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Invitro biological evaluation of 2- substituted benzimidazole derivatives

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ABSTRACTS
2-substituted benzimidazole derivatives having promising activities like anticancer, antibacterial, anti-inflammatory, antiparasitic, antihistamine, antiulcerative, antineoplastic, antifungal, antitubercular, anti HIV etc. In this view of these observation it was thought of interest to undertake the synthesis of 2- substituted Benzimidazole moiety and evaluate their biological activity. Forinvitroantibacterial activity of prepared compounds was studied against gram negative bacteria, Escherichia coliand gram positive bacteria, Staphylococcus species. Forinvitroanti-inflammatory activity by HRBC membrane stabilization method. ForInvitro antioxidant activity by DPPH method. Synthesized compound exhibited good activity compared with standards.
Keywords: HRBC methods, DPPH methods, Gentamicin, Diclofenac sodium, Ascorbic acid

I. INTRODUCTION

Benzimidazole derivatives are versatile nitrogen containing heterocyclic compounds which have long been known as a promising class of biologically active compounds possessing wide variety of biological and pharmacological activities like antibacterial, anti-inflammatory, anti-ulcer, anti-diabetic etc. Scientists have elucidated that Benzimidazole system possesses the variable sites like position 2 and 5 which can be suitably modified to yield potent therapeutic agents.The present review covers the chemistry and pharmacological activities of substituted benzimidazole. Keywords: Benzimidazole, anti-fungal, anti-histaminic, anti-convulsant, anti-inflammatory, analgesic, anti-viral, anti-oxidant, anti-cancer and anti-ulcerative. Inflammation is a bodily response to injury, infection or destruction characterized by heat, redness, pain, swelling and disturbed physiological functions. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. It is the body response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair. It is triggered by the release of chemical mediators from injured tissue and migrating cells. Inflammation is usually classified according to its time course as acute or chronic inflammation and there is also a subtype of chronic inflammation, known as granulomatous inflammation. Granulomas may be of three subtypes, infectious, non-infectious and foreign-body granulomas. The infectious group includes the typical caseating granulomas of tuberculosis and some deep fungal infections. The non-infectious group includes the diseases of unknown aetiology such as sarcoidosis.[1]The foreign-body group includes granulomas that form around exogenous indigestible material such as surgical suture material, talc, inorganic dust, parasitic ova and splinters of wood and endogenous material such as keratin and cholesterol crystals. Molecular etiology of inflammation are inflammation mediatory compounds such as prostaglandins (PGs), leukotrienes (LTs),histamine and bradykinin, platelet-activating factor (PAF) and interleukin-1, eicosanoids etc.2-4

II. MATERIALS AND METHODS:

Chemicals and reagents

The chemicals and reagents used in the present work were of AR and LR grade, procured from Spectrum, Chemphasol, and Nice.

Apparatus and Glasswares

Heating mantle, filter flask assembly, beakers, pipette, glass rod, conical flask, round bottom flask, measuring cylinder, Petri dishes etc.

Analytical and instrumental techniques

Physical Data

The melting point of the synthesized compounds were determined by Thiel's melting point apparatus (open capillary tube method) and all the compounds gave sharp melting points.

Thin Layer Chromatography

Purity of the compounds were ascertained by thin layer chromatography using silica gel as stationary phase and appropriate mixtures of the following solvents as mobile phase: Hexane, Chloroform,

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Ethyl acetate. The spots resolved were visualized using iodine chamber.

NMR Spectra:

The NMR Spectra was recorded by NMR 400 MHZ Spectrometer Brucker.

Mass Spectra :

The Mass Spectra was recorded by Jeol G.C Mass spectrometer technique electron ionosation IIT Chennai.

Infrared Spectra

The IR spectra of the synthesized compounds were recorded on IR affinity-1 FTIR spectrophotometer Shimadzu in the range of 400-4000.

HRBC membrane stabilization method

The HRBC membrane stabilization has been used as a method to study the in-vitro anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce a various disorders. The extra cellular activity of these enzymes are said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. Principle behind the membrane stabilization iswhen hypotonic solution was added to HRBC suspension the lysis of RBCs occurs.[6]

Percentage protection = 100 – (OD sample / OD control) X 10

Protein denaturation method

Denaturation of tissue proteins is one of the well-documented causes of inflammatory disorders. Protein Denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteinslose their biological function when denatured. Denaturation of proteins is a well-documented cause of inflammation Production of auto antigens in certain inflammatory conditions may be due to denaturation of proteins. Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development.[7-8]

% inhibition =100*(1-A2/A1)

Antimicrobial

Antimicrobial agents may be categorized on the basis of their antibacterial activity as both bacteriostatic and bactericidal. Bacteriostatic drugs suppress the growth of microorganisms without actually killing existing microbes. The invading microorganisms are removed by the host defence mechanism. Bactericidal drugs are capable of directly destroying organisms, especially those in an active state of replication. In sub therapeutic doses, bactericidal drugs are merely bacteriostatic, and conversely, at very high doses some bacteriostatic drugs may exert a bactericidal action. Nevertheless, even the most potent bactericidal drugs is usually incapable of totally eliminating an infection without intervention of the patients own natural defence mechanisms, such as antibody production, phagocytosis, and leucocytes proliferation.[9-10]

Antioxidant activity

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause. Free radical damage may lead to cancer.[11-12]

DPPH assay (2, 2-diphenyl -1-picrylhydrazyl)

1,1-diphenyl-2-picryl hydrazyl is a stable free radical with red colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

DPPH + [H-A] = DPPH-H + (A)

Biological screening:

Antibacterial studies[13-14]

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Procedure

Sample preparation

1 mg of the isolated compound was dissolved in 10 ml of DMSO to prepare 100µg/ml solution. 0.25ml, 0.5ml and 0.1ml of the stock solution was used to prepare 25µg, 50µg and 100µg of the test disc respectively

Standard : - Gentamycin 10µg disc

Test organisms

The activity of compound PI was studied against two gram negative bacteria, Escherichia coli and one gram positive bacteria, Staphylococcus species. The pure clinical isolates were obtained from the Microbiology lab, Department of MLT, University College of Medical Education, Puthupally. All the clinical isolates were checked for purity and maintained on nutrient agar at 40 C in the refrigerator until required for use.

Preparation of bacterial culture media

Nutrient agar medium

The medium was prepared by dissolving 2.8 g of the commercially available Nutrient Agar Medium (HiMedia) in 100ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°Cfor 15 minutes.

Nutrient broth

FORMULA

Peptone -0.5g

Beef extract -0.5g

Sodium chloride -0.25g

Distilled water -50ml

Nutrient broth was prepared by dissolving the specified quantity of peptone, beef extract and sodium chloride in distilled water and boiled to dissolve the medium completely. The medium was sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

Sterilization of media and glass ware

The media used in the present study, nutrient agar and nutrient broth, were sterilized in conical flask of suitable capacity by autoclaving at 15 lbs pressure for about 20 minutes. Petri dishes, beakers, test tubes and pipettes were sterilized in hot air oven at 1600 C for an hour.

Preparation of subculture

One day prior to test, the microorganisms were inoculated into the sterilized nutrient broth tubes and incubated at 370 C for 24 hours.

Procedure for antimicrobial screening

1. Prepared microbial inoculums with required quantity or suspension of test organism in nutrient broth
2. Nutrient agar medium was prepared and sterilized
3. Microbial suspension were mixed aseptically with the nutrient agar medium in ratio 0.25:100 ml and mixed it
4. Poured 15ml of medium to cleaned sterilized Petri dish and kept it for solidification.
5. Filter paper disc was sterilized by exposing both sides under sterilized lamp
6. Sterilized paper disc were soaked in the solution for few minutes.
7. Standard disc and imbibed paper disc were placed in the previously prepared agar plates. Each disc was pressed down to ensure complete contact with agar surface and distributed evenly.
8. The agar plates were then incubated at 370 C
9. After 16 to 18 h of incubation the plates were examined.
10. Measured the diameter of zone of inhibition of isolated compound in

Millimeters and compared with that of the standard.

Anti-inflammatory activity[15]

Protein denaturation assay

Procedure

A solution of 0.2% w/v BSA was prepared in Tris HCl buffer saline (10mM Tris pH 8,150mM NaCl and distilled water)and PH was adjusted to 6.8 using glacial acetic acid.Stock solutions of 10000µg/ml of all extracts were prepared by using methanol as a solvent. From these stock solutions of 5 different concentrations of 100,200,300,400 and 500µg/ml were prepared by using methanol as a solvent.5 ml of 0.2% w/v BSA was added to all the above tubes. The control consists of 5ml 0.2% w/v BSA solution with 50µl methanol. The standard consists of100µg/ml of prednisolone drug in methanol with 5ml 0.2% w/v BSA solution. The test tubes were heated at 72°C for 5 minutes and then cooled for 10 minutes. The absorbance of these solutions was determined using spectrophotometer at a wavelength of 660nm.The percentage of denaturation of protein was determined on a % basis relative to the control using the formula.

HRBC Membrane stabilization methods:-

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Preparation of Human Red Blood cells (HRBCs) suspension:-

The blood was collected from Kottayam Medical College Blood Bank and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 minutes and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal

Hypotonicity-induced Hemolysis

Different concentration of synthesized compound (100-500µg/ml), reference standard (25-500µg/ml) and control were separately mixed with 1ml of phosphate buffer, 2ml of hyposaline and 0.5ml of HRBC suspension. Diclofenac sodium (25- 500µg/ml) was used as standard drug. All the assay mixtures were incubated at 370C for 30 minutes and centrifuged at 3000rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by a spectrophotometer at 560nm (Kar et al., 2012). The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

Antioxidant activity DPPH method

Preparation of standard and test solution

3 to 15 µg/ml solution of ascorbic acid was used as standard. 1mg/ml solution of the crude extract in methanol was prepared and then serially diluted to obtain lower concentration (10-400µg/ml)

Reagents preparation

0.1mM solution of DPPH was prepared by dissolving 1.95mg of DPPH in methanol. After dissolving completely the solution was made up to 50 ml with methanol.

Procedure [52]

DPPH in methanol (0.1Mm) was prepared and 1.0 mlof this solution was added to 3.0 ml of extract solution in methanol at different concentration. Thirty minutes later, the absorbance was measured at 517nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity The capability to scavenge the DPPH radical was calculated using the following equation

DPPH Scavenged (%) = Absorbance of Control – Absorbance of test X 100 Absorbance of control

The antioxidant activity of extract was expressed in terms of IC50 the concentration required to inhibit DPPH radical formation by 50%), calculated from the inhibition curve.

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III. RESULTS AND DISCUSSION:

1. Antibacterial activity

Table no:1antibactial activity of synthesized compounds and E.coli

Sl.no	Concentration (µg/ml)	Zone of inhibition in mm					
		Staphylococcus aureus			E.coli		
		BZ3	BZ4	BZ5	BZ3	BZ4	BZ5
1	25	10	16	8	1	3	1
2	50	13	19	12	7	12	8
3	100	18	21	15	12	18	13
4	Gentamicin 10 µg/ml	21			32		

Figure no: diagram of 2 substituted benzimidazole derivatives antibacterial activity against Staphylococcus aureus

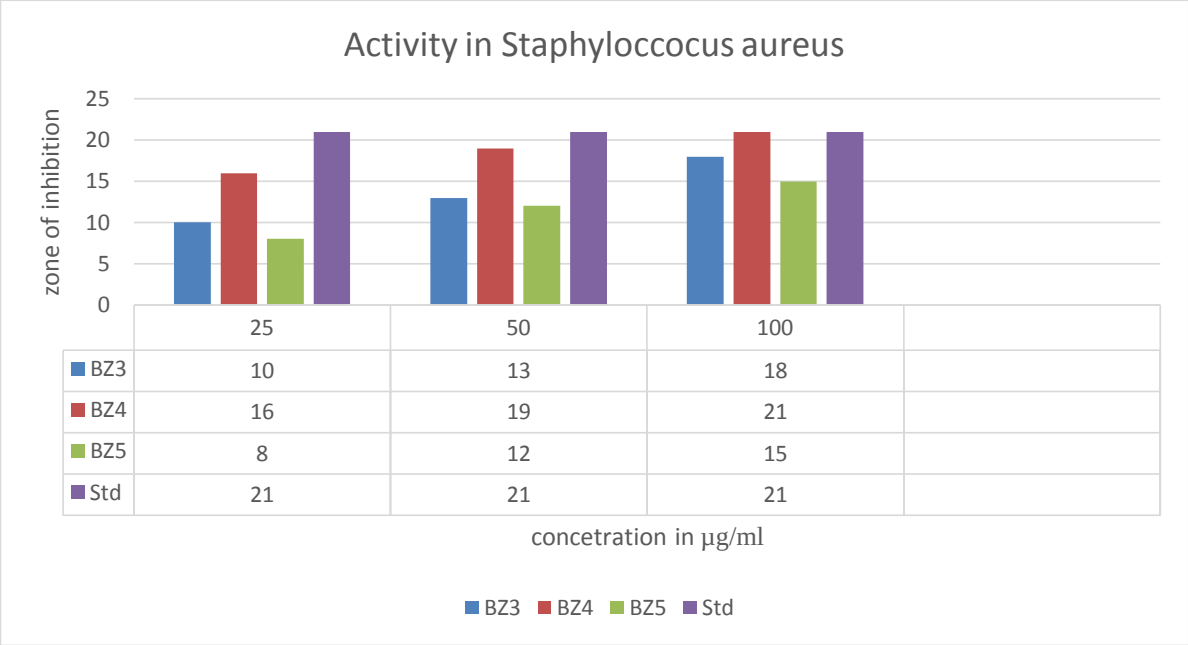

Figure 2: diagram of 2 substituted benzimidazole derivatives antibacterial activity against E.coli

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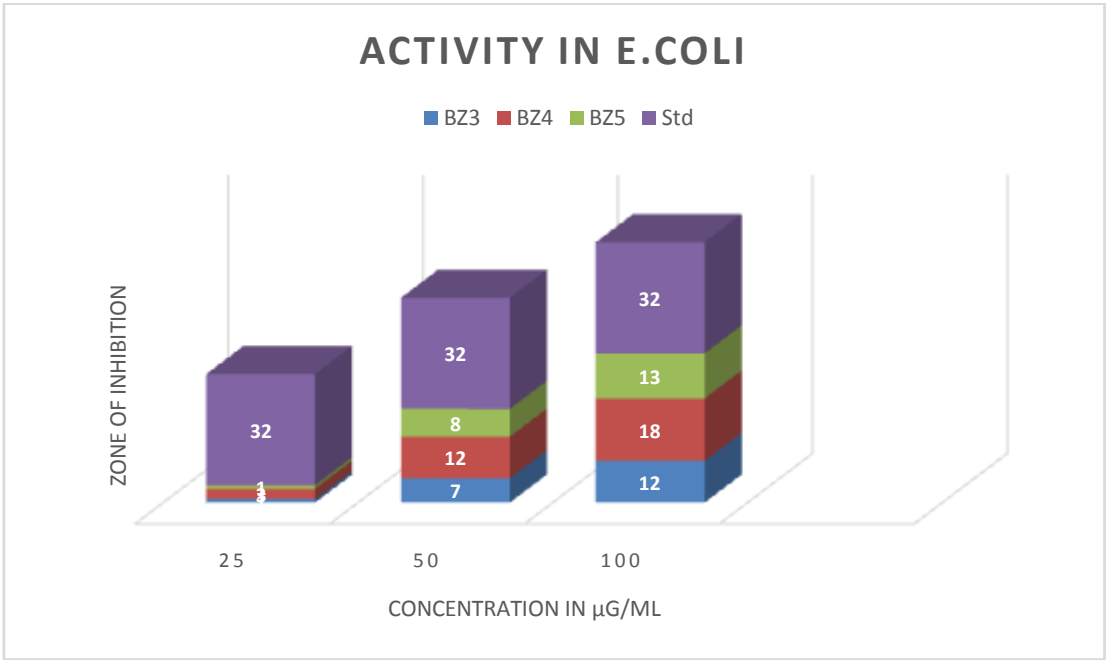

Figure no:3 Zone of inhibition of 2-substituted benzimidazole derivatives (a)E.coli (b) staphylococcus aureus

(a) **(b)**

Anti-inflammatory protein denaturation method

Absorption of control= 0.572

Table no: 2 anti -inflammatory study of 2- substituted benzimidazole derivatives

Sl no	concentration in µg/ml	Absorption		% Inhibition	
		BZ5	Standard	BZ5	Standard
1	100	0.356	0.511	37.76	38.66
2	200	0.320	0.371	44.05	46.13

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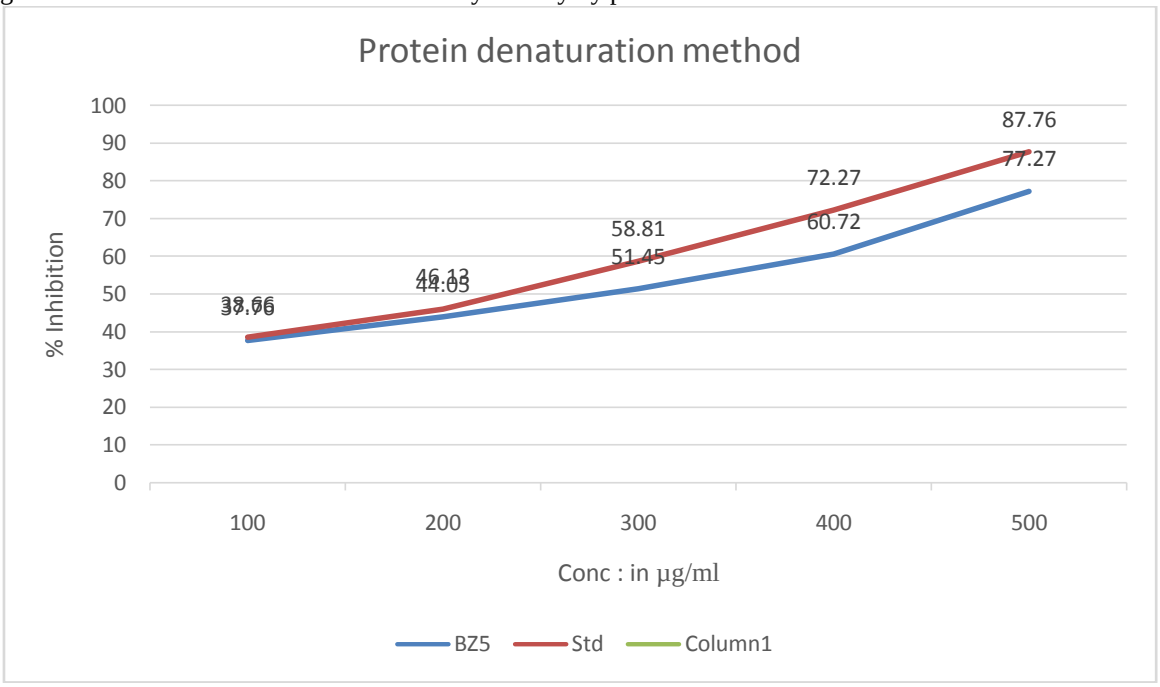
3	300	0.312	0.350	51.45	58.81
4	400	0.299	0.273	60.72	72.27
5	500	0.273	0.070	77.27	87.76

Figure no:4 % inhibition of anti- inflammatory activity by protein denaturation method



IC_{50}(drug= 280µg/ml and standard= 230µg/ml)

HRBC membrane stabilization method

The derivative was evaluated for the membrane stabilizing property. The derivatives was effective in inhibiting the hypotonicity induced hemolysis at different concentrations. Test compound (100-500µg/ml) inhibited the hypotonicity induced hemolysis of RBCs to varying degree as shown in table.

Table no: 3 Effect of the synthesized derivatives on membrane stabilization values represent in the results are mean±SD of five replicates.

SL NO	concentrationµg/ml	% inhibition					
		BZ1	BZ2	BZ3	BZ4	BZ5	Standard
	Control	0	0	0	0	0	0
	100	28.02	25.16	20.10	27.02	28.45	65.39
	200	40.23	32.76	29.59	36.26	29.23	70.65
	300	46.11	44.50	48.36	52.29	48.28	75.66
	400	58.66	54.33	55.26	51.21	56.75	80.70
	500	68.36	57.09	60.24	60.97	63.86	86.23

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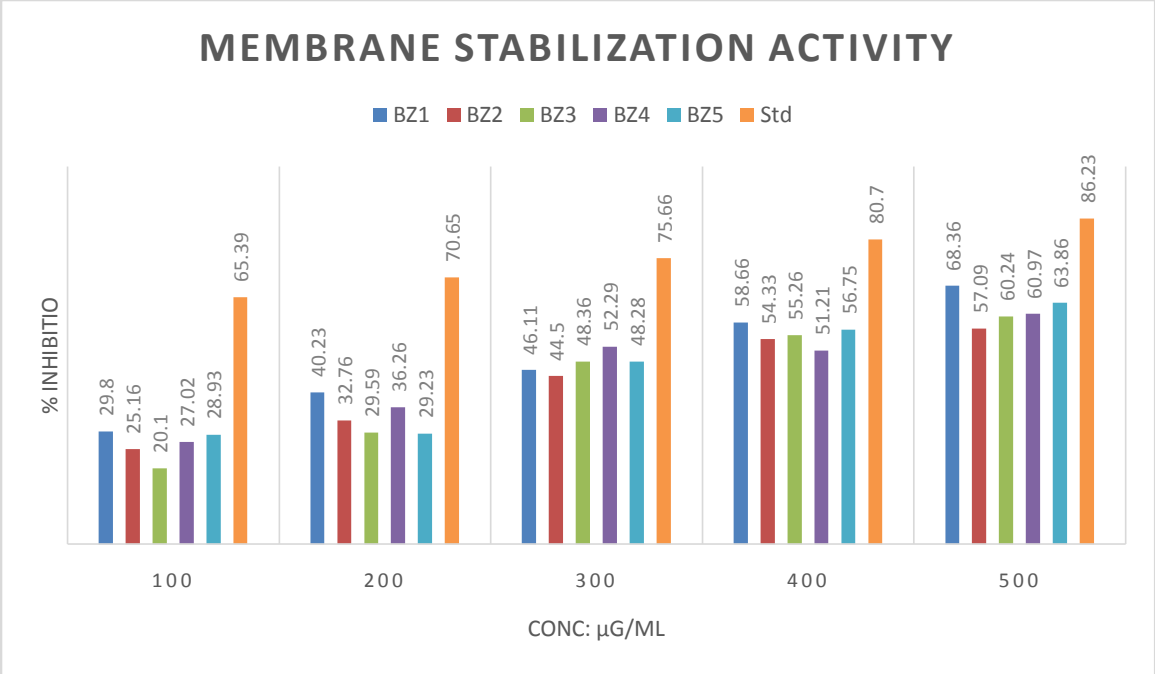
Figure no: 5 % inhibitionof synthesized compounds by HRBC method

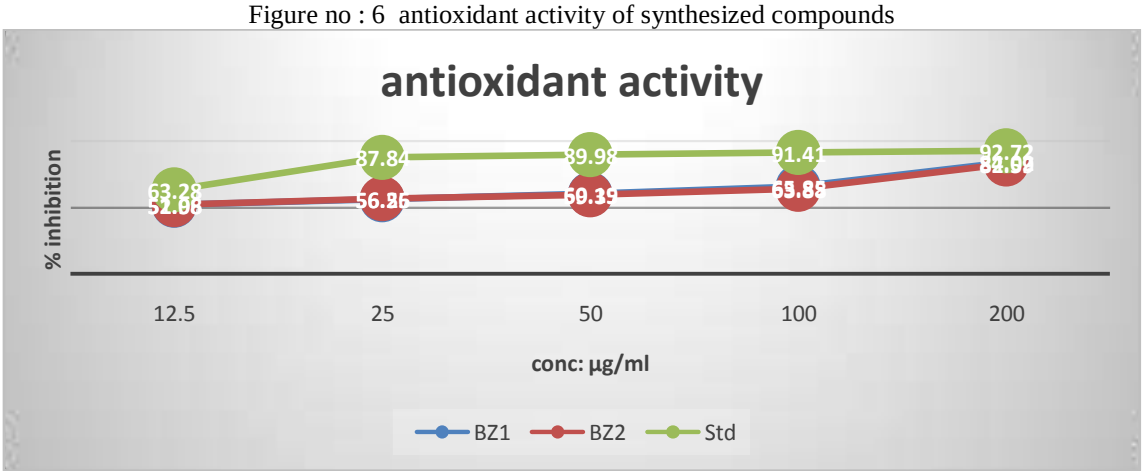


Antioxidant activity

Table no: 4 antioxidant activity of benzimidazole derivatives
Absorbance of controle= 0.839

Sl no	Concentration µg/ml	Absorbance at 517nm			% inhibition		
		BZ1	BZ2	Standard	BZ1	BZ2	Standard
1	12.5	0.403	0.402	0.308	51.96	52.08	63.28
2	25	0.367	0.398	0.102	56.25	56.56	87.84
3	50	0.334	0.341	0.084	60.19	59.35	89.98
4	100	0.320	0.303	0.072	65.85	63.88	91.41
5	200	0.134	0.146	0.061	84.02	82.59	92.72

Figure no : 6 antioxidant activity of synthesized compounds



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IV. DISCUSSION

1. Different 2- substituted benzimidazole analogues synthesized by conventional and microwave procedures. The purity of the synthesized molecules was ascertained routinely by TLC, and melting point determination.
2. The biological evaluations are antioxidant activity,Anti-inflammatory and Antibacterial activity.
3. Purity of the compound was done routinely by TLC and melting points. Ethyl acetate: Chloroform(2: 3) system was found to be ideal system for the development of compounds in TLC. The characterizations of the derivatives were carried out by various spectroscopic methods such as FTIR and NMR, UV and mass spectroscopy.
4. A substituents in the 2nd position of benzimidazole seem to enhance the biological activity. Synthesized compound BZ3, BZ4, BZ5 were screened for antibacterial activity, some of the tested compound exhibited good antibacterial activity against Staphylococcus aureus (gram +) than E-coli (gram-) when compared to standard Gentamycin.Among the 2 compound BZ1,BZ2 tested for the in vitro antioxidant activity using the DPPH method. The antioxidant activity with IC50 values of compound are 17.5µg/ml, 22.5µg/ml respectively. The IC50 value of standard 15.85 µg/ml. The result indicate that the antioxidant activity of the test compound showed good activity when compared with standard.
5. Synthesized compound were tested for anti-inflammatory activity using protein denaturation and HRBC membrane stabilization method.The result was confirmed by IC50 value. Better activity with the IC50 concentration in protein denaturation 280µg/ml for test compound,230µg/ml for standard compound, inHRBC method compound BZ4IC50 value 230 µg/ml,IC 50 of standard drug 210µg/ml. Anti-inflammatory activity of test compound good activity when compared with standard.

V. CONCLUSION

1. The present investigation was designed and extensive interest has been shown in 2- methyl benzimidazole containing compounds in search of potential drugs.
2. The objective of the present work was synthesis, characterization and biological activity studies of newly synthesized derivatives.
3. Five different benzimidazole analogues were synthesized by both conventional and microwave method.
4. The synthesized compounds were ascertained by consistency in the M.P
5. Purity of the compounds was checked by TLC.
6. The structure of the newly synthesized compounds were confirmed by IR, NMR, UV and Mass spectra.
7. Preliminary pharmacological screening of the synthesized analogues were performed activity likeantibacterial, anti-inflammatory and antioxidant activity. From this study it can be concluded that 2 – substituted benzimidazole derivatives act as biologically active compound.

Future scope of the study

1. All the synthesized compounds can be further explored for structural modifications to improve their activity so that they can be converted to prospective drugs.
2. In vivo toxicity studies
3. In vivo bioassay studies
4. Clinical trials
5. Since benzimidazole having various biological activities, these derivatives may be future leads for screening of other activities.

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