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SYNTHESIS AND INVESTIGATION OF BIOLOGICAL PROPERTIES OF THE 2-SUBSTITUTED BENZIMIDAZOLE DERIVATIVES

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ABSTRACT

2-(3-cyano-phenyl)-1H-Benzimidazole [MCB], 2-(4-hydroxy-phenyl)-1H-Benzimidazole [MHB] and 2-(4-nitro-phenyl)-1H-Benzimidazole [MNB] were synthesized from o-phenylene diamine and different aldehydes to investigate the biological (i.e. antimicrobial and cytotoxicity) studies as 2-substituted benzimidazole derivatives have promising biological activities. The structures of the synthesized molecules were confirmed through analysis of FTIR, ^{1}H-NMR, ^{13}C-NMR, and ^{13}C-DEPT NMR spectra. In-vitro antibacterial assay was studied against both gram-positive and gram-negative bacterial strains. *B. subtilis* was the most susceptible bacteria against all the synthesized benzimidazole derivatives under investigation. MNB showed highest antibacterial activity in contrast to MHB & MCB whereas MCB showed least activity. *A. niger* was the resistant fungal strains to all the test materials under analysis. In term of cytotoxicity based on the brine shrimp lethality bioassay, MNB showed highest toxicity (lowest LC_{50} value, 6.13 µg/mL) while MHB reflected least toxicity (LC_{50} value, 28.18 µg/mL).

KEYWORDS: 2-substituted benzimidazole derivatives, antimicrobial activity, cytotoxicity, LC_{50} value.

INTRODUCTION

The well-known and important biomolecules, like adenine and guanine, two of the four nucleic acid bases, caffeine and uric acid contain the same basic ‘6+5’ heterocyclic fused ring

structure like benzimidazole, which is one of the key indication of important bioactivity.[1] It is notable that benzimidazole containing molecules and benzimidazole derivatives have been recognized to be biologically active molecules like different antimicrobial, antiparasitic, and even antitumor agents reported.[1,2] Benzimidazoles are curiously effective compounds based on their favorable selectivity ratio and their inhibitory activity. Extensive biochemical and pharmacological studies have confirmed that benzimidazole molecules are effective against various strains of microorganisms. Currently infectious microbial diseases are causing problems world-wide, because of resistance to number of antimicrobial agents (quinolones, β-lactam antibiotics, macrolides, and vancomycin). A variety of clinically significant species of microorganisms has become health problem globally.[3] The advance of novel antimicrobial agents and effective usage of the available marketed antibiotics could be the ways to fight with this challenge.[4] 2-substituted benzimidazole derivatives compared to other benzimidazoles are reported as pharmacologically more potent and hence the design and synthesis of 2-substituted benzimidazoles are the focal area of research.[5-7] The present study was focused to synthesis 2-substituted benzimidazole derivatives and studies of their biological properties. Different synthetic procedures were reported in the literature for the synthesis of 2-substituted benzimidazole derivatives. However, the benzaldehyde derivative used in the present study in combination of o-phenylene diamine were not reported in the literature before. Though different 2-substituted benzimidazole derivatives were investigated for the antimicrobial and cytotoxicity studies (Jeyaprakash *et al.*, 2009[8] and Priyal Jain *et al.*, 2011[9]) but the compound listed in this study were not reported yet.

MATERIALS AND METHODS

Chemicals: All reagents & solvents were of synthetic grade purchased from commercial suppliers and used as received without further purification. O-Phenylene-di-amine, 3-cyano-benzaldehyde, 4-hydroxy-benzaldehyde, and 4-nitro-benzaldehyde were purchased from Merk Germany. Toluene, Dioxane, Di-methyl-formamide (DMF), Di-methyl-sulfoxide (DMSO), Ethanol, Ethyl-acetate, Di-chloro-methane (DCM), Chloroform, Acetone, and Methanol were purchased from Alfa Aesar.

Microbial strain: All the microbial strains were collected from the Food Lab of the Centre for Advance Research in Sciences (CARS), University of Dhaka. Both gram-positive (*Bacillus subtilis, Staphylococcus aureus, Staphylococcus aureus, Listeria monocytes)* and gram-negative bacteria (*E. coli-0157, Salmonella Typhimurium, Salmonella enteric,*

Klebsiella pneumonia) were used for investigation of microbial properties. In addition, only fungal agent used was *Aspergillus niger.*

Synthesis

General procedure: The method was adapted and modified from the reported procedure by Songnian Lin *et al.*, 2005.[10] Equimolar amounts of o-phenylene-di-amine and benzaldehyde derivatives were heated to 100°C in presence of air.

Synthesis of 2-(3-cyano-phenyl)-1H-Benzimidazole [MCB]): o-phenylene-di-amine (0.5 mmol) and 3-cyano-benzaldehyde (0.5 mmol) were added to 1 mL of solvent under stirring. Reaction mixture was flushed with air, capped, and heated to100°C for 18h. A pale yellow solid product formed was filtered followed by n-hexane wash. Purification was done by recrystallization technique using the solvent ET-O-Ac. This reaction was performed using 3 different solvents (Toluene, DMF, Dioxane) to check the solvent effect. FTIR (υ_{max}, cm^{-1}) = 742.64 (CH-bending aromatic), 1271.06 (CN-stretching aromatic), 1431.54 (C=C stretching aromatic), 1621.82 (C=N stretching imines), 2227.20 (CN-stretching nitrile), 3058.65 (CH stretching aromatic), and 3450.513 (NH-stretching secondary amine). ^{1}H NMR (500 MHz, DMSO-d_6) δ_H = 7.31-7.22 (2H, m), 7.65-7.64 (2H, m), 7.78 (1H, t, *J=7.8Hz*), 7.97 (1H, dd, *J=7.5, 1.4 Hz*), 8.50 (1H, dd, *J= 8.0, 1.5Hz*), 8.56 (1H, s), 13.11 (1H, s). ^{13}C NMR (125 MHz, DMSO-d_6) δ_C = 113, 119, 130, 131, 131, 132, 133, 150.

Synthesis of 2-(4-hydroxy-phenyl)-1H-Benzimidazole [MHB]): A very similar procedure to MCB was followed and instead of 3-cyano-benzaldehyde 4-hydroxy benzaldehyde was used. After completion of the reaction the solid product (color- off white) formed was filtered and followed wash with n-hexane. Purification was done by recrystallization technique using the solvent ET-OH. This reaction was performed using two solvents (Toluene & Dioxane) to check the solvent effect. FTIR (υ_{max}, cm^{-1}) = 736.144 (CH-bending aromatic), 1261.043 (CN-stretching aromatic), 1449.50 (C=C stretching aromatic), 1601.278 (C=N stretching imines), 3049.491 (CH stretching aromatic), and 3265.774 (NH-stretching secondary amine).^{1}H NMR (500 MHz, DMSO-d_6) δ_H = 6.94-6.84 (2H, m), 7.13 (2H, d, J=5.8Hz), 7.62-7.40 (2H, m), 8.03-7.93 (2H, m), 12.6 (1H, s). ^{13}C NMR (125 MHz, DMSO-d_6) δ_C = 40, 40,40, 40 40, 68, 116,122, 129,152, 159.

Synthesis of 2-(4-nitro-phenyl)-1H-Benzimidazole [MNB]): A similar procedure to MCB was followed and instead of 3-cyano-benzaldehyde 4-nitro-benzaldehyde was used. The

progress of reaction was monitored by TLC. After completion of the reaction the solid product (color- mahogany brown) formed was filtered and followed wash with n-hexane. Purification was done by recrystallization technique using the solvent ET-OH. FTIR (υ_{max}, cm^{-1}) = 721.569 (CH-bending aromatic), 1224.80 (CN-stretching aromatic), 1435.04 (C=C stretching aromatic), 1330.435 & 1510.089 (NO_2 aromatic), 1664.57 (C=N stretching imines), 3064.89 (CH stretching aromatic), and 3437.15 (NH-stretching secondary amine). ^{1}H NMR (500 MHz, DMSO-d_6) δ_H =7.33 – 7.20 (2H m), 7.58 (1H, d, *J = 7.9 Hz*), 7.72 (1H, d, *J = 8.0 Hz*), δ 8.40 (4H, d, *J = 1.1 Hz*), 13.27 (1H, s). ^{13}C NMR (125 MHz, DMSO-d_6) δ_C = 112, 120, 123, 124, 125, 128, 136, 148, 149.

Characterization

Antibacterial Sensitivity Test Method

The antibacterial sensitivity of synthesized benzimidazole derivatives were investigated by Kirby-Bauer method reported by Prescott *et al*., 2005.[11] Selected bacterial lawns were prepared on Mueller-Hinton Agar (MHA) plates by dipping sterile swabs with reference bacterial cultures. Stock solutions of synthesized benzimidazole derivatives were prepared in DMSO. First cultured bacterial strains were swabbed using cotton bud into the agar plates and then sample solutions (20 µL) followed by the standard disc were injected with a sterile borer. The agar petri plates were incubated at 37^0C for 18 h and the inhibition zone diameter appeared were recorded. Nalidixic acid 30 µg/mL discs were used as the antimicrobial positive control and solvent DMSO were used as the negative control for all these assays.

Antifungal Sensitivity Test

Kirby-Bauer method was applied for determination of antifungal properties of the synthesized novel benzimidazole derivatives. *Aspergillus niger* was cultured in both tryptic soya broth (TSB) and Potatoes dextrose agar (PDA) plates. The sample solutions (20 µL, 5.8 mg/L in DMSO) were injected into the PDA plate containing the fungal agent and kept for 3 days for the growth. After 3 days the test plates were inspected, and the data were recorded.

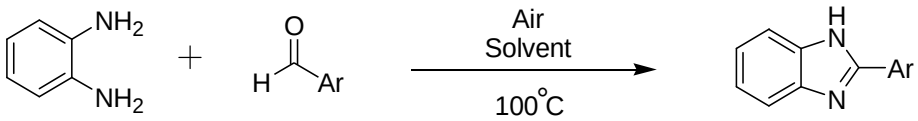
Brine-Shrimp Lethality Bioassay

Brine shrimp lethality bioassay technique was applied for determination of general toxic properties of the synthesized novel benzimidazole derivatives according to the reported procedure by Meyer *et al*., 1982.[12] In this method, brine shrimp eggs are hatched in simulated sea water (3.8% w/v sea salt in distilled water) to get *nauplii*. Sample stock solutions of varying concentrations (such as- 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563

and 0.78125 µg/mL) were prepared by dissolving sample materials in DMSO by serial dilution technique. Then the sample solutions of different concentrations were added to the pre-marked test vials containing 10 live hatched brine shrimp *nauplii* in 5 mL simulated sea water. After 24 hours, each test vials were inspected visually and the number of the survived *nauplii* was counted. These data are processed and analyzed to estimate LC_{50} values for the comparisons of potencies. The median lethal concentration (LC_{50}) was calculated by Probit Method.[13] In the present study vincristine sulphate (cytotoxic agent) was used as a positive control and 100 µL of DMSO was used as negative control. The % mortality was calculated as: Mortality rate (%) = [*nauplii* killed (=test-control)/*nauplii* taken] × 100.

RESULTS AND DISCUSSION

In the present work solvent effect was investigated in the synthesis procedure of 2-substituted benzimidazole derivatives using toluene, dimethyl formamide (DMF) and 1,4-dioxane. The solvent effect was compared based on yield and easy separation method (scheme-1 & table-1). In this level the structure of the synthesized products was compared using FTIR spectra (Figure-1). It was observed that highest isolated yield was found for the solvent toluene compared to DFM & dioxane. Synthesized benzimidazole derivatives are insoluble in toluene which facilitate easy separation and purification through filtration and recrystallization. The highest yield for the entry 1 (MCB**)** was seen 85% which is better than reported (82%) by Qui Dezhi *et al.*, 2014.[14] Though the yield of entry 4 (MHB) and 6 (MNB) have lower yield than reported in the literature, but this synthetic procedure and easy separation and purification were convincing.

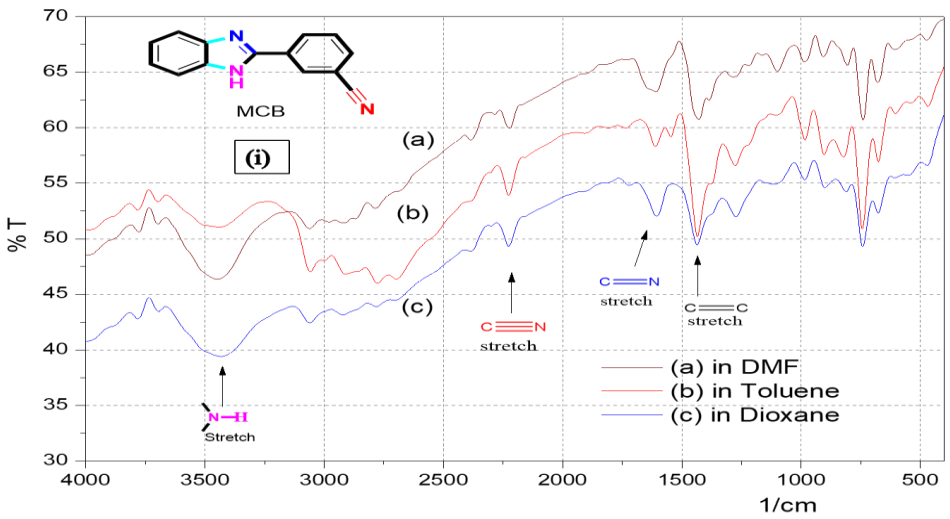


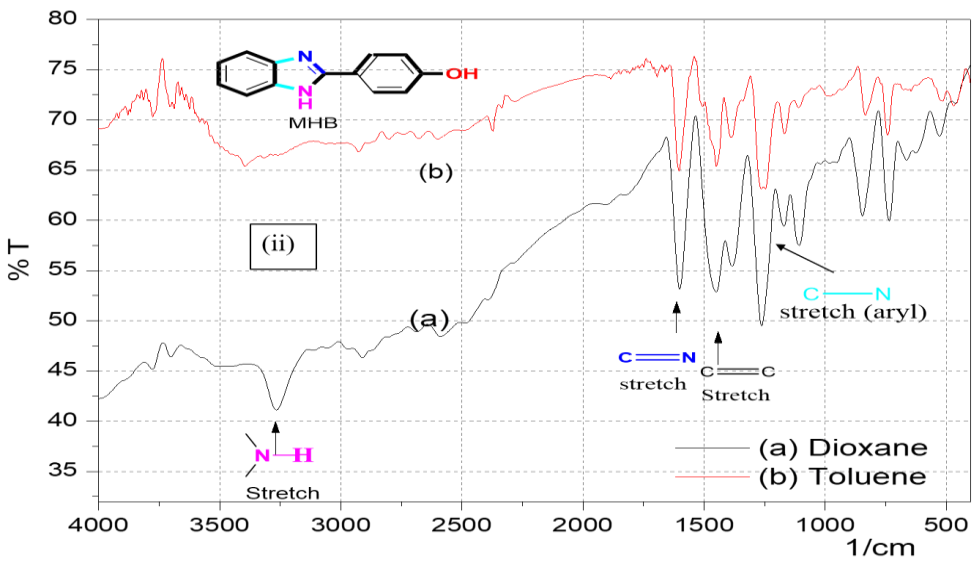
Scheme-1: General synthesis reaction of 2-substitutes benzimidazole derivatives.

Table-1: Solvent effect in the synthesis of 2-substituted benzimidazole derivatives.

Entry	Product	Solvent	Ar	Reaction time (h)	Condition	Crude Yield (Isolated) (%)
1.	MCB	Toluene	3-cyanophenyl	18	100°C	98.00 (85)
2.		DMF	3-cyanophenyl	18	100°C	99.92 (81)
3.		1,4-Dioxane	3-cyanophenyl	18	100°C	66.39 (49)
4.	MHB	Toluene	4-hydroxyphenyl	18	100°C	76.2 (61.5)
5.		1,4-Dioxane	4-hydroxyphenyl	24	100°C	73.66 (73.66)
6.	MNB	Toluene	4-nitrophenyl	45	100°C	58.00 (50)

The FTIR spectra (Figure-1) showed peaks at different regions of wavelengths for different molecular vibration of the functional groups. It is known that primary amines react readily with aldehydes and ketones to form imines. The characteristic peaks are observed at 1450.73, 1621.82 & 3450.51 cm^{-1} for the C=C or aromatic ring system, C=N stretching of imines & N-H stretching of secondary amine respectively which is comparable reported by Suwaiyan *et al*., 1990 & Mohan *et al*., 1991.[15-16] Form the figure it is evident that all spectra have similar FTIR peak and suggested that all the test samples were desired product of the synthesis reactions. However, the structures of all the synthesized 2-substituted benzimidazole derivatives were determined using ^{1}H-NMR, ^{13}C NMR & ^{13}C-DEPT NMR analysis. The NMR spectra of MCB, MHB & MNB are comparable to the work of Qui Dezhi *et al*., 2014, Shirini, Farhad *et al*., 2010 and Zakaria *et al*., 2016.[14,17-18] The plausible mechanism of the synthesis reaction was described by Songnian Lin *et al*., 2005.[10]





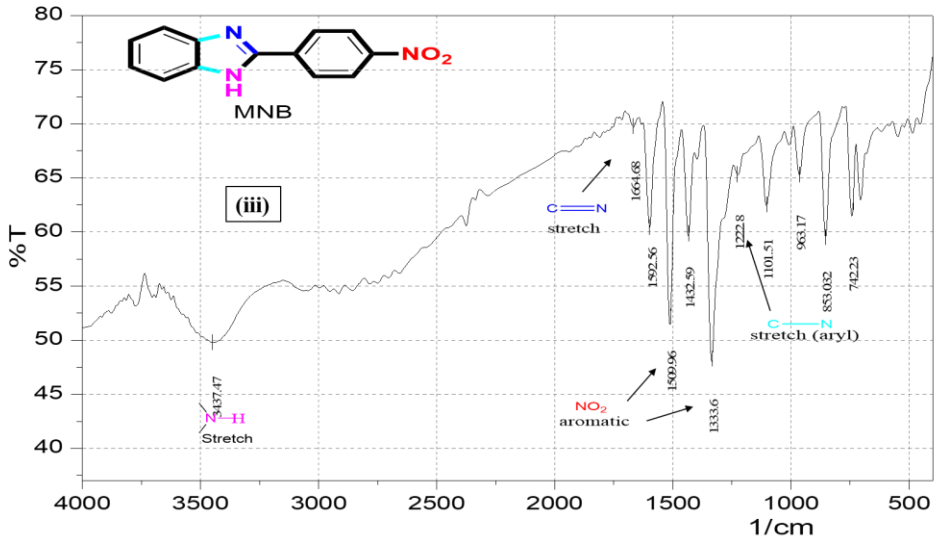


Figure-1: FTIR spectra of (i) MCB produced (a) in DMF, (b) in Toluene, and (c) in Dioxane solvent; (ii) MHB produced (a) Dioxane, (b) Toluene; (iii) MNB in Toluene.

Antibacterial Sensitivity Test

The antimicrobial activities of MCB, MNB, and MHB were analyzed against seven bacterial strains including both gram-positive and gram-negative bacteria, and the susceptibility pattern were assessed by Mueller-Hinton agar disk diffusion susceptibility testing method according to NCCLS (National Committee for Clinical Laboratory Standards) and international guidelines. The concentration of the samples was 5.8 mg/L in DMSO and 20 µL amount was used for the assay. The zones of growth inhibition were compared with standard drug (Nalidixic acid) loaded disks. Table-2 shows the antibacterial activity of synthesized 2-substituted benzimidazole derivatives. From the results it is evident that MHB shows maximum positive results against 4 bacterial strains compared to MCB & MNB.

Table-2: Quantitative measurement mean zone of inhibition of the synthesized 2-substituted benzimidazole derivatives against several bacterial strains.

Bacterial Strain	Mean zone of inhibition of 2-substituted benzimidazole derivatives (20µL; 5.8mg/ml), mm			DMSO (20µL), mm	Nalidixic Acid (30µg/disc), mm
	MCB	MNB	MHB		
B. subtilis	12.3 ± 0. 11	28.21 ± 0.05	15.6 ± 0.05	0.00	17.1 ± 0.23
S. aureus	0.00	0.00	14.4 ± 0.25	0.00	17.8 ± 0.15
E. coli-0157	0.00	37.7 ± 0.00	18.2 ± 0.17	0.00	16.2 ± 0.09
S. typhimurium	0.00	0.00	16.8 ± 0.07	0.00	18.9 ± 0.13
S. enterica	0.00	0.00	0.00	0.00	17.7 ± 0.21
K. pneumonia	11.5 ± 0.08	0.00	0.00	0.00	17.7 ± 0.06
L. monocytes	10.1 ± 0.15	26.1 ± 0.10	0.00	0.00	0.00

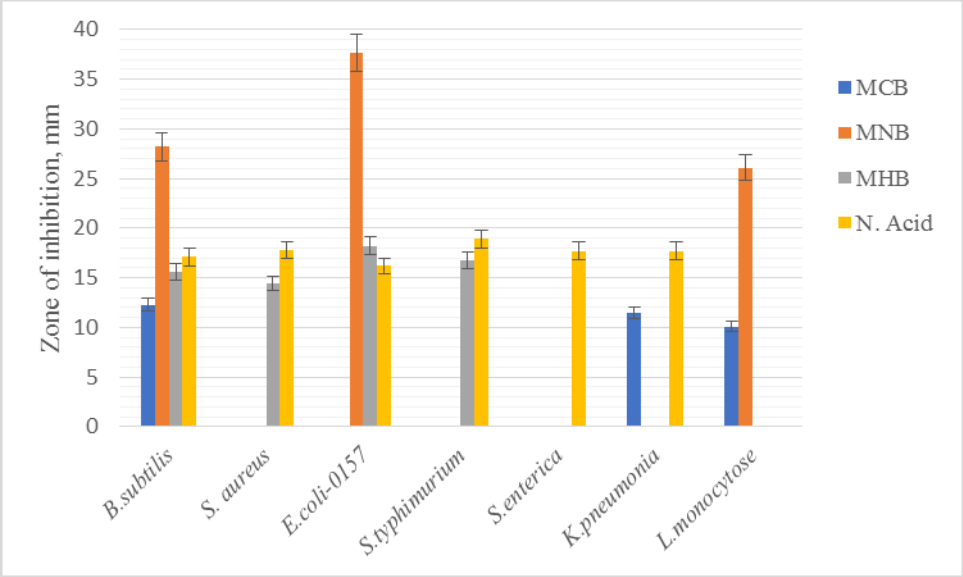


Figure-2: Comparison of the mean zone of inhibition of synthesized 2-substituted benzimidazole derivatives against seven bacterial strains.

The *S.enterica* was the only bacteria under study showed resistance against all the test samples, whereas *B. subtilis* was most susceptible bacteria. MNB showed most antibacterial activity compared to other test samples including the standard. However, the antibiotic activity of MHB was comparable to the standard nalidixic acid. MCB was the least active agent among the synthesized benzimidazole derivatives

Antifungal sensitivity Test

The antifungal activity of MCB, MNB, and MHB was investigated against *Aspergillus niger* only due to the limitation of fungal agent. *A. niger* was resistant to all the synthesized benzimidazole derivatives.

Brine-Shrimp Lethality Bioassay

In vitro cytotoxicity test of synthesized novel benzimidazole derivatives was performed by brine shrimp lethality bioassay technique. Each of the samples showed different mortality rates at different concentrations. Varying degree of lethality to synthesized benzimidazole derivatives was observed with exposure to different dose levels of the test samples. The degree of lethality was directly proportional to the concentration of the test samples ranging from significant with the lowest concentration (0.781 µg/mL) to highly significant with the highest concentration (400 µg/mL). Maximum mortalities took place at a concentration of 400-200 µg/mL, whereas least mortalities were at 0.781 µg/mL concentration (Table-3).

Table-3: Results of the brine shrimp lethality bioassay of synthesized novel benzimidazole derivatives.

Conc. (C), µg/ml	log C	% Mortality			LC_{50} (µg/ml)		
		MCB	MHB	MNB	MCB	MHB	MNB
400	2.60206	100	100	100	7.94	28.18	6.31
200	2.30103	100	100	100			
100	2.00000	100	80	100			
50	1.69897	100	60	100			
25	1.39794	100	30	100			
12.5	1.09691	60	20	100			
6.25	0.79588	40	20	90			
3.125	0.49485	30	0	0			
1.56	0.19313	10	0	0			
0.78	-0.10791	0	0	0			

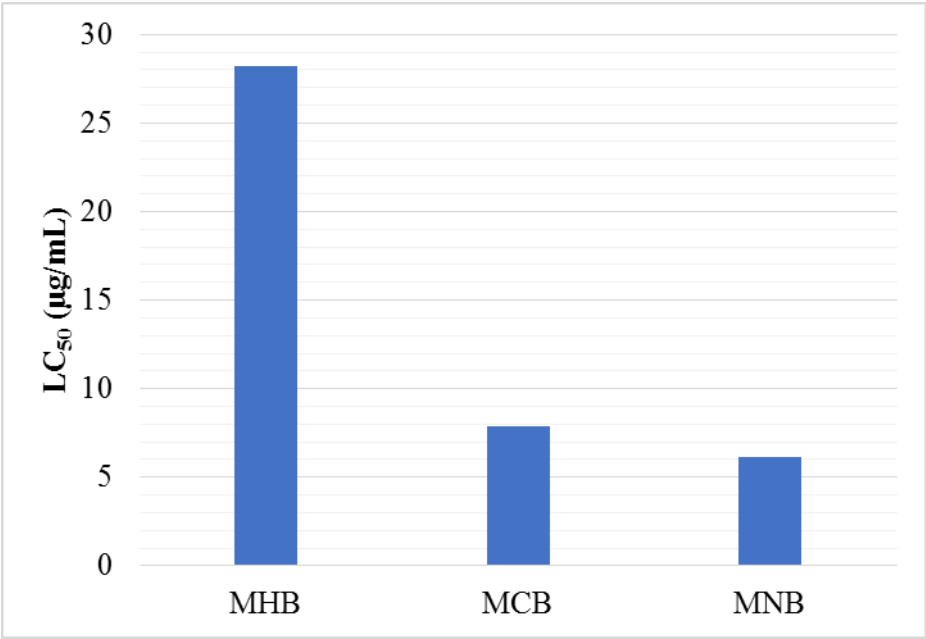


Figure-3: Comparison of brine shrimp lethality bioassay of synthesized novel benzimidazole derivatives.

MHB reflected highest LC_{50} value (28.18 µg/mL) where MNB showed least LC_{50} value (6.13 µg/mL) (Figure-3). Lowest the LC_{50} value highest the cytotoxicity revealed that, MNB was the most toxic compound among the 3 benzimidazole derivatives and MHB was least toxic compound. However, MCB showed moderate toxicity among the other benzimidazole derivatives under investigation.

CONCLUSION

2-(3-cyano-phenyl)-1H-Benzimidazole [MCB] showed best yield (> 99%), among other 2-substituted benzimidazole derivatives e.g. 2-(4-hydroxy-phenyl)-1H-Benzimidazole [MHB] (73.66%) & 2-(4-nitro-phenyl)-1H-Benzimidazole [MNB] (58%) during synthesis in direct one step air oxidation procedure. The MNB showed best antibacterial activity and rest of have significant activity. The cytotoxicity test showed that MNB was the highest toxic than others. The LC_{50} (6.13 µg/mL) value of MNB suggested that it requires further study for antitumor as well as antiparasitic activity.

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