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Original article

Synthesis, characterization and *in vitro*, *in vivo*, *in silico* biological evaluations of substituted benzimidazole derivatives

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ABSTRACT

A series of substituted benzimidazole derivatives were synthesized by reacting *O*-phenylenediamine with various aromatic aldehydes or glycolic acid using various inexpensive reagents in aqueous media. Synthesized compounds were characterized and elucidated by IR, ^{1}H NMR, ESI-MS spectra. Resultant compounds were screened for *in vitro* antimicrobial, cytotoxic, antioxidant, lipid peroxidation and cholinesterase inhibitory activities, *in vivo* analgesic and anti-inflammatory, and *in silico* anti-acetylcholinesterase and anti-butyrylcholinesterase activities. Among the synthesized compounds, compound **3b** showed most promising central analgesic effect (46.15%) compared to morphine (48.08%), whereas compounds **6**, **3c** and **3a** showed significant peripheral analgesic activity at two different dose levels (25 mg/kg and 50 mg/kg). Compounds **3b** and **3a** at the dose of 100 mg/kg showed significant anti-inflammatory effects from the first hour and onward, whereas compounds **6** and **3b** showed moderate cytotoxic activities. In addition, compound **3a** showed significant antioxidant activity having IC_{50} value of 16.73 μg/ml compared to 14.44 μg/ml for the standard BHT. Compound **6, 3a** and **3b** exhibited mild to moderate cholinesterase inhibitory activity. *In silico* studies revealed that compound **3a** and **3b** might be suitable for cholinesterase inhibitory activity. A comprehensive computational and experimental data suggested compounds **3b** and **3a** as the best possible candidates for pharmacological activity. All the experimental data were statistically significant ($p < 0.01$ level).

1. Introduction

Benzimidazole and its derivatives constitute a promising class of nitrogen-containing heterocyclic compounds in medicinal chemistry (Divya et al., 2012; Gao et al., 2007; Reddy, 2009, 2010; Wright, 1951). Extensive biochemical and pharmacological studies have revealed that derivatives of benzimidazole are effective as antiulcer (Husain et al. 2011, Welage and Berardi, 2000), anticancer (Refaat, 2010), cytotoxic and antitumor (Brana et al. 1994), DNA binding (Chodosh et al. 1989), antihypertensive (Sharma et al. 2010, Billups and Carter, 1998, Jones and Karly, 2011), antifungal (VanMeel et al. 1996), antibacterial (Ghannoum and Rice, 1999), anthelmintic (Kohli and Wakode, 2012, Chavarria et al. 1973, Seah, 1976), enzyme inhibition, anticonvulsant, CNS depressant, androgen receptor antagonist, antitubercular and HIV-1-induced cytopathic inhibitor (Navarro et al., 2009, Spasov et al., 1999, Kern et al., 1979, Poddar et al., 2016, Vogel et al., 2006, Buu-Hoi et al., 1963). Due to these immense and diverse bioactivities, organic and medicinal chemists have always put a huge concern in the synthesis of substituted derivatives of these heterocycles.

A vast number of synthetic pathways have been reported in various literature. For the synthesis of substituted benzimidazoles, commonly encountered catalysts are $ZrCl_4$ (Rao et al., 2002), CuCl (Lashkari et al., 2018), H_2O_2/HCl (Jinsong et al., 2011), iodine (Dixit et al., 2013), oxalic acid (Theerthagiri and Lalitha, 2013), SiO_2/$SOCl_2$ (Rezayati et al., 2016), sulphamic acid (Qin et al., 2014), *p*-toluene sulphonic acid (Qin et al., 2014), and Zeolite (Zhang et al., 2007). However, a number of reported routes have encountered noteworthy drawbacks, for instance, extreme

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reaction conditions, poor yield, prolonged reaction time, by-product formation, use of hazardous & expensive catalysts, and toxic organic solvents. Considering the immense importance of the ring system and its synthetic pitfalls, our group has previously reported the selective synthesis of several disubstituted and trisubstituted benzimidazoles using NH_4Cl in chloroform and investigated some preliminary biological activity (Brishty et al., 2020). To avoid toxic organic solvents and extensive biological screening, herein, we have synthesized several substituted benzimidazole derivatives using inexpensive reagents ($SiO_2/ZnCl_2$, iodine, boric acid, and benzyl Chloride as catalysts) in environment-friendly conditions and medium.

As useful drug molecules are limited in several dreaded diseases, drug synthesis and repurposing of existing drug might be the best solution to overcome the existing situations (Hossain and Rahman, 2021). The versatile benzimidazole group is a big target for modifying its structure and converting into potential molecules for further exploration in different ailments. Although a huge number of benzimidazole derivatives have been synthesized and their various pharmacological activities are evaluated, very few examples are found regarding their analgesic, antiinflammatory, anticholiesterase and lipid peroxidation properties (Akhtar et al., 2017). Some research groups have proposed benzimidazoles as emerging scafold for anti-nociceptive and antiinflammatory activities (Achar et al., 2010, Gaba et al., 2014). There are some instances in literature for testing its potential against acetylcholinesterase and butyrylcholinesterase to treat neurodegeneration diseases (Coban et al., 2016, Faraji et al., 2017). Some benzimidazole derivatives also have exhibited antioxidant, antimicrobial and lipid peroxidation properties (Bektas et al., 2018, Ayhan-Kılcıgil et al., 2012). Based on the above reports, we became interested to investigate the various pharmacological activities of the synthesized benzimidazole derivatives

Synthethic compounds are often tested by *in-silico* molecular docking and ADME/T analysis to support the *in-vivo* results and benzimidazole is not an exception (Alpan et al., 2013, Türkan, 2021). In this study, *in-silico* anti-acetylcholinesterase and anti-butyrylcholinesterase studies were performed to find the correlation with i*n-vitro* anti-acetylcholinesterase and anti-butyrylcholinesterase studies using homogenized rat brain.

2. Materials and method

2.1. Chemical:

All reagents were purchased from Sigma Aldrich or ROTH, Germany. Solvents were purified by distillation over calcium hydride. Reactions were performed by using well-dried glassware with the help of a reflux condenser. A rotary evaporator was used to remove the solvents under reduced pressure. The reaction progress was continuously monitored using Thin Layer Chromatography (TLC) followed by visual confirmation with UV lamp and *P*-anisaldehyde and heating at high temperature (120 °C). Purification of the compounds were accomplished by Clomun chromatography. Silica gel 60 (0.06–0.2 mm, ROTH) was employed for column chromatography. Purity of the compounds were examined by TLC. FTIR spectra were recorded by using Shimadzu FTIR spectrophotometer (Model: 8400S) in the range 4000–400 cm^{-1} at Central Instrument Lab, Faculty of Pharmacy, University of Dhaka. ^1H NMR (400 MHz) was recorded in $CDCl_3$ and methanol on JEOL alpha 400 spectrophotometer at Bangladesh Council of Scientific and Industrial Research (BCSIR). ESI-MS was recorded and obtained from Robert W. Coon Education Building, Marshall University, U.S.A.

2.1.1. General procedure for the synthesis of 1-(4-hydroxy benzyl)-2-(4-hydroxyphenyl)-1H-benzimidazole (3a) 1-(4-chlorobenzyl)-2-(4-chlorophenyl)-1H-benzimidazole (3b)

O-phenylenediamine (1 gm, 12.2 mmol), 4-chlorobenzaldehyde (2.81 gm, 20.0 mmol), and iodine (0.013 gm) were taken. The flask containing the mixture was taken on an oil bath and was stirred at 80–90 °C over a magnetic stirrer. The progress of the reaction was monitored by TLC and visualized under UV light. Then the TLC plate was further soaked into *P*-anisaldehyde / H_2SO_4 solution and subsequent heating on a hot plate above 120 °C for about 2 min. After 1.5 h, the reaction was completed. Then the reaction mixture was cooled to room temperature and diluted with ice-cold water (50 ml). The solid precipitate was collected by filtration, washed with cold water, and then dried to give corresponding crude 1-(4-chlorobenzyl)-2-(4-chlorophenyl)-1H-benzimidazole. Then the crude product was further purified by column chromatography on silica gel using ethyl acetate and n-hexane to afford the desired compound. The solvent system for TLC was ethyl acetate:*n*-hexane (3:5). The crude product was purified by column chromatography, and the solvent system was ethyl acetate: n-hexane (1:9). 1-(4-hydroxy benzyl)-2-(4-hydroxyphenyl)-1H-benzimidazole (Compound **3a**) was synthesized by condensing *O*-phenyldiamine and 4-hydroxybenzaldehyde using same equivalent of reagents and procedure.

2.1.1.1. 1-(4-hydroxybenzyl)-2-(4-hydroxyphenyl)-1H-benzimidazole (3a). Yield: 74%. White colored solid. IR (KBr, cm-1): 3031 (C-H stretching of aromatic ring), 3420, 3446 (O-H stretching of phenol). 1H NMR (400 MHz, TMS, $CDCl_3$, δ/ppm): 7.66 (d, 1H, *J* = 8 Hz), 7.50 (d, 2H, *J* = 8.4 Hz), 7.35 (d, 1H, *J* = 8 Hz), 7.24 (m, 2H), 6.89 (d, 2H, *J* = 8.4 Hz), 6.86 (d, 2H, *J* = 8.4 Hz), 6.69 (d, 2H, *J* = 8.4 Hz), 5.39 (s, 2H), 4.85 (s, 2H). ESI-MS: Calculated *m*/*z* (%): 316.12. Found *m*/*z* (%): 317.20 (M + 1).

2.1.1.2. 1-(4-chlorobenzyl)-2-(4-chlorophenyl)-1H-benzimidazole (3b). Yield: 90%. White colored crystal. IR (KBr, cm-1): 2929 (C-H stretching), 303, 3078 (C-H stretching of aromatic ring). 1H NMR (400 MHz, TMS, $CDCl_3$, δ/ppm): 7.93 (dd, 1H, *J* = 7.2 Hz, 2.4 Hz), 7.62–7.68 (m, 2H), 7.46 (dd, 2H, *J* = 8.4 Hz, 1.2 Hz), 7.35–7.41 (m, 1H), 7.32 (d, 3H, *J* = 8.4 Hz), 7.23 (d, 1H, *J* = 8.4 Hz), 7.01 (d, 2H, *J* = 8.4 Hz), 5.44 (s, 2H). ESI-MS: Calculated *m*/*z* (%): 352.05. Found *m*/*z* (%): 353.10 (M + 1).

2.1.2. Synthesis of 1-(4-methoxybenzyl)-2-(4-methoxyphenyl)-1H-benzimidazole (3c)

O-phenylenediamine (1 gm), *P*-anisaldehyde (1.126 ml), and $SiO_2/ZnCl_2$ (0.120 gm, 25%) were taken, and the mixture was stirred at room temperature over a magnetic stirrer. The progress of the reaction was examined by TLC and visualized under UV light. The TLC plate was further soaked into *P*-anisaldehyde / H_2SO_4 solution and subsequent heating on a hot plate above 120 °C for about 2 min. After stirring for 30 mins, the reaction was completed. Then ethyl acetate (10 ml) was added. The organic solution was separated, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography over silica gel. The solvent system for TLC was a mixture of ethyl acetate and *n*-hexane in a ratio of 3:5, respectively. After the solvent removal, the crude product was purified by column chromatography. The solvent system for column chromatography was ethyl acetate and *n*-hexane mixture in a ratio of 1:1.

2.1.2.1. 1-(4-methoxybenzyl)-2-(4-methoxyphenyl)-1H-benzimidazole (3c). Yield: 84%. White colored solid. IR (KBr, cm-1): 1245 (C-N stretching of aromatic amine), 1458, 1507, 1609 (C = C stretching of aromatic ring), 3010 (C-H stretching of aromatic ring), 1030 (C-O stretching). 1H NMR (400 MHz, TMS, $CDCl_3$, δ/ppm):

7.99 (d, 1H, *J* = 8 Hz), 7.84 (d, 1H, *J* = 7.6 Hz), 7.6 (d, 2H, *J* = 7.6 Hz), 7.1–7.3 (m, 2H), 7.01 (d, 2H, *J* = 7.6 Hz), 6.93 (d, 2H, *J* = 7.6 Hz), 6.84 (d, 2H *J* = 8 Hz), 5.37 (s, 2H), 3.81 (s, 3H), 3.77 (s, 3H). ESI-MS: Calculated *m*/*z* (%): 344.15. Found *m*/*z* (%): 345.20 (M + 1).

2.1.3. Synthesis of 2-(4-chlorophenyl)-1H-benzimidazole (4)

O-phenylenediamine (1 gm), 4-chlorobenzaldehyde (1.30 gm), and boric acid (0.572 gm) were added in 5 ml of water into a 250 ml round bottom flask at room temperature. Then the mixture was stirred over a magnetic stirrer for about 45 min. Progress of the reaction was monitored by TLC and visualized using UV light. Then the TLC plate was further soaked into *P*-anisaldehyde / H_2SO_4 solution and subsequent heating on a hot plate above 120 °C for about 2 min. After completion of the reaction, the solid product was collected by simple filtration and washed with water. The crude product was purified by recrystallization from ethanol.

Yield: 86%. Yellow colored solid. IR (KBr, cm-1): 3031 (C-H stretching of aromatic ring), 1558 (C = C stretching of aromatic ring), 668 (C-Cl stretching). 1H NMR (400 MHz, TMS, $CDCl_3$, δ/ppm): 8.49 (s, 1H), 7.48 (d, 2H, *J* = 8.4 Hz), 7.43 (d, 2H, *J* = 8.4 Hz), 7.04–7.09 (m, 2H), 6.71–6.78 (m, 2H). ESI-MS: Calculated *m*/*z* (%): 228.05. Found *m*/*z* (%): 229.00 (M + 1).

2.1.4. Synthesis of 2-((benzyloxy)-methyl)-1H-benzimidazole (6)

O-phenylenediamine (1 gm), glycolic acid (1.41 gm), and dimethylformamide (6 ml) mixture were taken into a 250 ml round bottom flask. The mixture was refluxed in an oil bath overnight. The temperature was increased slowly from 50 to 90 °C. The completion of the reaction was monitored by TLC and visualized under UV light. Then the TLC plate was further soaked into *P*-anisaldehyde / H_2SO_4 solution and subsequent heating on a hot plate above 120 °C for about 2 min. After that, the reaction mixture was diluted with water, and $NaHCO_3$ solution was added to neutralize the residual acid reactants. The mixture was then extracted with ethyl acetate. The organic layer was washed with brine solution, layers were separated, and the organic layer was dehydrated over anhydrous sodium sulfate. The solvent was removed under reduced pressure using a rotary evaporator. The crude product was purified by column chromatography to yield compound **5** (1.7 g, 94% yield). After that, 336 mg of purified **5** (2-methyl hydroxy-1H-benzimidazole) was allowed to react with 0.532 ml of benzyl chloride in the presence of 110.9 mg of sodium hydride in 100 ml round bottom flask under nitrogen atmosphere overnight at room temperature to yield compound **6** which was purified by column chromatography.

Yield: 77%. White colored solid. IR (KBr, cm-1): 3080 (C-H stretching of aromatic ring), 1493 (C = C stretching of aromatic ring), 1266 (C-N stretching of imidazole ring), 746 (C-H stretch). 1H NMR (400 MHz, TMS, $CDCl_3$, δ/ppm): 7.497 (s, 1H), 7.82 (d, 1H, *J* = 8 Hz), 7.25–7.33 (m, 4H), 7.17 (d, 2H, J = 8), 5.34 (s, 4H). The spectral data of the compounds were compared with reported data (Gao et al., 2017).

2.2. Biological:

A total of nine biological tests were performed to assess the biological effects produced by the synthesized compounds.

2.2.1. Experimental animals

Swiss-albino mice (*Mus musculus*), weighing about 25 g each of either sex, and Sprague Dawley rat of 150 g each approx. were collected from the animal house of Jahangirnagar University to perform the *in vivo* investigations. Clean polypropylene cages were used to keep the experimental animal at controlled room temperature (24 ± 2 °C) and relative humidity (60–70%). Formulated rodent food was provided, and the leftover food was withdrawn 12 hourly. Animals were treated as per the ethical guidelines set by the faculty office at the University of Dhaka. For each investigation, mice or rats (where applicable) were categorized randomly in three groups namely, Control, Standard, and Test groups consisting of five animals in each group. The Control group was given placebo only, the Standard group was given an appropriate standard drug and the two Test groups (lower dose −25 mg/kg, higher dose −50 mg/kg) were given two separate doses of the synthesized compounds under investigation. Each mouse/rat was weighed properly prior to any treatment, and administration of the doses of the test samples and control materials were adjusted accordingly. The animals were individualized by marking them as M_1, M_2, M_3, M_4 and M_5 for easy and accurate management.

2.2.2. Evaluation of central anti-nociception activity

Central anti-nociception activity was measured by the radiant heat tail-flick method. Changes in sensitivity of test animals were evaluated based on the response of analgesic activity observed (Hegedüs et al., 2006). With the help of a feeding needle, test samples and standard were given orally. After 30 min' intervals, the tail flicking time was measured using an analgesiometer (Medicraft, India). The site of application of the radiant heat was maintained at 2.5 cm in the tail, and a cut off time of 10 s was imposed to prevent any injury. The analgesic response was expressed as % MPE (maximum possible anti-nociceptive effect) described by Ahmed *et al.* (Ahmed et al., 2001). The central analgesic activity of the test samples was compared to standard Morphine.

$$\%\text{time elongation} = \frac{(Average\ time\ of\ tail\ flicking\ of\ test\ samples - Average\ time\ of\ tail\ flicking\ of\ the\ control\ group)}{\text{Average time of tail flicking of the control group}}$$

2.2.3. Evaluation of peripheral anti-nociception activity

The acetic acid-induced writhing method is the most commonly used method to evaluate the analgesic activity of the test samples (Cao and Li, 1994). In this method, experimental animals were subjected to the administration of acetic acid (0.7%) intra-peritoneally for the induction of pain sensation (Koster et al., 1959). In order to administer the synthesized benzimidazole derivatives at a dose of 25 mg/kg and 50 mg/kg body weight of mice, 3.125 mg and 6.25 mg of samples of each of the derivatives were measured. Then they were triturated by adding 2–3 drops of Tween-80 in a unidirectional way, and 1–2 drops of DMSO was also added as the solubilizing agent. The final volume of the suspension was adjusted to 4 ml by the addition of normal saline water. With the vortex mixture, the solution was stirred well and stabilized. As standard, diclofenac sodium (Intafenac, Incepta Pharmaceutical Ltd.) was used as 25 mg/kg of body weight dose. The total number of writhing was counted for a duration of 15 min for each mouse, and the data were evaluated statistically to find a correlation.

2.2.4. Evaluation of anti-inflammatory activity

To assess the activity of the synthesized compounds against acute inflammation, Carrageenan-induced rat hind paw oedema was utilized (Singh et al., 1990). Carrageenan was injected in the sub-plantar region of the rat's hind paw to induce the localized inflammation. As a result, it leads to the formation of oedema in situ. The volume of the rat's paw oedema was measured using a plethysmometer (37140, Ugo Basile, Italy) per hour interval. The percentage (%) of increase in paw volume with time was calculated and compared using the following equation:

$$\%\text{inhibition} = \frac{Vc - Vt}{Vt} \times 100$$

where Vc and Vt represent the average oedema volume of control and treated animals, respectively.

2.2.5. Evaluation of antimicrobial activity

The disc diffusion method is a classical method in which antibiotics diffuse from a confined source through nutrient agar gel (Zajac et al., 2008, Nagumanthri et al., 2012, McLaughlin et al., 1991, Adib et al., 2021). In a strict aseptic condition, the test organisms were carefully transferred from the pure culture to the agar slants with the help of a sterile inoculating loop in a zigzag pattern. At 37° C for 24 h, the seeded strains were incubated to ensure optimum growth. To evaluate the sensitivity test, the test organisms were then transferred from the subculture to the test tubes containing prefilled 10 ml of melted and sterilized agar medium. In this investigation, sterilized metrical (Whatman No.1) filter paper discs were used to ensure the targeted delivery of samples, and Ciprofloxacin (5 μg/disc) was used as the reference (Adib et al., 2021). Then the discs were soaked with 10 μl of the solution of the test sample and dried. The antimicrobial effects of the synthesized compounds on various microorganisms were observed.

2.2.6. Brine shrimp lethality (BSL) bioassay

BSLT is considered an important preliminary assessment of cytotoxicity for analyzing the synthesized compounds (Michael et al., 1956, Vanhaecke et al., 1981, Sleet and Brendel, 1983, Solis et al., 2007, Harwig and Scott, 1971). Following the serial dilution method, different solutions of various concentrations were prepared from the stock solution. In each case, 100 μl of sample and fresh 100 μl DMSO were mixed. As a positive control, Vincristine sulfate was used. After that, all these solutions were transferred to the pre-marked test tubes where there were ten living brine shrimp nauplii in simulated seawater (5 ml). After 24 h, the test tubes were visually inspected using a powerful magnifying glass to assess the number of viable shrimp nauplii. The per cent (%) mortality was calculated and analyzed statistically. By using probit analysis and linear regression in Microsoft Excel, experimental data were evaluated. The effectiveness of the concentration-mortality relationships of compounds was expressed as (LC_{50}) values (Martinez et al., 1999, Jaki et al., 1999, Barahona and Sanchez-Fortun, 1999).

2.2.7. Evaluation of antioxidant activity

The antioxidant activity of the synthesized compounds was assessed in accordance with the method provided by Brand-Williams *et al*., based on the free radical scavenging capacity of the stable 1,1 diphenyl-2-picrylhydrazyl (DPPH) (Meyer et al., 1982, Prakash et al., 2007, Ardestani and Yazdanparast, 2007, Valco et al., 2007, Brand-Williams et al., 1995, Desmarchelier et al., 1997, Sharmin et al., 2018). This principle is most popularly utilized for the determination of the antioxidant capacity of any newly discovered drug. As a positive control, a potential antioxidant, *tert*-butyl-1-hydroxytoluene (BHT), was incorporated into the system. Different concentrations ranging from 1 to 500 μg/ml were produced by applying the serial dilution technique. The reaction mixture was kept in the dark place for about 25–30 min, and absorbance was measured at the wavelength of 517 nm using the UV-Spectrophotometer. The following general equation was utilized to measure the inhibition of free radical by test samples and DPPH:

$$(\boldsymbol{I}\%) = \left(\mathbf{1} - \boldsymbol{A_{sample}}/\boldsymbol{A_{blank}}\right) \times \mathbf{100}$$

where A_{blank} is the absorbance of the control reaction in methanol, *inhibition* concentration (IC_{50}) was calculated from the graph.

2.2.8. Evaluation of in-vitro lipid peroxidation inhibitory studies

In the Fenton reaction, Fe^{3+}-ascorbate-EDTA-H_2O_2 system produces $OH^{\cdot}$ Radical and the ferryl–perferryl complex initiates the lipid peroxidation in the system. During the oxidation of polyunsaturated fatty acids, Malondialdehyde (MDA), a low molecular weight end product, is formed. While MDA reacts with thiobarbituric acid (TBA), eventually, a pinkish-red chromogen developed (Raka et al., 2019). Estimation of the thiobarbituric acid-reactive substances (TBARS) provides an assessment of the degree of lipid peroxidation that occurred. The stock solution was serially diluted to prepare sample solutions having concentrations of 50, 100, 200, 400, 800 μg/ml, respectively. The adult rats (weight: 150 gm each) were anaesthetized using standard anaesthetics, for instance, sodium phenobarbitone. After the treatment, the rats were sacrificed, and the brains were dissected and homogenized using a homogenizer. A 5 ml of ice-cold phosphate buffer (50 mM, pH 7.4) was used to produce a 1/10 homogenate. The obtained homogenate was centrifuged to collect the supernatant at 10,000 rpm for 20 min at 4 °C since the supernatant was used as the liposome in this experiment.

2.2.9. Evaluation of in-vitro anti-acetylcholinesterase and anti-butyrylcholinesterase activity

In this present study, the anti-acetylcholinesterase (AchE) and antibutylchloinesterase assay were performed, and the colourimetric method of Ellman et al. (1961). was used, where acetylthiocholine iodide acted as a substrate. The stock solution was serially diluted to prepare a sample solution having concentrations of 50, 100, 200, 400, 800 μg/ml, respectively. The rate of hydrolysis by acetylcholinesterase and butyrylcholinesterase was monitored spectrophotometrically. After a gentle mixer of the standard solution (500 μl) with the enzyme solution (500 μl), the mixed solution was subjected to incubation for 15 min at 37 °C. About 3.5 ml of Ellman's reaction mixture further was mixed with sodium phosphate buffer (50 mM, pH 8.0), and absorbance was measured immediately at 405 nm. To confirm whether the reaction occurred linearly or not, reading was taken for 10 min at 2 min intervals. At the same time, a blank sample was also measured by substituting the enzyme with saline solution. The following formula was used to calculate the percentage of enzyme inhibition (McLughilin and Rogers, 1998):

$$\%\ inhibition = 100 - (A_t/A_c \times 100)$$

where A_t is the absorbance of the tested sample and A_c is the absorbance of standard control.

2.3. In-silico studies:

2.3.1. In-silico consensus molecular docking

The crystal structure of the targeted protein was retrieved from PDB (Protein Data Bank), AChE (code: 4pqe) and BChE(code: 6esy) in PDB format. All the heteroatoms and water crystals (>5 Armstrong) were removed from the protein structure to avoid possible interference in ligand binding. Protein preparation wizard of Schrodinger suites used to preprocess the missing atoms/residues, polar hydrogens addition and charges (Gasteiger) to the proteins downloaded in the workspace. 3D binding pockets were analyzed by Molsoft ICM-Pro software and further validated through AutoDock tools. Molecular docking studies were carried out using the software AutoDock vina, Chimera and Achilles docking server (Rahman et al., 2020).

2.3.2. In-silico ADMET analysis

To predict the *in vivo* pharmacokinetic properties of the synthesized compounds, *in silico* ADME/T studies were performed (Rahman et al., 2018). Importance was given to screening the

physicochemical properties such as MW (molecular weight), TPSA (Topological polar surface area), HBD & HBA (hydrogen bond donor and acceptor), lipophilicity, water-solubility, pharmacokinetics, drug-likeness and so on. *In silico* toxicity predictors such as admetSAR and SwissADME servers were utilized to meticulously predict the possible AMES toxicity, oral acute toxicity, and carcinogenicity.

2.4. Statistical analysis

The resultant data were subjected to statistical analysis using Anova test to find out whether the result is significant or not. Standard Error Mean (SEM) was also calculated and represented in the data Table 1–3.

3. Results

3.1. Chemical

Several substituted benzimidazole derivatives were synthesized from *O*-phenyldiamine as shown in Scheme 1. The reaction between *O*-phenylenediamine (Compound **1)** and *P*-chloro or *P*-hydroxy benzaldehyde or *P*-anisaldehyde (Compound **2)** in the presence of $SiO_2/ZnCl_2$ or iodine/water furnished corresponding disubstituted benzimidazoles **3a-3c** in good yields. Compound **3c** was synthesized using SiO_2, $ZnCl_2$ catalyst in a solvent free condition. Although both the synthetic methods can be applied for the synthesis of all disubstituted derivatives, we observed higher yields of **3a** aand **3b** using iodine water. In another instance, when the reaction was conducted with boric acid, monosubstituted derivativere **4** was obtained exclusively. As we were interested with disubstituted derivatives, we did not synthesize the other monosubstituted derivatives in this series. However, we also synthesized benzimidazole benzyl ether (compound **6**) by condensing *O*-phenyldiamine with glycolic acid followed by benzylation. Reaction of compound **1** with glycolic acid under reflux condition, provided 2-methyl hydroxy-1H-benzimidazole (Compound **5**), which was finally treated with benzyl chloride in the presence of sodium hydride in tetrahydrofuran to deliver 2-((benzyloxy)-methyl)-1H-benzimidazole (Compound **6**) in good yield **(**Scheme 1**)**.

All synthesized compounds were purified by column chromatography and the purity of all fractions were ascertained by using TLC. Finally, the purity of the molecule was again confirmed by 1H NMR spectroscopy. These purified samples were used for biological investigation.

Table 2
Comparison of % of inhibition of writhing by control, standard, and synthesized compound.

Sample code	Dose (mg/kg)	Number of Writhing (Mean ± SEM)	% of Inhibition of Writhing
Control	0	26.8 ± 2.131	–
Standard	0.1 ml/10 g	2.2 ± 0.583***	91.79
3a	25	13.6 ± 0.430***	49.25
	50	19.0 ± 1.649***	29.10
3b	25	19.6 ± 0.797***	26.87
	50	23.2 ± 0.464	13.43
3c	25	12.3 ± 0.831***	54.10
	50	15.4 ± 0.697***	42.54
4	25	26.0 ± 1.001	2.99
	50	23.1 ± 0.843	13.81
6	25	16.7 ± 0.889***	37.69
	50	11.9 ± 0.458***	55.60

Note: Each value represents the Mean ± SEM, (n = 5). ***P < 0.001, **P < 0.01 compared with
control (one-way ANOVA followed by Dunnett's test)

3.2. Biological

3.2.1. Evaluation of central anti-nociception activity

The central anti-nociceptive activity of the synthesized compounds was evaluated by the radiant heat tail-flick method, and the results are summarized in Table 1. The synthesized compounds elongated the reaction time in a dose-dependent manner. After 30 min of oral administration, **3b** showed promising analgesic activity having 90.70% (P < 0.001) elongation time at 25 mg/kg and 69.63% (P < 0.001) at 50 mg/kg dose. After 60 min of oral administration, compound **6** showed moderate anti-nociception activity having 45% (P < 0.05) elongation at 25 mg/kg and promising anti-nociception activity (87.5% elongation; P < 0.001) at 50 mg/kg dose compared to standard morphine (82.5% elongation). Compound **3a** also exhibited remarkable activity after 60 min of oral administration (79% elongation; P < 0.001). After 90 min of oral administration, the central anti-nociception activity of **3b** and **3c** at 50 mg/kg was 46.15% (P < 0.01) and 44.23% (P < 0.01) respectively which was compareable to that of standard morphine (48.08%).

3.2.2. Evaluation of peripheral anti-nociception activity

Acetic acid-induced writhing, a commonly used method, was utilized to determine the peripheral analgesic activity of the synthesized compounds. Among the compounds, compound **6**, **3c** and **3a** showed significant analgesic activity (P < 0.001) at 25 mg/kg and 50 mg/kg dose level with writhing inhibition values

Table 1
Comparison of the central anti-nociceptive activity of synthesized compounds and standard morphine.

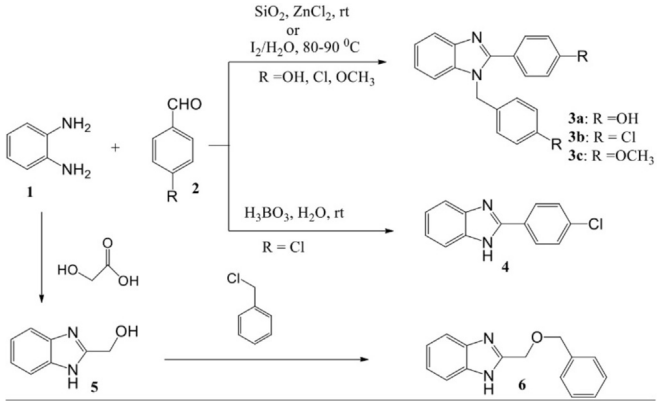
Group	Reaction time				% Elongation value			
	0 min	30 min.	60 min.	90 min.	0 min	30 min.	60 min.	90 min.
Control	7 ± 0.55	8.6 ± 0.93	8 ± 0.55	10.4 ± 0.51	–	–	–	–
Standard	11 ± 0.9*	22.6 ± 1.03***	14.6 ± 0.93***	15.4 ± 0.81***	57.14	162.79	82.5	48.08
3a (25)	12.4 ± 0.93***	13.2 ± 0.74**	14.4 ± 0.93***	13 ± 0.84*	77.14	52.56	79	25.10
3a (50)	11.6 ± 0.93**	9.8 ± 1.03	13.2 ± 0.86***	13.6 ± 0.68*	65.71	33.95	65	31.54
3b (25)	10 ± 0.71	16.4 ± 0.93***	10 ± 0.71	12.2 ± 0.66	42.86	90.70	25	17.31
.3b (50)	14.6 ± 0.93***	15 ± 0.55***	10.8 ± 0.97	15.2 ± 0.86**	108.57	69.63	35	46.15
3c (25)	9.8 ± 0.86	10.2 ± 0.86	10.6 ± 0.68	11.8 ± 0.66	40.00	18.60	32.5	13.46
3c (50)	10 ± 0.707	12.4 ± 0.93*	12 ± 0.71**	15 ± 0.71**	42.86	44.19	50	44.23
4 (25)	9.4 ± 0.93	8.8 ± 1.24	10.6 ± 0.68	12.6 ± 0.93	34.29	2.33	32.5	21.15
4 (50)	9.6 ± 0.93	9.4 ± 0.68	11.2 ± 0.86*	14.4 ± 0.93*	37.14	7.6	40	38.46
6 (25)	10 ± 0.71	14 ± 0.71**	11.6 ± 0.81*	11.6 ± 0.87	42.86	62.79	45	11.54
6 (50)	19.8 ± 0.86***	12 ± 0.89	15 ± 0.71***	13.8 ± 0.58*	182.86	39.53	87.5	32.69

Note: Each value represents the Mean ± SEM, (n = 5). ***P < 0.001, **P < 0.01, *P < 0.05 compared with control (one-way ANOVA follwed by Dunnett's test). single dose (25 mg/kg), double dose (50 mg/kg).

Table 3
Mean paw edema and percent inhibition of paw edema at different time intervals.

Group	Mean paw edema (volume in ml) ± SEM				% Paw edema inhibition			
	1st hour	2nd hour	3rd hour	4th hour	1st hour	2nd hour	3rd hour	4th hour
Control	1.18 ± 0.556	1.22 ± 0.068	1.31 ± 0.069	1.41 ± 0.075	–	–	–	–
Standard	0.902 ± 0.059**	0.84 ± 0.065***	0.80 ± 0.067***	0.77 ± 0.044***	23.73	31.15	38.93	45.39
3a	0.768 ± 0.0575***	0.78 ± 0.023***	0.80 ± 0.043***	0.88 ± 0.034***	34.75	36.07	38.93	37.59
3b	0.724 ± 0.057***	0.73 ± 0.029***	0.76 ± 0.048***	0.78 ± 0.056***	38.98	40.16	41.98	44.68
3c	0.758 ± 0.035***	0.79 ± 0.043***	0.82 ± 0.022***	0.88 ± 0.046***	35.59	35.25	37.40	37.59
4	0.838 ± 0.051**	0.88 ± 0.027***	0.92 ± 0.017***	0.89 ± 0.033***	28.81	27.87	29.77	36.88
6	0.906 ± 0.047**	0.93 ± 0.028***	0.98 ± 0.018***	0.96 ± 0.039***	22.88	23.77	25.19	31.91

Each value represents the Mean ± SEM, (n = 5). ***P < 0.001, **P < 0.01, *P < 0.01 compared with control (one-way ANOVA follwed by Dunnett's test)



Scheme 1. .

Table 4
Diameter of zone of inhibition of standard and test compounds.

Test organisms	Diameter of zone of inhibition (mm)		
	Standard	4	6
Gram (+) Positive Bacteria			
Bacillus cereus	48	7	8
Bacillus megaterium	50	9	7
Bacillus subtilis	42	9	10
Sarcina lutea	44	7	7
Staphylococcus aureus	50	0	7
Gram (-) Negative Bacteria			
Escherichia coli	43	10	0
Pseudomonas aeruginosa	43	8	7
Salmonella paratyphi	43	0	8
Salmonella typhi	45	9	8
Shigella boydii	44	9	7
Shigella dysenteriae	48	8	0
Vibrio mimicus	47	0	9
Vibrio parahaemolyticus	45	7	10
Saccharomyces cerevisiae	45	9	7
Fungi			
Candida albicans	49	0	0
Aspergillus niger	48	10	0

of 37.69% and 55.60%, 54.10% and 42.54%, 49.25% and 29.10% respectively (Table 2) compared to 91.79% writing inhibition for the standard Diclofenac. Compound **3b** showed moderate analgesic activity at 25 mg/kg dose level with a value of 26.87% of inhibition (P < 0.001). Compound **4** did not show any peripheral analgesic activity.

3.2.3. Evaluation of anti-inflammatory activity

The per cent (%) paw oedema inhibition of standard Diclofenac sodium was 23.73%, 31.15%, 38.93%, 45.39% in 1st, 2nd, 3rd, and 4th hour respectively (Table 3). From the statistical evaluation, it is evident that the **3b** and **3a** at 100 mg/kg dose showed significant anti-inflammatory effects from the first hour and onward. The % paw edema inhibition of **3b** was 38.98%, 40.16%, 41.98%, 44.68% and that of **3a** was 34.75%, 36.07%, 38.93%, 37.59% in 1st, 2nd, 3rd and 4th hour respectively. On the other hand, **3c** at 100 mg/kg dose showed moderate anti-inflammatory effect from the first hour and onward (Table 3). Compound **4** and **6** exhibited mild anti-inflammatory actions.

3.2.4. Evaluation of antimicrobial activi ty

The possible antimicrobial activity of the synthesized benzimidazole derivatives was evaluated by the disc diffusion method. Among them, mild antimicrobial activity was found with the zone of inhibition ranging from 7 to 10 mm in the case of compounds **4** and **6**, while the zone of inhibition of standard Ciprofloxacin was 40–50 mm (Table 4). Both compounds showed mild activity against the gram negative bacteria and gram positive bacterial strains. Compounds **3a**, **3b** and **3c** did not show any antimicrobial activity against the microbial strains.

3.2.5. Evaluation of brine shrimp lethality bioassay

The synthesized chemical compounds were tested for cytotoxicity using Brine Shrimp Lethality Bioassay and the results are presented in Fig. 1. Compounds **6**, **3c**, and **3b** showed moderate cytotoxic activities with the LC_{50} values of 16.68 μg/ml, 35.99 μg/ml and 21.9 μg/ml, respectively compared to the standard vincristine sulfate having the LC_{50} value of 1.283 μg/ ml. On the other hand, compounds **3a** and **4** are less cytotoxic having LC_{50} values of 116.17 and 148.48 μg/ml, respectively.

3.2.6. Evaluation of antioxidant activity

Antioxidant activity was evaluated by DPPH scavenging assay method and the results are depicted in Fig. 2. Among the synthesized compounds, **3a** showed very strong antioxidant property

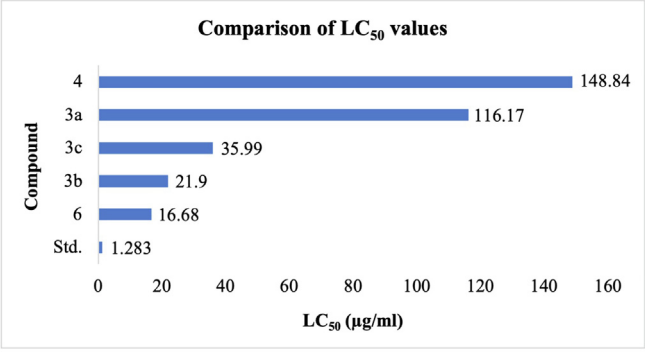


Fig. 1. Comparison of LC_{50} values between standard and synthesized compounds.

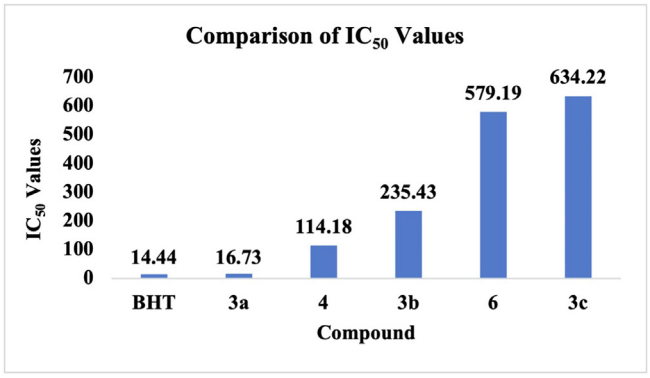


Fig. 2. Comparison of antioxidant activities (IC_{50} Values) of Standard and Synthesized Compounds.

(IC_{50} = 16.73 μg/ml) similar to that of standard BHT (14.44 μg/ml). Compounds, **4** and **3b** displayed moderate antioxidant activity having IC_{50} values of 114.18 and 253.43 μg/ml, respectively. On the other hand, Compounds **6** and **3c** exhibited very low antioxidant profiles (IC_{50} values of 579.19 and 634.22 μg/ml respectively).

3.2.7. Evaluation of in-vitro lipid peroxidation inhibitory studies

All the synthesized benzimidazole derivatives were subjected to observe the reactivity against non-enzymatic lipid peroxidation using rat brain homogenate. Lipid peroxidation level increased with the addition of Fe^{2+}-ascorbate in the prepared brain homogenate. The inhibitory activity of the synthesized compounds was found to increase with increasing concentration (Fig. 3). The highest activity was obtained at the dose of 800 μg/ml. The IC_{50} values of compounds **3a** and **4** were found to be 75.53 μg/ml and 98.55 μg/ml, respectively, whereas the value for standard (Catechin) was 59.36 μg/ml (Fig. 3). Compound **3a** showed moderate inhibitory and compound **4** showed mild inhibitory activity in lipid peroxidation assay.

3.2.8. Evaluation of In-vitro Anti-acetylcholinesterase activity

The results of the anti-acetylcholinesterase activity is summarized in Fig. 4**.** The inhibitory activity of synthesized compounds increased with increasing the concentration, and the highest activity was found at 800 μg/ml concentration. Compound **6** showed moderate inhibitory activity against the enzyme acetylcholinesterase (IC_{50} = 29.64 μg/ml), whereas **3b** and **3a** exhibited mild inhibitory activity (IC_{50} = 66.03 μg/ml and 73.15 μg/ml respectively). The IC_{50} value of standard drug donepezil was 9.54 μg/ml.

3.2.9. Evaluation of in-vitro anti-butyrylcholinesterase activity

Among the synthesized compounds, **3b** and **3a** showed moderate inhibitory activity against butyrylcholinesterase compared to donepezil. The IC_{50} value of donepezil (standard) was 9.54 μg/ml.

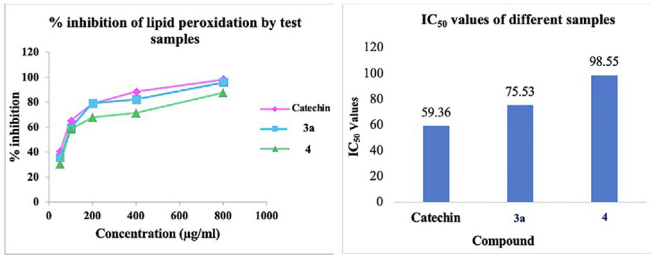


Fig. 3. Lipid peroxidation of Catechin (standard) and synthesized compounds at different concentrations.

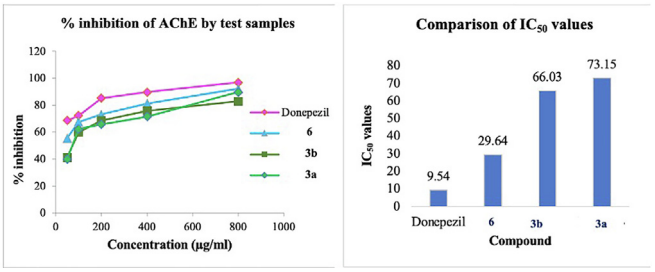


Fig. 4. Comparison of the anti-acetylcholinesterase activity of Donepezil (standard) and test samples.

The IC_{50} values of **3b** and **3a** were 23.42 μg/ml and 15.53 μg/ml, respectively (Fig. 5).

3.3. In-silico studies:

3.3.1. Evaluation of In-silico anti-acetylcholinesterase & anti-butyrylcholinesterase activity

Molecular docking studies of the synthesized compounds was performed against acetylcholinesterase (AchE) and butylcholinesterase (BChE) enzymes. Based on the consensus docking scores, compounds **3b**, **3a** & **4** showed the higher affinity to bind in the active pocket of AChE, and compounds **4**, **3a** & **3c** showed greater affinity for BChE. The compound **3b** interacted with the AChE through three hydrophobic interactions (alkyl, Pi-Alkyl, & Pi-Cation), whereas according to the binding score, **3a** was found to elicit strong interaction through hydrogen and hydrophobic bonds (Table 5 and Figs. 6 and 7). In the case of BChE, **4** and **3a** showed promising interactions.

3.3.2. Comprehensive ADMET analysis of the synthesized compounds

In- vitro ADMET/T studies of the synthesized compounds were accomplished and the scores based on several parameters are tabulated in Table 6. Considering the various parameters such as molecular weight, hydrogen bond acceptor & donor, topological surface area, lipophilicity, BBB penetration property, violation of Lipinski rule, drug-likeness and synthetic accessibility, **3a** and **4** outweighed the other synthesized molecules (Table 6**)**.

4. Discussion

A number of substituted benzimidazole derivatives were synthesized using environment-friendly aqueous medium. Since the catalysts were non-toxic, inexpensive, recyclable and ionic liquids; the synthesis scheme can be considered as green and economical. Chemoselectivity of the reactions is very high. Therefre, our synthetic protocol is simple, inexpensive and avoids use of hazardous organic solvnts. All synthesized compounds were purified by column chromatography and the purity of all fractions were ascertained by using TLC. Finally, the purity of the molecule was again

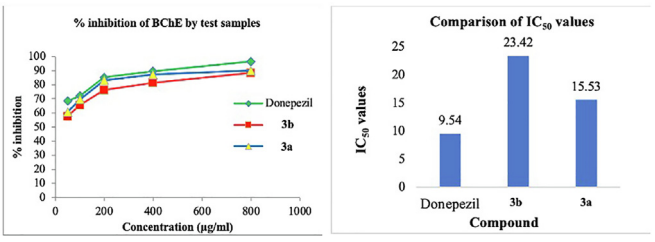


Fig. 5. Comparison of the anti-butyrylcholinesterase activity of Donepezil (standard) and test samples.

Table 5
Consensus docking score and intermolecular interactions.

Target	Product Code	Consensus Docking Scores (Kcal/mol)	Hydrogen bond	Hydrophobic interactions				Van Der Waals interaction
				Alkyl	Pi-Alkyl	Pi-Cation	Pi-Pi Stacked	
AChE	**3a**	– **8.37**	GLU A:181	–	PRO A:51	ARG A:9	–	–
	3b	– **8.50**	–	LEU A:4, ILE A:16	LEU A:4, ILE A:16	ARG A:14	–	–
	3c	– **7.67**	–	–	–	ARG A:14	–	–
	4	– **8.07**	–	LEU A:44, ALA A:163, VAL A:260	LEU A:44, ALA A:163, VAL A:260	ASP A:256	–	–
	6	– **6.90**	–	–	–	ARG A:14	–	VAL A:55
BChE	**3a**	– **8.63**	–	–	LEU A:123	–	HIS A:75	–
	3b	– **8.23**	–	–	LEU A:123	–	HIS A:75	–
	3c	– **8.60**	LEU A:123	–	–	–	LEU A:123	HIS A:124
	4	– **9.20**	LEU A:123	LEU A:127	–	–	LEU A:123	LEU A:123, HIS A: 124, ASN A:94
	6	– **7.93**	GLU A:78	–	LEU A:123	–	HIS A:75	–

Note: Glu = Glutamic acid, LEU = Leucine, ILE = Isoleucine, VAL = Valine, ARG = Arginine, ASP = Aspartic acid, HIS = Histidine, A = Chain A.

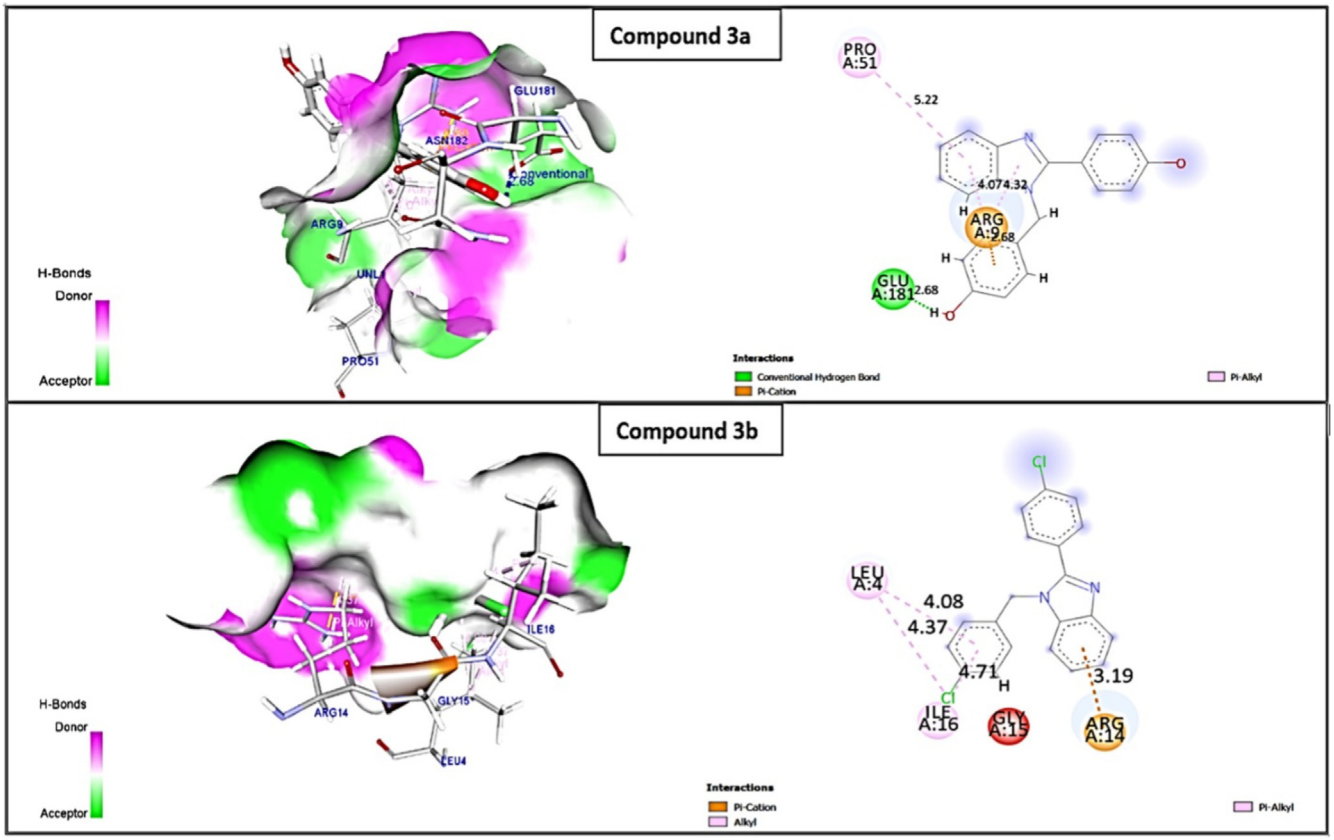


Fig. 6. Docking pose and molecular interaction of the synthesized compounds with AChE.

confirmed by 1H NMR spectroscopy. These purified samples were used for several biological investigation. Though the compounds are already available in the literature (Gao et al., 2017; Jian et al., 2006), we have synthesized the compounds in better yields by a new simple, inexpensive methods without utilizing hazardous organic solvents.

The scope of this work is more focused on the biological investigation and *in silico* studies as we have conducted a total of nine biological tests along with some *in silico* studies. In evaluation of central analgesic effect, disubstituted benzimidazole derivatives such as **3a**, **3b** and **3c** showed prominent activity compared to the monosubstituted analogues. Compound **3b** and **3c** reflected a promising central analgesic effect (46.15% and 44.23% at 50 mg/kg respectively) compared to standard morphine (48.08%). Compound **3a** also exhibited significant peripheral analgesic activity ($P < 0.001$) after 60 min. The data from peripheral analgesic study also reveals that the disubstituted benzimidazoles and the ether derivative compound **6** showed superior analgesic property. Therefore, the results of the peripheral analgesic activity correlate with that of the central analgesic activity indicating that the observed activity is closely related to the structural features of the synthesized derivatives. Acetic acid induced analgesia occurs due to

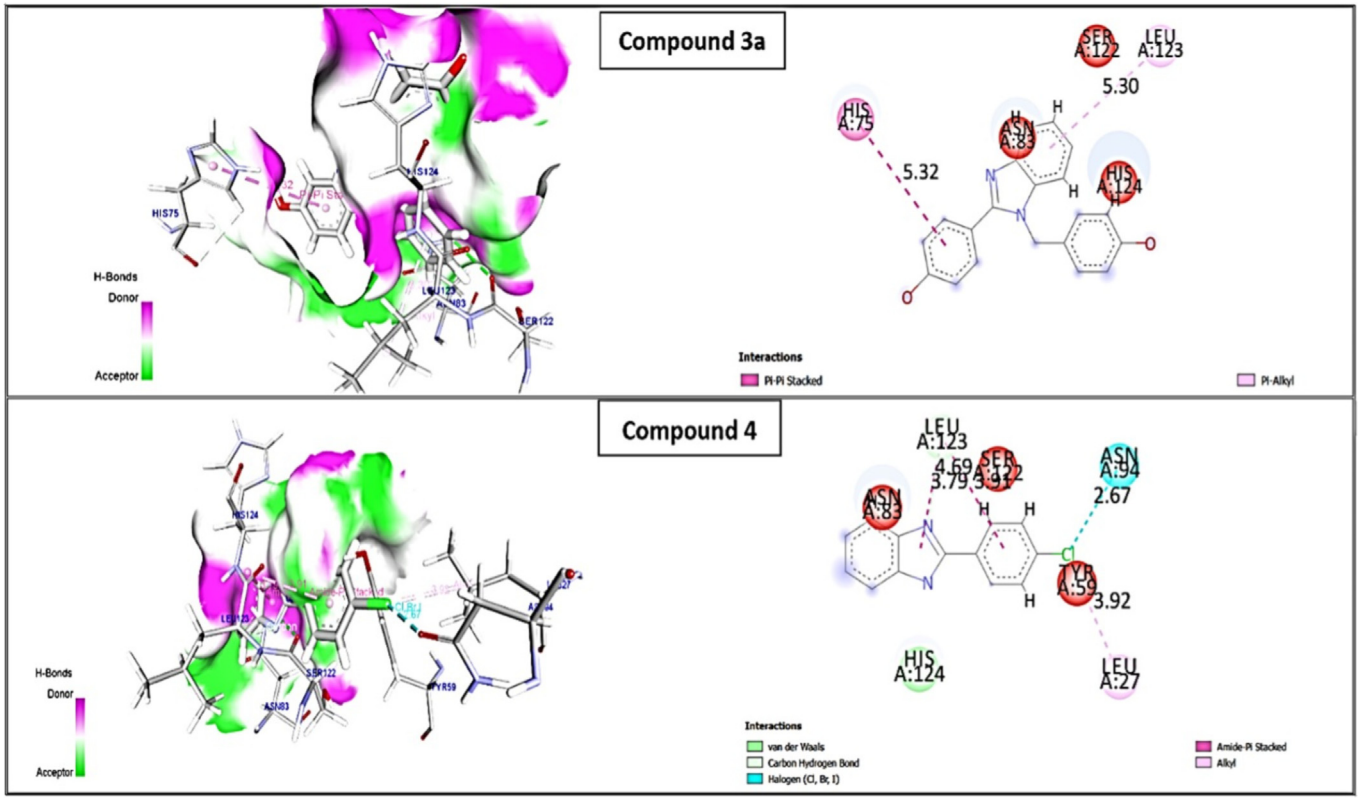


Fig. 7. Docking pose and molecular interaction of synthesized compounds with BChE.

Table 6
In silico ADME/T studies of the synthesized compounds.

Parameters	3a	3b	3c	4	6
MW (⟨450)	316.35	353.24	344.41	228.68	238.28
#Rotatable bonds (<5)	3	3	5	1	4
#H-bond acceptors (<7)	3	1	3	1	2
#H-bond donors (<3)	2	0	0	1	1
TPSA (<90)	58.28	17.82	36.28	28.68	37.91
WLOGP	4.16	6.06	4.77	3.88	2.98
MLOGP (1.5–2.7)	3.1	5.28	3.54	3.34	2.18
Silicos-IT class	Poorly soluble	Poorly soluble	Poorly soluble	Poorly soluble	Moderately soluble
GI absorption	High	High	High	High	High
BBB permeant	Yes	No	Yesss	Yes	Yes
Pgp substrate	Yes	Yes	Yes	Yes	Yes
log Kp (cm/s)	−5.4	−4.23	−5.11	−4.95	−5.79
Lipinski #violations	0	1	0	0	0
Bioavailability Score	0.55	0.55	0.55	0.55	0.55
PAINS #alerts	0	0	0	0	0
Leadlikeness #violations	1	2	1	2	1
Synthetic Accessibility	2.23	2.26	2.44	1.67	2.13
AMES Toxicity	AMES toxic	AMES toxic	AMES toxic	AMES toxic	Non-AMES toxic
Carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens
Acute Oral Toxicity	III	II	III	III	III
Human Intestinal Absorption	+	+	+	+	+
Caco-2 Permeability	+	+	+	+	+
Eye corrosion	–	–	–	–	–
Eye irritation	+	–	–	+	+
Ames mutagenesis	–	–	–	–	–

Note: Bioavailability score (range: 0–1, close to 1 better), Synthetic accessibility (range: 1–10, lowest value preferable), Acute oral toxicity (range: I-V, III = moderate toxicity), (+) = positive activity, (-) = no effect. (http://www.swissadme.ch/index.php).

liberation of several chemical mediators generated by cyclooxygenase pathway (Ribeiro et al., 2000). Therefore, the recorded analgesic activity might be due to the inhibition of cyclooxygenase pathway as perceived for standard analgesic drug diclofenac (Cashman, 1996).

In anti-inflammatory screening (Table 3), the disubstituted derivatives again showed superior anti-inflammatory activity compared to monosubstituted analogues. This might be due to the fact that analgesic and anti-inflammatory drugs work in the same mechanisms. Therefore, we may assume that the present activity

of the synthesized compounds might results from either interference of the biosynthesis of inflammatory mediators or direct interaction with the mediators (Spriha et al., 2021). In accordance to our previous results obtained with some monosubstituted benzimidazole derivatives (Poddar et al., 2016), the synthesized derivatives did not show notable antimicrobial properties. In cytotoxicity evaluation by BSL bioassay, varying degree of lethality was observed at different dose levels of the synthesized compounds. The degree of lethality was directly proportional to the concentration of the compound used. It was found that compound **6** and **3b** showed moderate cytotoxic activities (LC_{50} values:16.68 and 21.9 μg/ml respectively) compared to vincristine sulphate (LC_{50} value: 1.283 μg/ ml). At the present level of understanding, it is very difficult to predict the definite mechanism of cytotoxic activity; however, several target proteins responsible for cytotoxic activity might be involved (Ahmed et al., 2021).

Presence of the phenolic group has been found to augment the antioxidant property of several natural and synthesized compounds (Al-Mamary et al., 2021). In our study, the disubstituted benzimidazole **3a** containing the phenolic OH group possessed highest antioxidant property which is as strong as that of standard BHT (16.73 and 14.44 μg/ml. for **3a** and BHT respecticvely, Fig. 2*)*. This result is consistent with one of our previous studies, where we found potential antioxidant property of some synthesized benzimidazole derivatives (Brishty SR 2020). In lipid peroxidase inhibition assay, compound **3a** also showed highest lipid peroxidaae inhibition activity among the synthesized compounds which further elucidates its antioxidant profiles.

Among the synthesized compounds, both **3a** and **3b** exhibited mild to moderate AChE and BChE inhibitory activity (Figs. 4 and 5). Compound **6** also showed moderate AChE inhibitory activity. A great correlation was observed in *in silico* studies where compounds **3a** and **3b** showed most favourable binding interactions against the target enzymes. In comprehensive ADMET analysis (Table 6), most of the compounds showed favourable pharmacokinetic, drug likeliness and toxicity profiles; among which compound **3a** and **4** were the best suited compounds. Comparing the experimental data with the computational data (Table 6), compounds **3b** and **3a** were found the best possible candidate for AChE and BChE inhibition, respectively. Compounds **6** and **4** are backed due to the presence of unfavourable bumps in the binding loop.

Considering all the above discussions, it can be concluded that disubstituted benzimidazole derivatives especially compound **3a** and **3b** possessed most favourable pharmacological profiles in respect to several *in vitro*, *in vivo* and *in silico* computational studies.

5. Conclusion

A number of substituted benzimidazole derivatives were synthesized in very good yields using a simple, inexpensive and environment-friendly aqueous medium. Synthesized compounds were subjected to several *in vitro*, *in vivo* and *in silico* biological evaluations. Disubstituted benzimidazoles such as **3a**, **3b** and **3c** showed significant analgesic and anti-inflammatory activities. Compound **6** also possessed remarkable analgesic property. Compound **6** and **3b** exhibited moderate cytotoxic activities. Compound **3a** showed significant antioxidant activity in DPPH scavenging and lipid peroxidation assay. Compound **6**, **3a** and **3b** showed mild to moderate cholinesterase inhibitory effect which was further clarified by *in silico* studies. Considering the all experimental results and computational ADMET/T studies, it can be assumed that compound **3a** and **3b** might be the suitable candidates for further development as therapeutic agents.

6. Authors' Contributions

SCR conducted all the tests to conceive the project. FH guided and helped to carry out the synthesis and *in-vitro* studies. SCR & AR designed and performed the computational analysis. SMAR conceptualized the research work and supervised the entire project. SCR, AR and SMAR wrote the manuscript. All the authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.08.082.

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