium perfluorooctane sulfonates, lead tetracetate, iodine, nitrobenzene, copper (II) acetate, indium perfluorooctane sulfonates, and even the normal air can also be used for this aim[8]. Moreover, several other catalysts, namely boron trifluoride diethyl etherate, zirconyl (IV) chloride, iodine, hydrogen peroxide, zeolite, and L-proline have been effectively used for the synthesis of benzimidazole derivatives[9-14]. Despite the high effectiveness of the various synthetic methods, some of the methods still need to be improved for long reaction times, high reaction temperature, toxic solvents and high cost catalysts[15]. Therefore, development of mild, efficient and environment friendly protocols for the design and synthesis of benzimidazoles is still a hot topic for the researchers. After the first report of microwave-assisted reaction in synthetic chemistry in the year 1986, microwave-assisted reaction has become popular from the last two decades owing to its short reaction times, high yields and purity of the synthesized products[16]. Till date, several microwave-assisted methodologies for the synthesis of benzimidazoles have been reported. On the basis of these reports, microwave assisted synthesis of novel benzimidazole compounds were performed and screened for anthelmintic, anticoagulant and antioxidant activity. Substitution at 2-phenyl benzimidazole especially at 2nd, 5th and 6th position appears to be an important scaffold for anti-anthelmintic, antioxidant and anticoagulant activities[17]. In the present study substituted 2-phenyl benzimidazole derivatives were synthesized using microwave irradiated methodology by treating different substituted phenylenediamines with ethyl benzoyl acetate. In-silico parameters such as Molecular docking, ADMET and Adverse effect prediction of the synthesized compounds were carried out to study the pharmacokinetics, bioavailability and toxicity of these molecules as drugs. Various Benzimidazole derivatives along with structure and biological activity are shown in the figure below[18]:

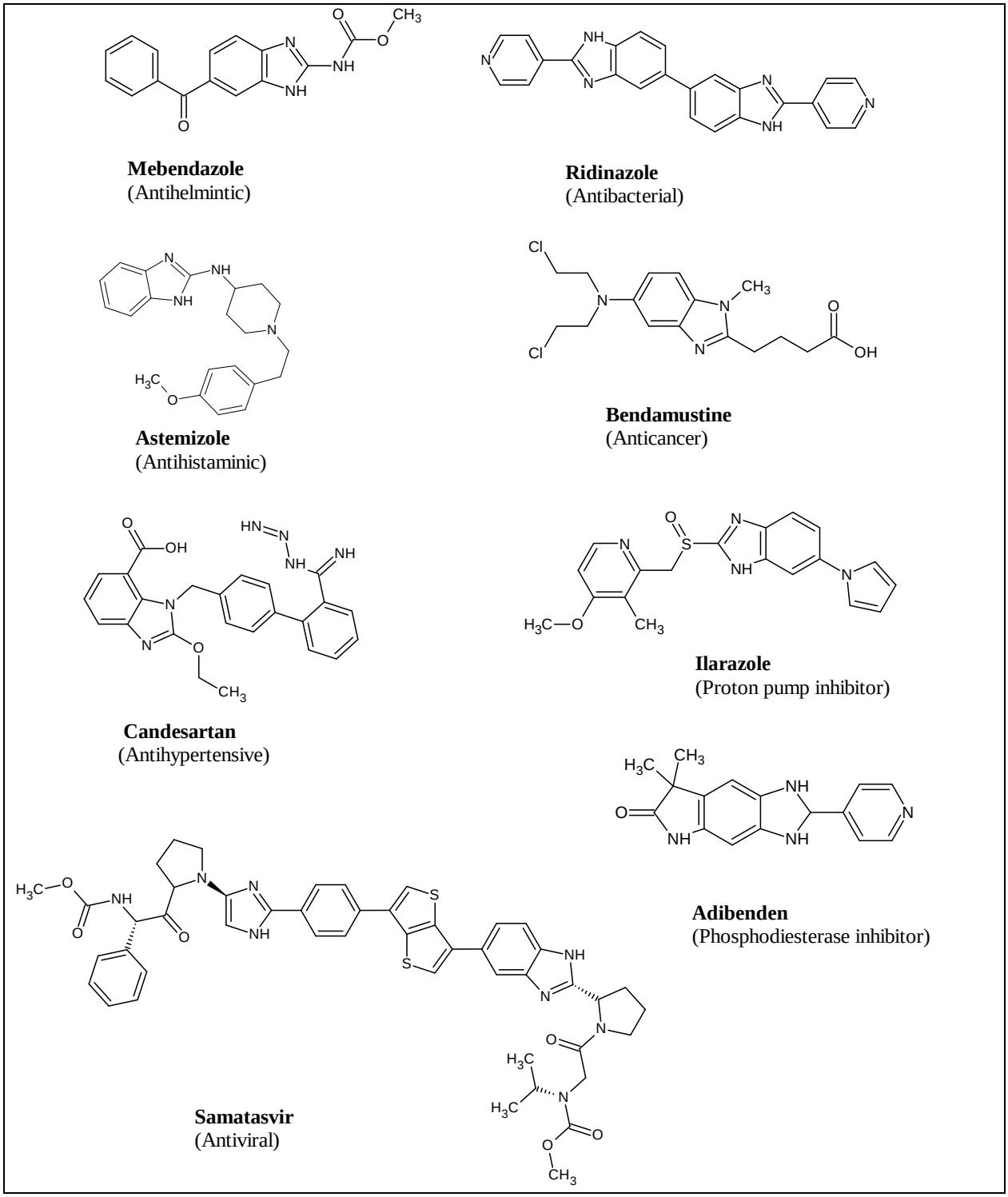


Figure 1: Commercially available drugs with Benzimidazole as core moiety

MATERIALS AND METHODS

All chemicals and reagents which used in this protocol were purchased from Sigma Aldrich. The purity of chemicals and reagents which were employed for synthesis of intermediate and benzimidazole derivatives were checked by TLC analysis. Melting point of the synthesized

compounds were determined in open capillary tube Moreover, the structures of all derivatives were confirmed *via* spectroscopic techniques like IR, ^{1}H NMR, ^{13}C NMR, LC-MS & elemental analysis respectively.

a. Docking study protocol

Docking study was done targeting the crystal structure of target proteins in order to reveal the binding modes of synthesized derivatives[19]. For the purpose of docking studies, protein preparation module in BIOVIA discovery studio was used to optimize the crystal structure of target protein[20]. From protein data bank (PDB), crystal structure of target proteins was retrieved and furthermore, structure was optimized by removing chains, hetero-atoms and H_2O molecule.

b. ADMET Prediction

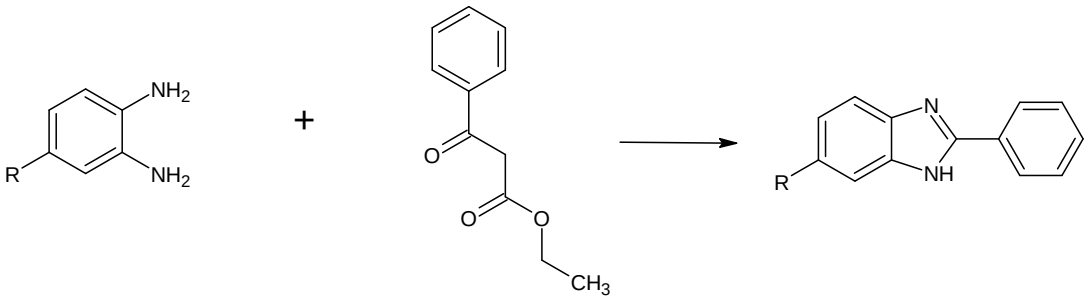
For a molecule to be considered as a drug candidate it should obey the Lipinski, Veber and Igan rules which are based on evaluation of ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) parameters and bioavailability of molecules by oral administration[21].The ADME properties were determined with Swiss ADME which is an online ADME prediction software tool (http://www.swissadme.ch.) The various type of toxicity parameters can be measured by means of online Protox II software tool.

c. Adverse effects prediction

The virtual prediction of biological activity along with adverse effects can be predicted using the online software tool PASS Online which predicts over 4000 kinds of biological activity, including pharmacological effects, interaction with metabolic enzymes and transporters, influence on gene expression mechanisms of action, toxic and adverse effects.

d. Chemistry

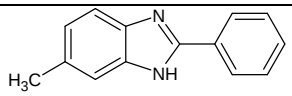
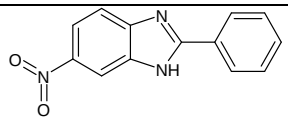
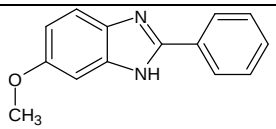
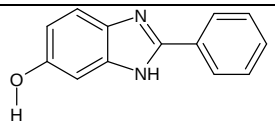
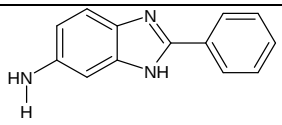
General procedure: An equimolar mixture (0.01mole) of 4-substituted-o-phenyldiamine and Ethyl benzoyl acetate was dissolved in Polyethylene glycol-400 and few drops of dilute hydrochloric acid was added (10%). The reaction mixture was stirred for 2hrs and irradiated with microwave radiation for few minutes (2-5 minutes) at 140W output power. The reaction progress was examined with thin layer chromatography (TLC). After completion of the reaction, the mixture was cooled at room temperature and then poured into ice cold water. Dilute ammonia was added dropwise and stirring was continued for few minutes with till the reaction gets neutralized. The precipitated product was recrystallized with hot ethanol.



4-substituted diamino benzene ethyl benzoyl acetate Substituted benzimidazole derivatives

Scheme 1: Synthesis of substituted benzimidazole derivatives

Table 1: Structure of the compounds and their reaction time

Compound code	Structure	Reaction time (minutes)
1a		2.3
1b		2.7
1c		3.3
1d		2.5
1e		2.9

f. Physical and Spectral data

6-Methyl-2-phenyl-1H-benzimidazole(1a): Percentage yield 92%; Melting point 203-205°C; IR (KBr, cm^{-1}): 3247(-NH stretch),1567(-C=C, aromatic),1402(-CH3 stretch),1666(C=N),1277(C-N),3012(=C-H, aromatic); ^{1}H NMR (DMSO, 400 MHz, δ (ppm) : 9.81(s, 1H) ,2.51(s,3H), 7.40-7.61(m,3H), 7.65-8.15(m,5H); ^{13}C NMR (DMSO, 400 MHz), δ ppm: 156.1,139.1, 137.4, 136.5, 135.2, 132.6, 129.2, 129.2, 128.3, 128.3, 118.6, 118.9, 23.4; Mass spectra $[M+H]^{+}$: 209.258; Elemental analysis-C(80.74%), H(5.81%) & N(13.45%); R_f 0.69 (n-Hexane, Ethyl acetate)

6-nitro-2-phenyl-1H-benzimidazole(1b): Percentage yield:94%; Melting point:213-215°C; IR (KBr, cm^{-1}): 3141(-NH stretch),1537(C=C, aromatic),1666(C=N),1523(-N=O stretch),1277(C-N), 3010(=C-H, aromatic); ^{1}H NMR (DMSO, 400 MHz), δ ppm: δ 12.49 (s,

1H), 7.57-7.85 (m, 3H), 7.23-8.25 (m, 5H); ^{13}C NMR (400 MHz, DMSO) δ ppm: 156.2, 147.9, 147.6, 141.3, 140.8, 139.5, 134.3, 131.4, 131.4,129.8, 129.8 119.5,118.9; Mass spectra $[M+H]^+$: 240.22; Elemental analysis-C(65.27%), H(3.79%), N(17.56%) & o(13.38%); R_f 0.67 (n-Hexane, Ethyl acetate)

6-methoxy-2-phenyl-1H-benzimidazole(1c): Percentage yield 88%; Melting point 233-235°C; IR (KBr, cm^{-1}) 3147(-NH stretch),1596(C=C, aromatic),1525(C=N),1212,1275(-OCH_3 stretch),1277(C-N), 3010(=C-H, aromatic); ^{1}H NMR (DMSO, 400 MHz), δ ppm: δ 11.64 (s, 1H), 7.31-7.53 (m, 3H), 8.33-8.36 (m, 2H), 3.89(s, 3H), 7.63-8.33(m, 5H); ^{13}C NMR (400 MHz, DMSO), δ ppm: 158.3, 154.9, 148.6, 144.3,144.1, 135.9, 134.5, 134.5, 134.9, 134.9,119.8, 117.8 106.5, 56.9; Mass spectra $[M+H]^+$: 315.33; Elemental analysis-C(74.98%), H(5.39%), N(12.49%) & o(7.13%); R_f 0.63 (n-Hexane, Ethyl acetate)

6-hydroxy-2-phenyl-1H-benzimidazole(1d): Percentage yield: 83%; Melting point: 205-207°C;IR (KBr, cm^{-1}):3522(-OH stretch),3478 (-NH stretch),1584(C=C, aromatic),1580(C=N), 1332(C-N), 3110(=C-H, aromatic); ^{1}H NMR (DMSO, 400 MHz), δ ppm: δ 11.14 (s, 1H), 7.31-7.53 (m, 3H), 8.33-8.36 (m, 2H), 5.86(s, 3H), 7.63-8.33(m, 5H); ^{13}C NMR (400 MHz, DMSO), δ ppm: 158.3, 154.9, 148.6, 144.3,144.1, 135.9, 134.5, 134.5, 134.9, 134.9,119.8, 117.8 106.5, 56.9; Mass spectra $[M+H]^+$: 315.33; Elemental analysis-C(74.28%), H(4.89%), N(13.39%) & O(7.63%); R_f 0.61 (n-Hexane, Ethyl acetate)

6-amino-2-phenyl-1H-benzimidazole(1e): Percentage yield: 81%; Melting point: 218-220°C;IR (KBr, cm^{-1}): 3502(-NH_2, amine), 3486 (-NH, imidazole),1584(C=C, aromatic),1580(C=N), 1338(C-N), 3116(=C-H, aromatic); ^{1}H NMR (DMSO, 400 MHz), δ ppm: δ 10.64 (s, 1H), 7.81-8.23 (m, 5H), 6.83-7.36 (m, 3H), 6.46(s, 2H); ^{13}C NMR (400 MHz, DMSO), δ ppm: 156.2, 149.9, 146.7, 141.6, 138.2, 136.6, 133.7, 132.5, 132.5, 12.9,129.9, 119.5 116.9, 102.9; Mass spectra $[M+H]^+$: 315.33; Elemental analysis-C(74.68%), H(5.49%), N(20.56%); R_f 0.66 (n-Hexane, Ethyl acetate)

g. Biological activity

1. *In-vitro* anthelmintic activity

Earth-worms (3-5 cm in length and 0.1-0.2 cm width) from moist soil were taken and washed with normal saline water. The earthworms were used in this study due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings[22]. All the test solutions and standard drug solution were prepared freshly before starting the experiment using 5% DMF and saline solutions. Six groups of earthworms of approximately equal size were taken and kept into 25 ml solutions of three different concentrations (25,50,100 mg/ml) in petri dishes containing 5% of DMF solution. Albendazole was used as reference drug for this study and saline as control. Determination of time of paralysis and time of death of the worm were noted down. The time for paralysis was noted when no movement was observed upon vigorous shaking of worms. Time for death of worms was recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water (50 °C) followed by fading away of their skin colours.

2. *In-vitro* Antioxidant activity (DPPH Free Radical Scavenging Assay)

The DPPH (2,2- diphenyl-1-picrylhydrazyl) solution is prepared by dissolving 4 mg of DPPH in 100 ml of Methanol and then the solution is stirred for 30 minutes. The standard solution of Ascorbic acid is prepared in Methanol in 10,20 and 30 µg/mL, while sample solutions was prepared in DMSO at concentrations of 10,20 and 30 µg/mL. After addition of 1.5 mL of DPPH solution to 0.75 mL of the sample and diluted/standard/control solutions, it is allowed to react for 30 min in the dark condition. Samples absorbance measurements were evaluated with a UV-VIS spectrophotometer at fixed wavelength of 517 nm. Blank sample was prepared adding methanol to DPPH solution[23-24]. After incubation, decrease in absorption was measured at a wavelength of 517 nm. Percent radical scavenging activity of samples was determined in comparison with a Methanol treated control group by using the following formula:

$AA\% = 100 * A_{Control} - A_{test} / A_{Control}$

$A_{Control}$: is the absorbance of the control

A_{test}: is the absorbance of the samples.

AA: is the antioxidant activity

3. *In-vitro* anticoagulant activity

Blood samples were drawn and placed separately in containers containing 3.8% trisodium citrate solution to prevent the clotting process. In order to separate the blood cells from plasma centrifugation (15 minutes at rate 3000 rpm) was carried out and pure platelet plasma (ppp) was obtained for prothrombin time test. After centrifugation 0.2 ml plasma, 0.1 ml of methanolic solution of different concentration of compounds and 0.3ml of $CaCl_2$ (25 mM) were added together in a clean fusion tube and incubated at 37^0C in water bath. Heparin is taken as a standard drug for the experiment. For control experiment methanolic solution of compounds was replaced by same volume of 0.9% saline water. The clotting time of blood was recorded with a stopwatch by tilting the test tubes every 5 seconds and it is called as prothrombin time.[25]

RESULTS AND DISCUSSIO

A. Molecular Docking study

Molecular docking studies of the designed compounds were docked by using PyRx virtual screening tool by taking target proteins with PDB ID: 2CDU, 2WS2 & 1AZX for antioxidant, anthelmintic and anticoagulant respectively which were identified from the literature studies. Reference drugs taken for antioxidant, anthelmintic and anticoagulant activities are Ascorbic acid, Albendazole and Heparin respectively. The binding energy with root mean square deviation (RMSD) equals to zero was selected as the docking score with best protein affinity and it is shown in Table 2.

Table 2: Binding affinity of the compounds with target proteins (PDB ID: 2CDU, 2WS2, 1AZX)

Compound code	2CDU	2WS2	1AZX
1a	-7.8	-7.4	-5.9

1b	-8.4	-7.2	-8.2
1c	-8.0	-7.3	-6.5
1d	-7.7	-7.5	-7.7
1e	-7.8	-7.3	-7.9
Reference Drugs	-6.5	-6.8	-9.1

b. ADMET Prediction

The results on predictive data for Gastrointestinal absorption, Blood Brain permeability, P-glycoprotein substrate, enzyme inhibitors (CYP450 2C9, CYP450 3A4, CYP450 2C19, CYP450 2D6 and CYP450 1A2) and Skin permeation(logKp) values of compounds are shown in table 3. All the compounds showed Gastrointestinal absorption Blood Brain Barrier permeability and the compounds are CYP450 1A2 enzyme inhibitors.

Table 3: ADME properties of the five designed compounds

Compound code	Gastrointestinal absorption	Blood Brain Barrier	P-glycoprotein substrate	CYP450 1A2 inhibitor	CYP 450 2C19 inhibitor	CYP 450 2C9 inhibitor	CYP 450 2D6 inhibitor	CYP 450 3A4 inhibitor	Skin permeation as log Kp(cm /s)
1a	High	Yes	Yes	Yes	No	No	Yes	Yes	-4.99
1b	High	Yes	No	Yes	Yes	Yes	No	No	-5.56
1c	High	Yes	Yes	Yes	No	No	Yes	Yes	-5.37
1d	High	Yes	Yes	Yes	No	No	Yes	Yes	-5.51
1e	High	Yes	Yes	Yes	No	No	Yes	Yes	-5.74

The prediction of bioavailability score, lipophilicity and water solubility values for all studied compounds are shown in table 4. All the compounds have bioavailability score of 0.55 and have lipophilicity: XLOGP3 value between normal range i.e. within -0.7 to +5.0 and solubility logS not higher than 6 which suggests that the compounds have good Bioavailability, lipophilicity and water solubility properties.

Table 4: Bioavailability, lipophilicity and water solubility prediction of the compounds

Compound code	Bioavailability score	Water solubility as log S	iLOG P	XLOGP3	WLOGP	MLOGP	SILICOS-IT
1a	0.55	-3.93	2.05	3.65	2.06	2.89	2.78
1b	0.55	-3.66	1.75	3.12	2.19	2.62	2.05
1c	0.55	-3.69	2.33	3.26	1.76	2.62	2.39
1d	0.55	-3.49	1.70	2.93	1.46	2.36	1.91
1e	0.55	-3.28	1.71	2.61	1.35	2.36	1.67

c. Toxicity prediction

The risk assessment and safety profile were predicted using Protox II web-based software which is used for prediction of hepatotoxicity, immunotoxicity, mutagenicity, cytotoxicity and carcinogenicity of the compounds. The Lethal dose (LD_{50}), Toxicity class, organ toxicity and toxicity end points of the compounds of the compounds are discussed below.

Table 11: Prediction of $LD_{50,}$ Toxicity class, organ toxicity and toxicity end points of the compounds

Compounds code	**Predicted LD_{50} (mg/kg)**	**Predicted Toxicity Class**	**Hepatoxicity**	**Carcinogenicity**	**Immunotoxicity**	**Mutagenicity**	**Cytotoxicity**
1a	9	2	Active	Inactive	Inactive	Active	Inactive
1b	67	3	Active	Active	Inactive	Active	Inactive
1c	41	2	Active	Inactive	Inactive	Active	Inactive
1d	55	3	Active	Inactive	Inactive	Active	Inactive
1e	67	3	Active	Active	Inactive	Active	Inactive

Biological activity

A. *In-vitro* anthelmintic activity

The synthesized compounds were evaluated for their in-vitro anthelmintic activity at different concentrations (25, 50,100 mg/ml) and compared against the standard drug Albendazole. The Observations were made for the time taken to paralysis and death of individual worms up to 1 hour of the test period. Among all the synthesized compounds, 1a & 1d has shown very good activity closer to the standard drug Albendazole. The results of the activity is discussed below in table 5.

Table 5: Anthelmintic activities of substituted benzimidazole derivatives

Compound Code	**Concentration(mg/ml)**	**Time taken (in minutes)**	
		Paralysis	**Death**
1a	25	33 ± 0.12	38 ± 0.16
	50	21 ± 0.53	29 ± 0.12
	100	11 ± 0.37	17 ± 0.53
1b	25	37 ± 0.17	41 ± 0.56
	50	26 ± 0.43	34 ± 0.32
	100	19 ± 0.27	25 ± 0.23
1c	25	36 ± 0.27	39 ± 0.36
	50	24 ± 0.23	31 ± 0.42
	100	15 ± 0.29	19 ± 0.33
1d	25	31 ± 0.52	36 ± 0.56
	50	20 ± 0.43	26 ± 0.52
	100	10 ± 0.56	15 ±0.55
1e	25	36 ± 0.57	39 ± 0.56
	50	24 ± 0.43	31 ± 0.52
	100	15 ± 0.59	19 ± 0.56
Albendazole	25	30 ± 0.22	35 ± 0.36
	50	19 ± 0.33	25 ± 0.42
	100	09 ± 0.26	14 ±0.43

b. *In-vitro* Antioxidant activity (DPPH Free Radical Scavenging Assay)

The result of the radical scavenging antioxidant activity is expressed in terms of half-inhibition concentration (IC_{50}) which determines the concentration of compounds required to scavenge 50% of DPPH radicals. Radical scavenging activity was calculated at a concentration of 10, 20, 30 µg/mL and IC_{50}(µM) values were ranging from 21.52, 22.09, 21.41, 21.57 and 21.52 respectively. All the synthesized compounds exhibited very good antioxidant activity when compared with the ascorbic acid. The result of the antioxidant activity is expressed in Table 6.

Table 6: Percentage of Inhibition of DPPH free radicals at different concentrations

Compounds code	**Concentration(µg/mL)**			**IC_{50} (µM)**
	10	20	30	
1a	39.70	43.20	47.53	21.52
1c	49.41	53.48	65.20	21.09
1c	47.38	51.30	62.19	21.41
1d	37.35	40.12	43.46	21.57
1e	39.59	43.35	47.61	21.55
Ascorbic acid	55.32	59.23	67.11	22.49

c. *In-vitro* anticoagulant activity

The anticoagulant activity of the compounds were determined at different concentrations (5,10,25µg/mL) and compared against the standard drug Heparin. The Observations were made for the time taken for inhibiting the coagulation of blood. Among all the synthesized compounds, 1a & 1d has shown very good activity closer to the standard drug Heparin. The results of the activity is discussed below in table 5.

Table 5: Determination of coagulation time

Compound code	**Concentration (µg/mL)**	**Amount of Extract solution(ml)**	**Amount of Plasma(ml)**	**$CaCl_2$ solution (ml)**	**Time of coagulation**
1a	5	0.1	0.2	0.3	3.6 ± 5.2
	10				9.6 ± 4.2
	25				36.3 ±1.2
1b	5	0.1	0.2	0.3	14.4 ± 5.4
	10				29.6 ± 2.7

	25				78.3 ± 2.2
1c	5	0.1	0.2	0.3	5.4 ± 8.2
	10				19.6 ± 4.7
	25				56.3 ± .2
1d	5	0.1	0.2	0.3	9.4 ± 8.4
	10				24.6 ± 2.9
	25				72.3 ± 2.8
1e	5	0.1	0.2	0.3	12.4 ± 5.8
	10				27.9 ± 2.7
	25				76.3 ± 2.1
Heparin	5	0.1	0.2	0.3	17.4 ± 4.4
	10				33.6 ± 1.7
	25				81.3 ± 2.2
Control (NaCl)	0.9%	0.1	0.2	0.3	1.45± 4.5

CONCLUSION:

In the present research, five benzimidazole derivatives were evaluated for *in-silico* docking studies with target proteins for anthelmintic, antioxidant and anticoagulant activity in order to validate their potency. *In-silico* ADMET and adverse effect studies have revealed that the drug molecules are less toxic and have good pharmacokinetic and absorption properties with oral bioavailability. The designed compounds were synthesized by microwave assisted reaction and were characterized by spectral techniques. Furthermore, *in-vitro* anthelmintic activity has revealed that the compounds 1a and 1d have very good activity against earthworms. For anticoagulant activity 1b and 1d has also showed good activity closer to standard drug Heparin whereas for antioxidant activity all the compounds have exhibited excellent results for radical scavenging activity. The outcome of this research work strengthens the relevancy of the synthesized compounds as promising drug candidates for anthelmintic, antioxidant and

anticoagulant activity. In summary, this contemporary research and its findings would be useful for future research directions on the pertinence of benzimidazole derivatives for development of drug molecules as potent anthelmintic, antioxidant and anticoagulant agents.

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