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Research Article

MICROWAVE ASSISTED SYNTHESIS, TOXICOLOGICAL ASSESSMENT USING BRINE SHRIMP LETHALITY ASSAY AND ANTIMICROBIAL POTENTIAL OF NEW SERIES OF BENZIMIDAZOLE DERIVATIVES

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A B S T R A C T

A new series of benzimidazole derivatives were synthesized under microwave irradiation and structures of the synthesized compounds were confirmed by IR, NMR and Mass spectral analysis. As benzimidazole are the vital pharmacophore and privileged sub-structures in chemistry of medicine. They have received much interest in drug discovery because benzimidazoles exhibited enormous significance. So attempts have been made to create repository of molecules and evaluate them for prospective inherent activity. They are extremely effective both with respect to their inhibitory activity and favorable selectivity ratio. The present studies widen the scope of the brine shrimp model that may prove quite helpful as a preliminary screen to determine toxic properties. In Brine shrimp lethality bioassay, compounds produced dose dependent cytotoxicity effect to brine shrimp nauplii and all the synthesized compounds were screened for antibacterial activity by disc diffusion method. Among synthesized derivatives, compound code 3d showed good antibacterial activity. The benzimidazole derivatives could be considered promising broad-spectrum antimicrobial candidates that deserve further study for potential therapeutic applications.

INTRODUCTION

Benzimidazole is a benzo-fused imidazole which constitutes an important class of heterocyclic compound in numerous natural and synthetic compounds in medicinal chemistry for new drug development[1]. The fact made scientists realize that benzimidazole-based compounds are both safe and effective at inhibiting the growth of bacteria[2-3]. It was demonstrated that benzimidazoles can be used in the clinic as antibacterial drugs. According to research results, the antibacterial mechanism of benzimidazoles is due to their structural similarity to purine. It is well known that purine plays an important role in the biosynthesis of nucleic acids and proteins in the bacterial cell wall. As competitive inhibitors, benzimidazoles can replace purine, thereby blocking the biosynthesis of key components, killing or inhibiting the growth of bacteria[4-5]. Infectious diseases caused by bacteria have led to a substantially high rate of mortality around the world in the past. Fortunately, since the introduction of penicillin as an active antibacterial agent in the 1940s, the deployment of a multitude of natural and synthetic antibiotics has provided an immeasurable benefit to human health. However, pathogenic bacteria have developed resistance against countless antibacterial agents, rapidly resulting in the widespread emergence of bacterial resistance, thereby severely limiting treatment options. Benzimidazole and its derivatives are important classes of active substances in the field of medicine and pesticides, having a wide range of biological activities such as antibacterial, antifungal, antiviral, and antiparasitic; they also show receptor antagonism and enzyme inhibitory activities. Benzimidazole and its derivatives are reported to be physiologically and pharmacologically active and some applications are found in the treatment of several diseases including epilepsy and diabetes. Benzimidazoles exhibit important applications such as possible Swish muscle fiber propagation inhibitors, antincancer agents, as a treatment for urinary infection and in various areas of chemistry. A targeted benzimidazole motif potently induced apoptosis via the c-Jun *N*-terminal kinase (JNK)-mediated death receptor 5 up-regulation in breast cancer cells. In similar manner, amino acid are utilized in living cells for protein synthesis under the control of genes are in a special category since they are fundamental to all life forms as building blocks for peptides and proteins[6-7]. Microwave assisted synthesis has become an important tool to the medicinal chemist for rapid organic synthesis. Application of microwave technology in organic synthesis has some of the major advantages like spectacular

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decrease in reaction in reaction time, improved conversions, clean product formation and wide scope for the development of the new reaction conditions. Microwave heating depends upon two major factors first is the pre-exponential factor 'A' which describe the molecular mobility and depends upon the frequency of vibrations of the molecule at reaction interface[8-11]. The other reason is the alteration in the exponential factors by affecting the free energy of activation. With microwave heating heat is directly applied to the sample not to the vessel or container that's why it increases the rate of reaction very quickly[12-16]. A microwave is a form of electromagnetic energy that falls at the lower frequency at the end of electromagnetic spectrum. Microwave heating is the best process due to the microwave couple directly with the molecule that are present in the mixture, leading to fast rise in temperature, faster reaction and cleaner chemistry[17-20]. The microwave is also called as green chemistry because it does not produce any hazardous material like gas fumes or heating using external energy source. Microwave uses electromagnetic radiation that passes through material and causes oscillation of molecule which produces heat[21-25]. Conventional method of organic synthesis usually requires longer heating time, tedious apparatus setup which result in higher cost of process and the excessive use of solvents or reagents lead to environmental pollution. Growth of green chemistry holds necessary potential for the reduction of by product, a reduction in the waste production and a lowering of energy costs[26-37]. Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells. This interaction may vary depending on the chemical properties of the toxicants and the cell membrane, as it may occur on the cell surface, within the cell body, or in the tissues beneath as well as at the extracellular matrix. The toxic effects may take place prior to the binding of the toxicants to the vital organs such as liver and kidneys. Hence, evaluation of toxic properties of a substance is crucial when considering for public health protection because exposure to chemicals can be hazardous and results to adverse effects on human being. In practice, the evaluation typically includes acute, sub-chronic, chronic, carcinogenic and reproductive effects. Brine Shrimp lethality bioassay is a rapid and comprehensive bioassay for the bioactive compounds of synthetic origin. The method is attractive because it is very simple, inexpensive, and low toxin amounts are sufficient to perform the test in microwell scale. In the present paper, we have explored substituted benzimidazole derivatives as antibacterial agents with the support of brine shrimp lethality test were applied to determine its toxic properties[37-38].

MATERIALS AND METHODS

All chemicals and solvents were procured from commercial sources, purified and dried using standard procedures from literature whenever required the regents were purchased from S.D fine, Research laboratory, mumbai and merck laboratory, Mumbai. The melting points of synthesized compound were determined by open capillary tube method and are uncorrected. Thin layer chromatography was used confirmation of reaction and the purity of the intermediate and the final compounds by applying a single spot on TLC plate (silica gel G) using various solvents such as butane, chloroform and water system. TLC plates were visualized under iodine chamber. IR spectra were recorded on FTIR. 1H NMR spectra were performed in DMSO solution using Bruker 300 MHz and their chemical shift are reported in δ unit with respect to TMS as internal standard. Mass spectra were recorded on Pe sciex (model no. API 2000) software analyst 1.4.2 mode: Q1MS Q1/AUTO INJECTION from diya lab, airoli, Mumbai.

General method for the Synthesis of 2-aminobenzimidazole (1a)

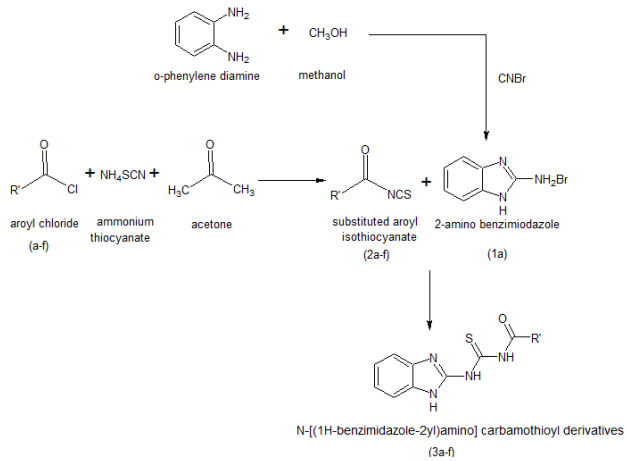
Mixture of o-Phenylenediamine 1.5 g, methanol 10 ml and cyanogen bromide 1 gm was irradiated for 15 min at 340 watt under microwave.

General method for the Synthesis of substituted aroyl isothiocyanate (2a-f)

A solution of substituted benzoyl chloride (5 mmol) in acetone (25 ml) was added dropwise to ammonium thiocyanate (5mmol) in acetone (15ml). The reaction mixture irradiated for 10 min at 340 watt under microwave. The reaction mixture was cooled at room temperature and the precipitate (NH_4Cl) was filtered off. Solution of aroyl isothiocyanate derivatives was prepared.

General method for the synthesis of substituted N-(1*H*-benzimidazole-2-yl-carbamothioyl) benzamide derivatives (3a-f)

A product of of 2-aminobenzimidazole (5mmol) was added in aroyl isothiocyanate and the reaction mixture irradiated for 10-15 min at 340 watt. The reaction was monitored by TLC using butane: chloroform: water (7:2:1) as mobile phase. The solid product was washed with water and recrystallized with methanol.

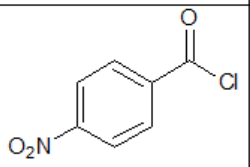
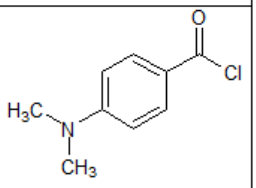
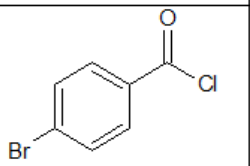
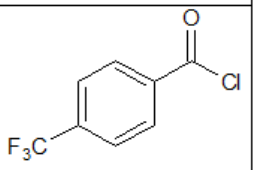
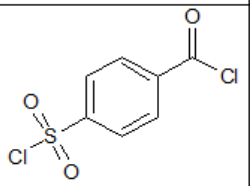
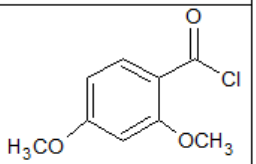


Scheme 1 Synthetic route for the preparation of the title compound (3a-f) where, R'=

Analytical Data of the Novel N-[(1H-benzimidazole-2yl)amino] carbamothioyl derivatives

3a.N-(1H-benzimidazol-2-ylcarbamothioyl)-4-nitrobenzamide

Yield 78%; m.p 154-156^{0}C; IR (KBr, cm-^{1}) 1710.11 (C=0), 1335.28 (Ar-NO_2), 3448.17 (C-NH), 1HNMR (DMSO, 300MHz, ppm): δ 7.35 (s, 1H), 7.57-7.66 (m, 4H, Ar-H), 7.70-7.78 (q, 4H, Ar-H), 8.97 (s, 1NH); mass m/z (M+) 341.4.

R'		R'	
a		d	
b		e	
c		f	

3b.*N*-(1*H*-benzimidazol-2-ylcarbamothioyl)-4-bromobenzamide

Yield 85%; m.p 161-163^{0}C; IR (KBr, cm-^{1}) 578.15 (R-Br), 1729.23 (C=0), 3428.41 (C-NH), 1HNMR (DMSO, 300MHz, ppm): δ 7.30 (s, 1H), 7.41-7.54 (m, 4H, Ar-H), 7.61-7.69 (q, 4H, Ar-H), 8.71 (s, 1NH); mass m/z (M+) 375.3.

3c.1-(1H-benzimidazol-2-yl)-4-[(chlorosulfanyl)carbonyl]benzamide

Yield 71%; m.p 143-145^{0}C; IR (KBr, cm-^{1}) 1754.83 (C=0), 3468.20 (C-NH), 1HNMR (DMSO, 300MHz, ppm): δ 7.54 (s, 1H), 7.64-7.78 (m, 4H, Ar-H), 7.82-7.89 (q, 4H, Ar-H), 8.51 (s, 1NH); mass m/z (M+) 286.8.

3d.N-(1H-benzimidazol-2-ylcarbamothioyl)-4-(dimethylamino)benzamide

Yield 69%; m.p 155-157^{0}C; IR (KBr, cm-^{1}) 1725.61 (C=0), 3412.20 (C-NH), 1HNMR (DMSO, 300MHz, ppm): δ 7.12 (s, 1H), 7.35-7.48 (m, 4H, Ar-H), 7.54-7.69 (q, 4H, d, Ar-H), 7.73-7.84, 8.78 (s, 1NH); mass m/z (M+) 339.5.

3e.N-(1H-benzimidazol-2-ylcarbamothioyl)-4-(trifluoromethyl)benzamide

Yield 76%; m.p 143-145^{0}C; IR (KBr, cm-^{1}) 1742.30 (C=0), 3420.61 (C-NH), 1HNMR (DMSO, 300MHz, ppm): δ 7.21 (s, 1H), 7.30-7.38 (q, 4H, Ar-H), 7.49-7.56 (m, 4H, d, Ar-H), 8.81 (s, 1NH); mass m/z (M+) 364.4.

3f.N-(1H-benzimidazol-2-ylcarbamothioyl)-2,4-dimethoxybenzamide

Yield 84%; m.p 147-149^{0}C; IR (KBr, cm-^{1}) 1140.51 (C=S), 1728.72 (C=0), 2872.10 (O-CH_3), 3471.61 (C-NH), ^{1}HNMR (DMSO, 300MHz, ppm): δ 7.28 (s, 1H), 7.49-7.54 (q, 4H, Ar-H), 7.70-7.71 (m, 4H, Ar-H), 8.70 (s, 1NH) mass m/z (M^+) 356.2.

Biological Evaluation

Brine Shrimp Lethality Assay

Brine shrimp lethality test has been used as a bioassay for a variety of toxic substances. A general bioassay that appears capable of detecting a broad spectrum of bioactivity, present in synthetic compounds, rather than more tedious and expensive *in-vitro* and *in-vivo* antitumor assays. Furthermore, it does not require animal serum as is needed for cytotoxicity.

Procedure

Preparation of seawater

38 gm sea salt (without iodine) was weighed, dissolved in one liter of distilled water and filtered off to get clear solution.

Hatching of Brine Shrimp

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank, and shrimp eggs were moved to one side of the tank, and sealed on this side. The shrimp was allowed to hatch for two days and be matured like nauplii. Constant supply of oxygen was rendered during the process of hatching. The hatched shrimps are drawn to the light (phototaxis) and so egg shell-free nauplii from the illuminated portion of the tank was collected. The nauplii was taken by a pipette from the fish tank and filtered to improve visibility in fresh clear sea water, and 10 nauplii was taken carefully by micropipette.

Preparation of test samples

In each experiment, 0.5mL of test compound of different concentration i.e (50, 100 and 150µg/mL) was added to brine solution and maintained at room temperature for 24hrs under the light and surviving larvae were counted. Vehicle treated used as control for the test. Test solutions were used in sets of three tubes per dose. Replicas should be maintained to get accurate results. Analysis of the data was performed by probit analysis to determine the lethal concentration to half of the test organisms (LC_{50})[39-40].

Antimicrobial Screening

Chemicals

All chemicals and solvent where procured from commercial sources, purified and sterilize using standard procedure from literature whenever required. MacConkey agar and nutrient agar medium used.

Dilution of compound

All the synthesized compound where dissolved in dimethyl sulphoxide [DMSO] so as to get concentration of 100µg/ml, 200µg/ml, and standard drug ciprofloxacin in DMSO as a concentration of 200µg/ml .

Sterilization of equipment and the chemicals

MacConkey agar, Nutrient agar medium [NO11], Normal saline solution where sterilized in autoclave. At 15 Ibs pressure [121^{0}C] for 150 min. Petri plates, Whatman filter paper, descant cotton swabs where sterilized in oven at 160^{0}C for 2 hours.

Preparation of MacConkey agar slant

MacConkey agar 206 mg was dissolved in 4ml of distilled water, boiled and poured test tube then plugged with cotton and sterilize in autoclave as 15 Ibs pressure 121°C for 15 min. After sterilization the tubes containing the MacConkeys agar were kept in inclined position from 30 min. Then on the surface of slants pure culture staphylococcus aureus where streaked in aseptic condition and incubated and 37°C for the 24 hrs.

Preparation of nutrient agar medium slant

Nutrient agar medium 112 mg and agar powder 100 mg was dissolved in 4ml distilled water, boiled and then poured in test tube then plugged with cotton and sterilized in autoclave at 15 Ibs pressure (121°C) for 15 min. After the sterilization the tubes containing the nutrient agar medium were kept in inclined position for 30 min. Then on the surface of slants pure culture of *E.coli* were streaked in aseptic condition and incubated at 37°C for 24 hours.

Preparation of suspension of test bacteria

Using the 24 hours old growth of test bacteria from the slant, suspension of bacteria was made separately in sterile normal saline solution (0.85% NaCl in distilled water) in aseptic condition, to get moderate turbidity. The turbidity of each suspension was compared adjusted with the turbidity of the solution resulting by mixing 0.5 ml of 1.175% of barium chloride and 99.5 mi of 36 N of H_2SO_4.

Method: Disc Diffusion Method

Preparation of culture media for antibacterial sensitivity test

MacConkey agar (50ml) and nutrient agar (100ml) was prepared as per the procedure given for preparation of slants respectively. Then it was sterilized in autoclave at 15lbs pressure (121^{0}C) for 15 min. after sterilization the media was cooled up to 45^{0}c, poured 20-25 ml in sterile Petri plates in aseptic condition and allowed to solidify.

Inoculation of suspension of bacteria on culture media

Sterile, non toxic swab were dipped into the standardized inoculums and then the entire agar surface of the plate was streaked with the swab three times, turning the plate at 60 angles between streaking. Then the streaked inoculum was allowed to dry for 5-15 min with lid. Sterile Whatman paper disc dipped separately into the solutions containing synthesized drug (100µg/ml and 200µg/ml) and standard drug ciprofloxacin (100µg/ml and 200µg/ml) in aseptic condition with the help of sterile forceps and placed on the surface of inoculated culture media after which the plates were kept in refrigeration for 30min. for the diffusion of the compound from the paper disc into the culture media. After 30min.the plates were incubated at 37^{0}C for 24 hrs. All the synthesized compounds (3a-f) were observed for antibacterial activity. Observation was recorded in tables by measuring the zone of inhibition in millimeters[41-45].

RESULTS AND DISCUSSION

Chemistry

In first step o-phenylenediamine, methanol and cyanogen bromide was irradiated for 15 min at 340 watt under microwave. In the subsequent step a solution of substituted benzoyl chloride in was added dropwise to ammonium thiocyanate in acetone. The reaction mixture irradiated for 10 min at 340 watt under microwave. The reaction mixture was cooled at room temperature and the precipitate (NH_4Cl) was filtered off. Solution of aroyl isothiocyanate derivative was prepared (2a-f). A product of of 2-aminobenzimidazole was added in aroyl isothiocyanate and the reaction mixture irradiated for 10-15 min at 340 watt. The reaction was monitored throughout by TLC using butane: chloroform: water (7:2:1) as mobile phase. The solid product was washed with water and purified by washing with methanol. The reaction sequence is shown in Scheme 1. By using green chemistry procedures, we can minimize the waste of materials, maintain the atom economy and prevent the use of hazardous chemicals. Thus, microwave-assisted synthesis has advantages over conventional technology: it is more energy efficient and it can lead to improved isolated yields of products with green synthesis. The synthesized compounds were characterized by spectral analysis and all the compounds were in full harmony with the proposed structures.

Brine shrimp lethality assay

The lethality of a test sample in a simple zoological organism such as the shrimp (*Artemia salina*) has been utilized in the Brine Shrimp cytotoxicity Test (BSCT). It is a very useful tool to screen a wide range of chemical compounds for their various bioactivities. It has been demonstrated that BSCT correlates reasonably well with cytotoxic and other biological properties. The brine shrimp bioassay has been established as a safe, practical and economic method for determination of bioactivities of synthetic compound as well as plant products. The significant correlation between the Brine shrimp assay and in vitro growth inhibition of human solid tumor cell lines demonstrated by the national cancer institute is significant because it shows the value of this bioassay as a pre-screening tool for antitumor drug research. The brine shrimp lethality bioassay also indicates antifungal effects, pesticidal effects, teratogenic effects, toxicity to environment and many more. Table 1 shows the lethality of different test sample to the Brine Shrimp nauplii. All the synthesized compounds (3a-f) were tested for cytotoxic activity by the brine shrimp lethality assay. Among them compounds 3b and 3d showed a dose dependent cytotoxic activity at concentrations of (3b) 1.20 µg/ml and (3d) 1.05µg/ml. The remaining compounds exhibited less activity when compared to the other compounds at various concentration levels. The degree of lethality is directly proportional to the concentration of the synthesized compounds.

Table 1 Brine shrimp lethality assay data novel of substituted N-(1*H*-benzimidazole-2-yl-carbamothioyl) benzamide derivatives (3a-f)

Sr. no	Compound code	LC_{50} (µg/ml)
1	3a	3.75
2	3b	1.20
3	3c	6.54
4	3d	1.05
5	3e	3.90
6	3f	2.24

Antimicrobial activity

Antibacterial activity of the newly synthesized compounds (3a-f) was evaluated by the disc diffusion method against Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus strains of bacteria. Compound code 3d were found to be highly active against all the tested strains of bacteria showing the broadest spectrum of antibacterial activity when compared with reference drugs ciprofloxacin.

Table 2 Antibacterial screening result of synthesized compounds measuring the zone of inhibition in millimeter

Sr. no	Compound Code	Diameter of zone of inhibition (mm)					
		E. coli		*P. aeruginosa*		*S.aureus*	
		100µg/ml	200µg/ml	100µg/ml	200µg/ml	100µg/ml	200µg/ml
1	3a	14	17	12	15	10	13
2	3b	11	15	14	18	12	17
3	3c	8	12	7	10	15	19
4	3d	18	21	19	24	21	25
5	3e	14	18	11	16	9	12
6	3f	12	16	8	15	13	18
7	ciprofloxacin	20	25	16	21	23	27

CONCLUSION

Substituted N-(1*H*-benzimidazole-2-yl-carbamothioyl) benzamide derivatives were synthesized by microwave method and it is a convenient way toward the goal of green chemistry. Microwaves irradiation can be used to in chemical synthesis as a heat source; it is very efficient and can be used to significantly reduce reaction times of numerous synthetically useful chemical transformations. Thus, microwave-assisted synthesis can lead to improved isolated yields of products with green synthesis. The derivatives characterized by IR, 1H NMR and mass spectra. The brine shrimp lethality bioassay is considered as a useful tool for the preliminary assessment of toxicity. As for the results of biological screening, the synthesized benzimidazole derivatives showed good antibacterial activity in comparison to standard drugs. It can be deduced that the synthesized benzimidazole hybrids were useful and potent compound that can be further modified to complex structures into useful therapeutic drugs.

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