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Research Article

SYNTHESIS, CHARACTERIZATION, *IN-VITRO* ANTI-OXIDANT AND CYTOTOXIC ACTIVITY OF N-MANNICH BASES OF 5-AMINO-4-[2-(6-BROMO-1, 3-BENZO THIAZOL-2-YL) HYDRAZINYLIDENE]-2, 4-DIHYDRO-3H-PYRAZOL-3-ONE

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ABSTRACT

In the present work twelve N-Mannich bases of 5-amino-4-[2-(6-bromo-1, 3-benzothiazol-2-yl) hydrazinylidene]-2,4-dihydro-3H-pyrazol-3-one were synthesized. Their structures have been elucidated on the basis of spectral studies (IR, ^{1}H-NMR, and MASS). These compounds were screened for their *in-vitro* anti-oxidant activity by DPPH, ABTS and FRAP methods and *in-vitro* cytotoxic activity against mouse embryonic fibroblasts cell line (NIH 3T3) by MTT assay method. The compounds B12N, B14N, B1Br, B12C, B1PB, B14C and B1AP showed significant effects with EC_{50} values 22.45, 22.91, 23.58, 24.15, 25.12,25.13 and 31.75 µg/ml level in the DPPH method, EC50 values 23.70, 24.30, 22.83, 22.05, 77.76, 22.49 and 30.97 µg/ml in the ABTS method, compounds B1P, B1PB, B1M showed significant effects in the FRAP method. All the synthesized compounds was found to be significant growth inhibition in mouse embryonic fibroblasts cell line (cytotoxic activity) with IC_{50} values at the range of 26–10 µM level. The compound B1PB was found to be the active *in–vitro* antioxidant in all three methods, and thus represent a new class of promising lead compounds.

Keywords: Benzothaizole, Pyrazolone, Mannich bases, antioxidant and cytotoxic activity.

1. INTRODUCTION

Cancer is still worldwide problem because of its significantly high rates in morbidity and mortality. In cancer, the cell growth regulatory processes have gone away, divide uncontrollably, consuming energy and losing both structure and function because of an inability to adequately differentiate. To add insult to injury, rampant cell division is accompanied by disabled cell-death processes, leading first to cellular immortality and, eventually, to genetic instability[1]. The cancers may cause by chemical, environmental, viral and mutagenic etc, On the other hand many reasons the cancer is more difficult to cure than bacterial infections.[2] a) There is qualitative difference between human and bacterial cells. b) Immune mechanisms and other host defenses are very important in killing bacteria and other foreign cells. c) Cancer cells have overcome the body's surveillance system; chemotherapeutic agents must kill every clonogenic malignant cell, because even one can reestablish the tumor. This kind of kill is extremely difficult to effect because antineoplastic agents kill cell lines in first-order kinetic. In order to gain new insights into the complexity of the disease, robust screening methods for evaluating different natural or synthetic drugs have been carried out from the science community. In this respect, thiazole ring system and its derivatives (mainly benzothiazole) are core structure in synthetic compounds displaying broad spectrum of biological actives. The first

natural antibiotic (penicillin), third generation cephalosporin's and thiamine (Vitamine B_1) structure containing sulphur and nitrogen in five member ring system which is similar to thiazole and its derivatives. The structure simplicity and synthetic accessibility of 2-substituted benzothiazole series belie remarkable cytotoxic activity[3].

Oxidation is a natural process and essential to many living organisms for the production of energy to fuel biological processes. In the human body, free radicals and other reactive oxygen species are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to UV light, ozone, cigarette smoking, environmental pollutants, certain drugs, pesticides and industrial chemicals[4].Sometime it cause harmful to health by production of highly reactive species such as reactive oxygen species (ROS), and free radical. The role of oxygen radicals has been implicated in several diseases, including cancer, diabetes and cardiovascular diseases, ageing, etc. This free radical is a chemical compound which contains an unpaired electron spinning on the peripheral layer around the nucleus. Free radicals are extremely reactive, almost electrically neutral (except some free radical ions), possess addition properties and paramagnetic. Types of free radicals include the hydroxyl radical (OH.), the superoxide radical ($O._2$), the nitric oxide radical (NO.) and the lipid peroxyl radical (LOO.). Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions. Cancer initiation and promotion is associated with chromosomal defects and oncogene activation. It is possible that endogenous free radical reactions, like those initiated by ionizing radiation, may result in tumour formation. Studies on atherosclerosis reveal the probability that the disease may be due to free radical reactions involving diet-derived lipids in the arterial wall and serum to yield peroxides and other substances. The human body has several mechanisms to counteract damage by free radicals and other reactive oxygen species. These act on different oxidants as well as in different cellular compartments a) One important line of defence is a system of enzymes, including glutathione peroxidases, superoxide dismutase's and catalase, which decrease concentrations of the most harmful oxidants in the tissues. b) The second line of defence against free radical damage is the presence of antioxidants. An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage.

Therefore, in order to generate new drugs for novel applications, many efforts have been devoted to design and synthesize molecules with diverse biological activities. Of particular interests, pyrazolone and its derivatives have attracted intense interest in recent years because of their potential application in medicinal chemistry as antibacterial[5], anti-inflammatory[6], antioxidant[7], analgesic[8], and cytotoxic agents[9]. As an example, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, has been recently shown to produce marked attenuation of brain damage caused by ischemia-reperfusion[10] and its pharmacological actions were attributed to its antioxidant activity, as a potent hydroxyl radical scavenger[11]. The biological profile of 2 substituted benzothiazole shown anti cancer activity and pyrazolone and its derivative have antioxidant activity. With this background the aim of our present study is to couple benzothiazole and pyrazolone through azomethine protons (-NHN=CH) linkage to constitute important new class of compound, have potent anti oxidant properties with less side effects.

2 EXPERIMENTAL

2.1.1 Material and methods

All chemicals used in the present study were purchased from SD Fine chemicals and E Merck. The spots were developed in iodine chamber and visualized. The infrared (IR) spectra were run as KBr disc on Jasco FTIR (4100). The ^{1}HNMR Spectra of the synthesized compounds were recorded by Bruker 300 MHz FT- NMR using TMS (Tetra Methyl Silane) as internal standard. The PMR (Proton Magnetic Resonance) spectroscopic values are measured in δ ppm in DMSO-d_6.

2.1.2. General procedure for synthesis of 6-bromobenzo[*d*]thiazol-2-amine (BTZ) [12]

To glacial acetic acid (150ml), precooled to 5˚C, ammonium thiocyanate (0.06 mol, 4.56gm) and 4-bromoaniline (0.06mol, 10.26gm) were added. The mixture was placed in dry ice then addition of bromine (0.02mol, 1ml of bromine dissolved in 10ml of glacial acetic acid) from a dropping funnel at such rate that temperature does not rise above 5˚C, stirring was continued for an additional 6 hrs at 0-10˚C and neutralized with aqueous ammonia solution, kept it in overnight, filtered, washed with water and dried, recrystallised from ethanol to obtain grey color precipitate of 6-bromo benzo [d] thiazol-2-amine (BTZ).

2.1.3. General procedure for synthesis of Ethyl [2-(6-bromo-1, 3-benzothiazol-2-yl) hydrazinylidene](cyano)acetate(BTZE) [13]

Dissolve 6-bromo benzo [d] thiazol-2-amine (BTZ) (0.05 mol, 11.45gm) in concentrated HCl (20ml) and water (10 ml), cooled to 0-5˚C under dry ice and precooled solution of sodium nitrite (1.5gm in 10ml of water) was added to it drop wise during 10mts. The reaction mixture was stirred for 40 minutes. The ice cold mixture of ethyl cyano acetate (0.01mol, 1.12gm) and saturated solution of sodium acetate (0.05mol, 4.1gm) in ethanol (50ml), was added drop wise with stirring to a solution of diazonium salt compound over 15 minutes. The stirring was continued for 30 minutes at 0-5˚C and the reaction mixture then stirred for 2.30 hrs at room temperature. The product was collected and recrystallised from ethanol to give grayish brown color solid of Ethyl [2-(6-bromo-1, 3-benzothiazol-2-yl) hydrazinylidene](cyano)acetate (BTZE).

2.1.4. General procedure for synthesis of 5-amino-4-[2-(6-bromo-1, 3-benzothiazol-2-yl) hydrazinylidene]-2, 4-dihydro-3H-pyrazol-3-one (BTZP) [13]

A mixture of ethyl cyano [2-(4-phenyl-1,3-thiazol-2-yl)hydrazinylidene]acetate, (0.005mole, 1.5gm) and hydrazine hydrate (0.01 mole, 0.32ml) in 30 ml of ethanol was heated under reflux for 6 hrs. The solvent was concentrated and the product obtained was allowed to cool, filtered, washed with water, dried and recrystallized from ethanol to get brownish red colour solid of 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinyl-idene]-2,4-dihydro-3H-pyrazol-3-one(BTZP). The physicochemical data of intermediates were given in the table no: 1.

2.1.5. General procedure for synthesis of Mannich Bases [14]

A mixture of 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydra-zinylidene]-2,4-dihydro-3H-pyrazol-3-one (0.05 mole, 1.73 gm) and 90% formaldehyde (6 ml) were refluxed with different aromatic amines (0.1mole) in ethanol (30 ml) for appropriate time and the reaction was monitored by TLC. The resulting mixture was poured in to crushed ice. The solid obtained was filtered, dried and recrystalized from ethanol. The physicochemical data of the derivatives were given in the table no: 2.

2.1.6 Spectral analysis.

2.1.6.1 Spectral analysis of intermediates

(Compound BTZ) 6-bromobenzo[*d*]thiazol-2-amine; Grey colour; solvent system: chloroform: methanol (7:3); Yield: 95%w/w, m.p. (˚C) 204-206, R_f: 0.785. IR (KBr pellets): cm^{-1} 3573.45 (NH_2 Stretching), 825.026 (Ar, CH=CHStreching), 1663.31 (C=NStretching), 669.178 (C-S-C Streching); ^1H NMR (DMSO-d_6): δ ppm 6.24-6.53 (m, 3H, Ar-H), 3.5, 4.0 (s, 2H, NH_2)

(Compound BTZE) Ethyl [2-(6-bromo-1, 3-benzothiazol-2-yl) hydrazinylidene](cyano) acetate; Greyishblack colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 57%w/w, m.p. (˚C) 174-176; R_f: 0.445. IR (KBr pellets): cm^{-1} 1527.35 (C=NStretching), 1400.07 (C≡NStretching), 3442.31 (-NH-Stretching), 639.84 (C-S-C Streching), 3032(CH str., aromatic), 1633.11 (C=O str., of carboxylic ester and acetyl carbonyl group); 1H NMR (DMSO-d_6): δ ppm 6.63(m, 3H, Ar-H), 6.60(s, 1H, NH =N- proton of hydrazone), 3.60(s, 3H, $-CH_3$ proton of ethyl).

(Compound BTZP) 5-amino-4-[2-(6-bromo-1, 3-benzothiazol-2-yl) hydrazinylidene]-2, 4-dihydro-3H-pyrazol-3-one; Brownish red color; solvent system benzene: chloroform: water (40:35:25) and Yield: 62%w/w, m.p. (˚C) 211-213; R_f: 0.688 IR (KBr pellets): cm^{-1} 3385.00 (NHStreching), 1676 (C=OStreching), 1528.38 (C=NStretching), 3385 (NH_2Streching), 615.43 (C-S-C Streching), 802(CH str.,aromatic); 1H NMR (DMSO-d_6): δ ppm 6.7-6.75 (m, 3H, Ar-H), 6.60 (s, 1H, NH=N- proton of hydrazone), 7.76 (s, 1H, NH proton of pyrazolone), 6.55(s, 2H, NH_2).

2.1.6.2 SPECTRAL ANALYSIS OF DERIVATIVES

(Compound B1P) 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-2-{[(4-hydroxyphenyl) amino] methyl}-2,4-dihydro-3H-pyrazol-3-one; Black colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 95%w/w, m.p. (˚C) 209-211; R_f: 0.463 (KBr pellets): cm^{-1} 1587.7 (C=NStretching),832.13,(Ar,CH=CHStretching) ,1598.7(C=OStretching), 3141.47 (NH aliphaticStretching), 1165.76 (-OH Stretching), 636.53 (C-Br Stretching); 1H NMR (DMSO-d_6): δ ppm 6.60(s, 1H, NH=N-proton of hydrazone), 4.8 (d, 2H, $-CH_2$- methylene), 7.5-7.7 (m, 3H, Ar- H), 6.63(s, 2H, NH_2), 9.1(s, 1H, OH).

(Compound B1PB) 4-[({3-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-5-oxo-4,5-dihydro-1H-pyrazol-1-yl}methyl)amino]benzoic acid;

Pale brown colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 72%w/w, m.p. (˚C) 189-191; R_f: 0.445 (KBr pellets): cm^{-1} 1588.09(C=N Stretching), 808.02(Ar,CH=CHStretching), 3127.01 (COOH Stretching), 691.35 (C-BrStretching); 1H NMR (DMSO-d_6): δ ppm 6.62(s, 1H, NH=N-proton of hydrazone), 7.78-8.04 (m, 3H, Ar-H), 6.92(s, 2H, NH_2), 4.72 (d, 2H -CH_2- methylene).

(Compound B1M) 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-2-{[(4-methoxyphenyl)amino]methyl}-2,4-dihydro-3H-pyrazol-3-one; Reddish brown colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 72%w/w, m.p. (˚C) 219-221; R_f: 0.724 (KBr pellets): cm^{-1} 675.92 (C-Br Stretching), 1526.33(C=N Stretching), 862.02 (Ar,CH=CH Stretching), 3118.33 (NH aliphatic Stretching); 1H NMR (DMSO-d_6): δ ppm 6.63(s, 1H, NH=N-proton of hydrazone), 3.3(s, 3H, (OCH_3), 7.73-8.14 (m, 3H, Ar-H), 6.73(s, 2H, NH_2), 4.8 (d, H, -CH_2- methylene).

(Compound B1Br) 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-2-{[(4-bromophenyl)amino]methyl}-2,4-dihydro-3H-pyrazol-3-one; Reddish brown colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 75%w/w, m.p. (˚C) 229-231; R_f: 0.663 (KBr pellets): cm^{-1} 676.17 (C-Br Stretching), 1660.63(C=N Stretching), 768.49(Ar CH=CH Stretching), 3121.22(NH aliphatic Stretching), 1674.87 (C=O Stretching). 1H NMR (DMSO-d_6): δ ppm 6.90(s, 1H, NH=N-proton of hydrazone), 7.5-7.7 (m, 3H, Ar-H), 6.7(s, 2H, NH_2), 4.95 (d, 2H, -CH_2- methylene).

(Compound B12C) 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-2-{[(2-chlorophenyl) amino]methyl}-2,4-dihydro-3H-pyrazol-3-one; Pale yellow colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 72%w/w, m.p. (˚C) 250-252; R_f: 0.746 (KBr pellets): cm^{-1} 771.38 (C-Cl Stretching), 697.12(ArCH=CH Stretching), 1676.8 (C=O Stretching); 1H NMR (DMSO-d_6) δ ppm: 6.60(s, 1H, NH=N-proton of hydrazone), 8.00 (m, 3H, Ar-H), 4.7(s, 2H, NH_2), 4.9 (d, 2H, -CH_2- methylene).

(Compound B14C) 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-2-{[(4-chlorophenyl)amino]methyl}-2,4-dihydro-3H-pyrazol-3-one; Pale yellow colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 72%w/w, m.p. (˚C) 219-221; R_f: 0.464 (KBr pellets): cm^{-1} 775.24 (ArCH=CH Stretching), 1676.80 (C=O Stretching), 697.01 (C-Cl Stretching);1H NMR (DMSO-d_6): δ ppm 6.40(s, 1H, NH=N-proton of hydrazone), 7.04-7.64 (m, 3H Ar-H), 6.5(s, 2H, NH_2), 4.62 (d, 2H, -CH_2- methylene),

(Compound B1AZ) 4-[({3-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-5-oxo-4,5-dihydro-1H-pyrazol-1-yl}methyl)amino]-2-phenyldiazene; Brownish red colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 69%w/w, m.p. (˚C) 240-242; R_f: 0.712 (KBr pellets): cm^{-1} 1491 (C=N Stretching), 830.20(Ar,CH=CH Stretching), 3363.25 (NH aliphatic Stretching), 1655.60 (C=O Stretching); 1H NMR (DMSO-d_6): δ ppm 6.80 (s,1H, NH=N-proton of hydrazone), 7.83-8.0 (m, 3H Ar-H), 4.72 (d, 2H -CH_2- methylene).

(Compound B12N) 4-[({3-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-5-oxo-4,5-dihydro-1H-pyrazol-1-yl}methyl)amino]benzoic acid; Yellow colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 77%w/w, m.p. (˚C) 239-241; R_f: 0.567 (KBr pellets): cm^{-1} 1669.09 (C=N Stretching), 840.81(Ar, CH=CH Stretching), 1603.52 (C=O Stretching), 3359.39(NH aliphatic Stretching), 1510.95 (-NO_2 Stretching); 1H NMR (DMSO-d_6): δ ppm 6.63(s, 1H, NH=N-proton of hydrazone), 7.78-8.02 (m, 3H Ar-H), 6.67(s, 2H, NH_2), 4.82 (d, -2H, CH_2- methylene).

(Compound B14N) 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-2-{[(4-nitrophenyl) amino]methyl}-2,4-dihydro-3H-pyrazol-3-one; Yellow colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 71%w/w, m.p. (˚C) 220-222; R_f: 0.432 (KBr pellets): cm^{-1} 1669.09 (C=N Stretching), 840.81 (Ar, CH=CH Stretching), 1603.52 (C=O Stretching), 3359.39 (NH aliphatic Stretching), 1510.95 (-NO_2); 1H NMR (DMSO-d_6): δ ppm 6.60(s, 1H, NH=N-proton of hydrazone), 7.73-8.14 (m, 3H Ar-H), 4.42 (d, 2H -CH_2- methylene).

(Compound B1AP) 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-2-[(pyridin-2-ylamino)methyl]-2,4-dihydro-3H-pyrazol-3-one; Black colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 72%w/w, m.p. (˚C) 221-223; R_f: 0.464 (KBr pellets): cm^{-1} 1593.6 (C=N Stretching), 843.42 (Ar CH=CH Stretching), 1671 (C=O Stretching); 1H NMR (DMSO-d_6) δ ppm: 6.56(s, 1H, NH=N-proton of hydrazone), 7.78-

8.04 (m, 3H Ar-H), 6.53(s, 2H, NH_2), 4.72 (d, 2H -CH_2- methylene).

(Compound B1T) 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-2-{[(2-methylphenyl)amino]methyl}-2,4-dihydro-3H-pyrazol-3-one; Brownish red colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 73%w/w, m.p. (˚C) 261-263; R_f: 0.723 (KBr pellets): cm^{-1} 926.54(Ar CH=CH Stretching), 1674.87 (C=O Stretching), 1600.63 (C=N Stretching), 1293.36 (C-CH_3 Stretching); 1H NMR (DMSO-d_6) δ ppm:6.5(s, 1H, NH=N-proton of hydrazone), 7.7-7.8 (m, 3H Ar-H), 6.6 (s, 2H, NH_2), 4.7 (d, 2H -CH_2- methylene), 3.5(s, 3H -CH_3).

(Compound B1B) 5-amino-4-[2-(6-bromo-1,3-benzothiazol-2-yl)hydrazinylidene]-2-{[(2-methylphenyl)amino methyl}-2,4-dihydro-3H-pyrazol-3-one; Grey colour; solvent system benzene: chloroform: water (40:35:25) and Yield: 77%w/w, m.p. (˚C) 190-192; R_f: 0.583 (KBr pellets): cm^{-1} 1511.11 (C=N Stretching), 3390.00 (NH aliphatic Stretching), 1673.91 (C=O Stretching), 686.17 (C-BrStreching); 1H NMR (DMSO-d_6): δ ppm 6.9(s, 1H, NH=N-proton of hydrazone), 7.78-8.04(m, 3H, ArH), 6.93(s, 2H, NH_2), 4.72 (d, 2H, -CH_2- methylene).

2.2 **Biological screening**

In-vitro anti-oxidant screening of synthesized compounds by DPPH, FRAP, ABTS methods[15].

2.2.1.1 Determination of DPPH (1-1-diphenyl 2-picryl hydrazyl) radical-scavenging activity

The free radical-scavenging activity of the synthesized compounds was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH. 0.1 mM solution of DPPH in ethanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in methanol at different concentrations (0.1–5 mg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Ascorbic acid was used as the reference compound. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\% \text{ inhibition} = (A_0 - A_t)/ A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without compounds) and A_t was the absorbance in the presence of the compounds. All the tests were performed in triplicate and the graph was plotted with the mean values.

2.2.1.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay is based on the ability of anti-oxidants to reduce Fe3+ to Fe2+ in the presence of 2,4,6-tri(2-pyridyl)- s-triazine (TPTZ), forming an intense blue Fe2+-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the anti-oxidant content (Benzie and Strain, 1996). 0.2 ml of the compound is added to 3.8 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM FeCl3. 6H2O solution) and the reaction mixture is incubated at 37°C for 30 min and the increase in absorbance at 593 nm is measured. $FeSO_4$ is used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of sample. BHT, BHA, ascorbic acid, quercetin, catechin or trolox can be used as a positive control.

2.2.1.3 2,2'-azino-di-(3-ethyl benzthiazoline-6-sulphonic acid(ABTS) radical scavenging assay

The ABTS assay was employed to measure the anti-oxidant activity of the derivatives. ABTS was dissolved in de-ionized water to 7 mM concentration, and potassium persulphate added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12–16 h) in the dark before usage. 0.5 ml of derivatives were diluted with 0.3 ml ABTS solution and made up to the volume with methanol. Absorbance was measured spectrophotometrically at 745 nm.

The assay was performed at least in triplicates. Fresh stocks of ABTS solution were prepared every five days due to self-degradation of the radical. The assay was first carried out on Ascorbic acid, which served as a standard. The percentage of inhibition was measured by the following formula:

$$\% \text{ inhibition} = (A_0 - A_t)/ A_0$$

Where A_0 was the absorbance of the control (blank, without compounds) and A_t was the

Absorbance in the presence of the compounds. All the tests were performed in triplicate and the graph was plotted with the mean values. The *in-vitro* antioxidant activity values of the synthesized compounds were given in the table no: 3.

2.2.2 *In-vitro* cytotoxicity screening by mtt assay[16,17]

The mouse embryonic fibroblasts cell line (NIH 3T3) was obtained from National Centre for Cell Science (NCCS), Pune, and grown in Dulbeccos modified Eagles medium containing 10% fetal bovine serum (FBS). All cells were maintained at 37^0C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

MTT assay

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilised in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC50 was determined using Graph Pad Prism software. The *in-vitro* cytotoxic activity IC_{50} values and figure were given in the table no: 4 and fig no:1.

3. RESULTS AND DISCUSSION

3.1Chemistry

The synthesis of the target compounds includes four steps briefly described as follows 1) synthesis of 6-bromobenzo[*d*]thiazol-2-amine 2) synthesis of Ethyl [2-(6-bromo-1, 3-benzothiazol-2-yl) hydraziny lidene](cyano)acetate 3)synthesis of 5-amino-4-[2-(6-bromo-1, 3-benzothiazol-2-yl) hydrazinylidene]-2, 4-dihydro-3H-pyrazol-3-one (BTZP) 4)synthesis of Mannich Bases with 12 different amines. The above steps are shown in Scheme-I. The yield was found to be 55-75% in all the stage. Thin layer chromatography was used to confirm the reaction time and purity of the synthesized compounds. Melting point was determined by one end open glass capillary tubes and was uncorrected. The interesting in this scheme is the key intermediate (BTZP) contain primary amino group at fifth position, question was mannich reaction take place in fifth position free amino group or the active hydrogen at second position of pyrazolone ring. The solution for the above, active hydrogen undergo mannich reaction. Mannich reaction defined as the condensation of an active hydrogen compound with a primary or a secondary amine and a non-enolizable aldehyde or ketone to afford β-amino carbonyl derivatives known as Mannich bases. Further, the free amino group in the title compounds at fifth position was confirmed by chemical test and spectral data. In IR spectra the structures of intermediates (BTZ) confirmed by the presence of characteristic peaks in the region 3573.45cm^{-1}, 1663.3 cm^{-1}, 669.17 cm^{-1} associated for $–NH_2$, C=N-, C-S-C stretching respectively. The compound (BTZE) confirmed by the stretching of ester group in the region 1702.84cm^{-1} for keto of acetyl group. The compound BTZP confirmed the presence of $–NH_2$ and –NH- groups by the peaks at 3385.42 cm^{-1} and 3385cm^{-1} respectively and also showed the disappearance of the characteristic bands of the acetyl carbonyl group and carboxylic acid ester. The UV spectrum of the title compounds values were observed between 400-800nm and IR spectra were exhibited 3340.23-3372.82cm^{-1} for associated NH of amines, 840-790cm^{-1} for Ar, CH=CH stretching, 1640-1620cm^{-1} and 1600-1400$cm^{-1}$for C=O stretching. The ^{1}H NMR spectra all the synthesized compounds showed multiplets in the range δ 6.3-7.9 for the protons of aromatic ring, singlets in the range δ 4.0-4.9 for the protons of the $–NH_2$, singlets in the range δ 6.2-6.9 for the protons of the –NH-N= hydrazone.

3.2 *In-vitro* anti oxidant activities

The DPPH radical is considered to be a model for a lipophilic radical. A chain in lipophilic radicals was initiated by the lipid autoxidation. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[18]. The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm, which is induced by anti-oxidants. Positive DPPH test suggests that the samples were free radical scavengers. The scavenging effect of

synthesized compounds and ascorbic acid on DPPH radical was compared. The result of *in-vitro* anti-oxidant activity by DPPH method indicates that among the screened compounds, compound B12N, B14N, B1Br, B12C, B1PB, B14C and B1AP have significant anti-oxidant activity with EC_{50} value 22.45, 22.9, 23.58, 24.15, 25.12, 25.13 and 31.75µg/ml, in ABTS method EC_{50} value 23.70, 24.30, 22.83, 22.05, 22.49, 22.62 and 30.97µg/ml respectively. The other synthesized compounds showed mild anti-oxidant activity. The DPPH and ABTS *in-vitro* anti-oxidant assay method result review that the synthesized compounds containing substitution of electron with drawing group attached at the para position of N-methyl benzenamine ring imparted significant anti-oxidant property to the resulting N-mannich base containing pyrazolone derivatives (B12N, B14N, B1Br, B12C, B1PB, B14C and B1AP). The highest anti-oxidant activity due to the compound with high lipophilic value, lowest electron withdrawing power, highly polarisability. In FRAP method among the synthesized derivatives B1P, B1PB, B1M, and B1AZ are having the R^2 value near to the standard ferrous sulphate acid showed good anti-oxidant activity.

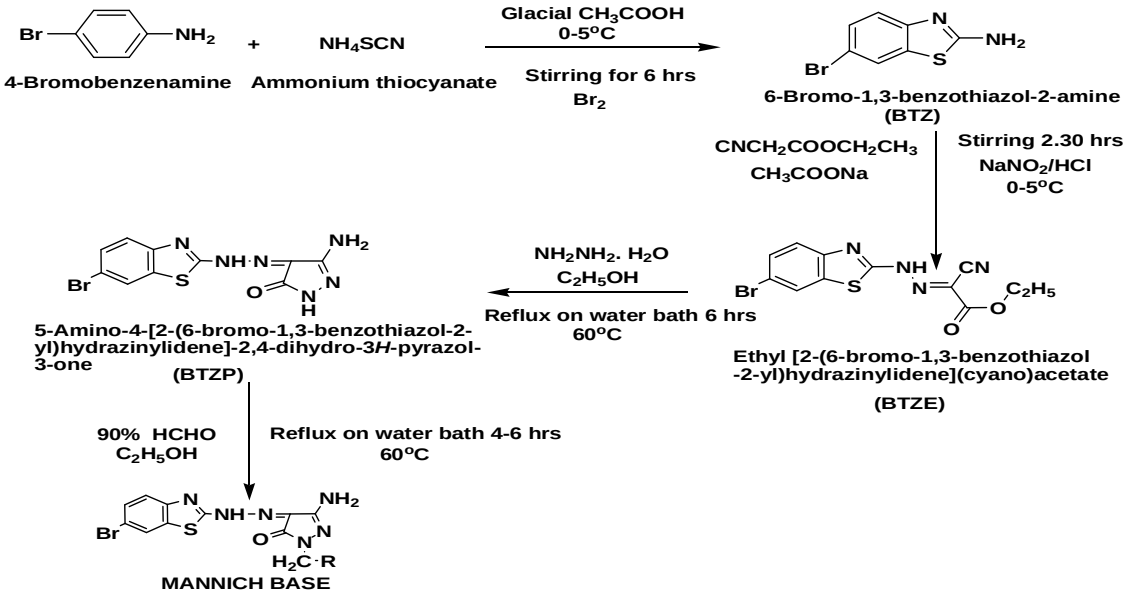
3.3 *In-vitro* cytotoxicity activity

The synthesized compounds were screened for *in-vitro* cytotoxicity against mouse embryonic fibroblasts cell line (NIH 3T3) by MTT assay. These results indicate that the synthesized compounds (B1P, B1PB,B1M,B1Br, B12C, B14C, B1AZ, B12N, B14N, B1AP B1Tand B1B) demonstrates high general cytotoxicity against normal cell line with IC_{50} values 26.1, 15.34, 24.56, 11.34, 13.76, 10.95, 20.24, 17.35, 18.54, 23.16, 14.88 and >100µM respectively.

4.1 CONCLUSION

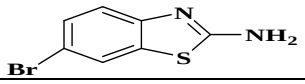
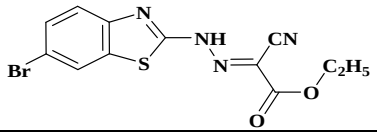
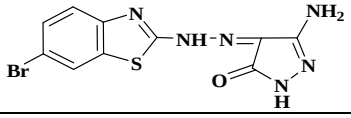
The following conclusions have been drawn from the results of these investigations:

- All the new N-mannich derivatives were evaluated for *in-vitro* anti-oxidant activity and in-vitro cytotoxicity activity, the results were found to be encouraging.
- Compound with electron with drawing group like nitro, chlorine, bromine attached at the para position of N-methyl benzenamine ring imparted significant anti-oxidant property.
- Compound with bulky substitution at the 2nd position of pyrazolone is having the less cytotoxicity activity.
- The promising results gave us scope for further work in this area. It has been felt necessary from the results of the present anti-oxidant and cytotoxicity activities that there is a need for further advanced studies, at least on the few of the test compounds which are found to be superior.



Scheme I:

Table 1: Physicochemical Properties of Intermediates

Cpd code	Structure	Molecular formula	Molecular weight	R_f Value	Melting point ˚C	ClogP	%Yield
BTZ		$C_7H_5BrN_2S$	229	0.78	204-206	2.68	95%
BTZE		$C_{12}H_9BrN_4O_2S$	353	0.44	174-176	4.78	57%
BTZP		$C_{10}H_7BrN_6OS$	339	0.68	211-212	1.02	62%

❖ Compound **B1P**

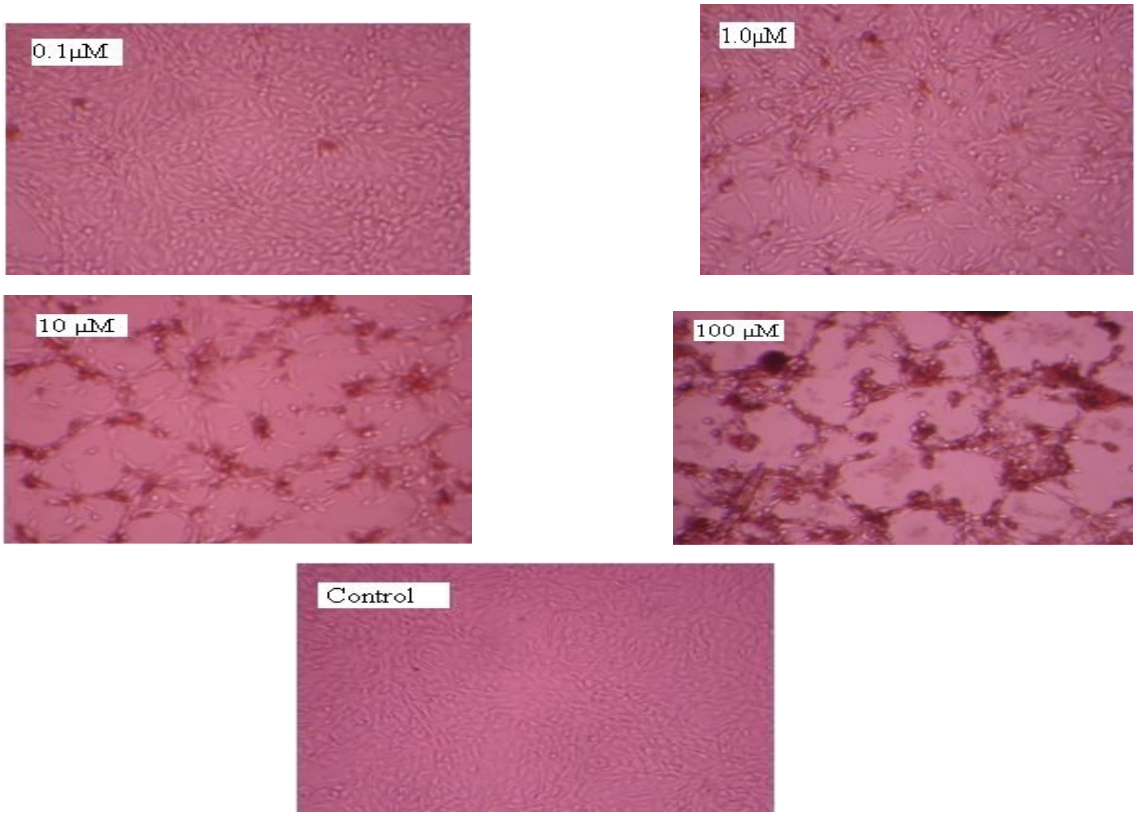


Fig. 1: Photographs of Mouse Embryonic Fibroblasts Cell Line (NIH 3T3) Inhibition By the Compound (0.1 µM -100 µM)

Table 2: Physicochemical Data of Synthesized Derivatives

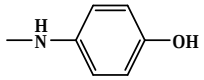
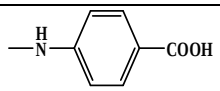
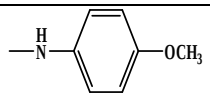
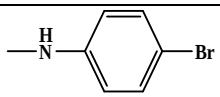
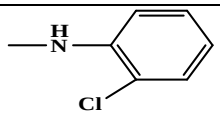
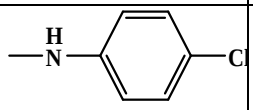
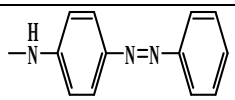
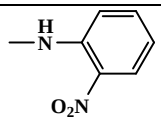
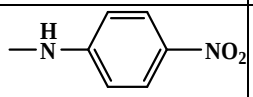
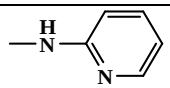
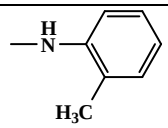
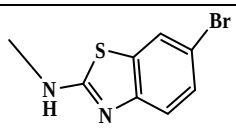
S.No	R	Molecular formula	Mol. Wt	R_f Value	Melting point˚C	ClogP	%Yield
B1P		$C_{17}H_{14}BrN_7O_2S$	460	0.46	209-211	2.53	95%
B1PB		$C_{18}H_{14}BrN_7O_3S$	488	0.44	189-191	3.28	72%
B1M		$C_{18}H_{16}BrN_7O_2S$	474	0.72	219-221	3.30	72%
B1Br		$C_{17}H_{13}Br_2N_7OS$	523	0.66	229-231	4.36	75%
B12C		$C_{17}H_{13}BrClN_7OS$	478	0.74	250-251	4.21	72%
B14C		$C_{17}H_{13}BrClN_7OS$	478	0.46	219-221	4.21	72%
B1AZ		$C_{23}H_{18}BrN_9OS$	548	0.71	240-242	5.69	69%
B12N		$C_{17}H_{13}BrN_8O_3S$	489	0.56	239-241	3.74	77%
B14N		$C_{17}H_{13}BrN_8O_3S$	489	0.43	220-222	3.59	71%
B1AP		$C_{16}H_{13}BrN_8OS$	445	0.46	221-223	2.67	72%
B1T		$C_{18}H_{16}BrN_7OS$	458	0.72	261-263	3.69	73%
B1B		$C_{18}H_{12}Br_2N_8OS_2$	580	0.58	190-192	5.05	77%

Table 3: *In-Vitro* Anti Oxidant Activity Dpph, Frap And Abts Methods of Synthesized Compounds

S.No	Compound code	DPPH EC_{50}(µg/ml)	FRAP R^2	ABTS EC_{50}(µg/ml)
1	B1P	77.78	0.949	24.62
2	B1PB	25.12	0.984	77.76
3	B1M	79.41	0.988	87.76
4	B1Br	23.58	0.788	22.83
5	B12C	24.15	0.793	22.05
6	B14C	25.13	0.734	22.49
7	B1AZ	99.76	0.885	97.80
8	B12N	22.45	0.793	23.70
9	B14N	22.91	0.793	24.30
10	B1AP	31.75	0.691	30.97
11	B1T	88.23	0.050	86.70
12	B1B	89.33	0.793	76.20
13	Standard	22.04 (Ascorbic acid)	0.996 (Ferrous sulphate)	21.55 (Ascorbic acid)

Table 4: *In-Vitro* Cytotoxic Activity By Mtt Assay Method And Ic_{50} Values Of Synthesized Compounds

S .No	Compound code	IC_{50} Values(µM)
1	B1P	26.1
2	B1PB	15.34
3	B1M	24.56
4	B1Br	11.34
5	B12C	13.76
6	B14C	10.95
7	B1AZ	20.24
8	B12N	17.35
9	B14N	18.54
10	B1AP	23.16
11	B1T	14.88
12	B1B	>100

Fig. 2

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