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Research Article

SYNTHESIS OF NOVEL 4, 6-DIPHENYLPYRIMIDINE SUBSTITUTED BENZAMIDE DERIVATIVES ITS CHARACTERIZATION AND EVALUATION FOR ANTI-OXIDANT AND ANTI-MICROBIAL ACTIVITIES

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ABSTRACT

The research work involves the estimation and evaluation of pharmacological activity of designed 4, 6-diphenylpyrimidine substituted benzamide derivatives that act as HDAC inhibitors. The Histone deacetylases inhibitors are a new class of cytostatic agents that exhibits promising results cancer treatment. The four HDAC inhibitors approved by FDA for cancer chemotherapy is SAHA (Vorinostat), Belinostat (PXD-101), Panobinostat (LBH-589), and Romidepsin (FK-228). The six novel 4, 6-diphenylpyrimidine substituted benzamide derivatives were synthesized, characterized and evaluated for anti-microbial and anti-oxidant activities. The designed molecule with cap group as substituted 4, 6-diphenylpyrimidine of functional groups adjacent to the metal-binding benzamide was evaluated for the pharmacological activity evaluation. Prior to this, the work was done on in-silico studies by molecular docking and confirmed the anti-cancer activity. The Anti-oxidant and Anti-microbial activity was evaluated as per standard methods. Anti-microbial activities involve the Anti-bacterial and Anti-fungal activity estimation. Hence, based on the pharmacological activity results, it is confirmed that the designed and characterized novel 4, 6-diphenylpyrimidine substituted benzamide exhibited the biological activity of Anti-oxidant and Anti-microbial activity.

Keywords: HDAC enzyme, HDAC inhibitor, Benzamide, 4, 6-diphenylpyrimidine, Anti-oxidant, Anti-microbial.

1. INTRODUCTION

Histone deacetylase inhibitors (HDACi) have revealed great efficacy as cancer therapeutics by blocking cell proliferation, programmed cell death, cellular differentiation, and inhibition of angiogenesis and cell migration [1]. Histone (de) acetylation is the most frequent epigenetic modification and has been shown to exert diverse effects on transcriptional activity, interactions between histones and DNA, changes in chromatin structure and the regulation of nucleosomes. HDACs are classified in four classes I, II, III, IV depending on sequence homology to the yeast enzymes Rpd3, Hdal and Sir2. Class I, II and IV are considered as "classical" HDACs, whereas class III enzymes are known as sirtuins. Classical HDACs and sirtuins differ in their catalytic mechanism. Classical HDACs carry a zinc ion catalytic pocket on their base and can be inhibited by zinc-binding chelating agents [2]. The enzymatic activity of HDAC is the deacetylation of histone proteins. Through this activity, HDACs control the interaction of positively charged histones with negatively charged DNA, thereby altering chromatin modulation, access to transcription enzymes therein and, consequently, transcriptional activity. Despite the structural diversity of HDACi, several features are common to most of the known HDAC inhibitors, including a functional group capable of binding a Zn^{+2} ion present in the active sites of HDAC enzymes. HDACIs cause accumulation of ROS in transformed cells, but not in normal cells. Free radical scavengers, such as N-acetylocysteine, reduce the HDACI-mediated apoptosis, suggesting that ROS production is an important factor in the death of cancer cells. It has been suggested that Histone deacetylase inhibition (HDACi) is a promising approach to bolster TLR-mediated induction of antimicrobial peptides such as human β-defensin 2 (hBD2)[3]. Only four molecules have been approved by US Food and Drug Administration for cancer treatment. Suberoylanilide hydroxamic acid SAHA)/Vorinostat (Zolinza) for the treatment of refractory and relapsed Cutaneous T-cell

lymphoma (CTCL) was approved in 2006 [1]. However, the current HDAC inhibitors have several limitations which include their ineffectiveness at micro or Nano concentrations in solid tumors and cardiac toxicity that limits their further clinical progress. Although classic HDAC inhibitors have both advantages and disadvantages, enormous efforts have been made for the next generation of HDACi with high selectivity, potency, and efficacy. Consequently, none of the molecules have gained access to clinical trials. Thioredoxin is a hydrogen donor that is required to activate various proteins including ribonucleotide reductases -which are necessary for DNA synthesis- and transcriptional factors such as NF-κB. Reduced thioredoxin is a scavenger of ROS. Vorinostat increases the expression of TBP-2 which binds and inhibits the activity of reduced thioredoxin, causing down regulation of thioredoxin to malignant but not to normal cells. Thioredoxin is a kinase 1 inhibitor, which regulates apoptosis [3].

1.1. Mechanism of action of HDAC inhibitor

There are two primary molecular mechanisms for epigenetic processes: DNA methylation and post-translational histone modifications. In epigenetic therapy, cancer is treated by targeting these epigenetic pathways. The basic concept of this therapy is to pharmacologically relieve the effects of DNA methylation and chromatin remodeling in malignant cells. Based on this, two classes of epigenetic drugs i.e. DNA methylation inhibitors and HDAC inhibitors (HDIs) have been approved by US FDA for the effective treatment of cancer [4].

From the docking study of HDAC, a conclusion was drawn that these Zn^{+2} dependent HADC inhibitors should possess three Pharmacophore motifs**,** (i) A cap group or surface recognition unit usually a hydrophobic or aromatic group interacting with the peripheral binding site adjacent to metal ion; (ii) a linker or spacer which is saturated or unsaturated with linear or cyclic. The structure that connects the surface recognition group and Zn^{+2} ion group; (iii) a Zn^{+2} binding group ZBG (or hydroxamic acid, benzamide, carboxylic acid groups), that chelate the Zn^{+2} ion by coordination bond formation with active sites (Fig. 1) [5].

The mechanism of actions of HDACi is represented under Fig. 2. Reactive oxygen species (ROS) induce oxidative damage to biomolecules like lipids, nucleic acids, proteins and carbohydrates. This damage causes the onset of many diseases such as rheumatoid arthritis, cirrhosis, arteriosclerosis, diabetes and cancer. Histone deacetylase inhibition activates transcription factor Nrf2. Specific antioxidative enzymes, including NAD (P) H: quinine oxidoreductase 1 (NQO1), heme oxygenase 1 (HO1), glutathione peroxidases, glutathione *S*-transferase, and glutamate cysteine ligase, protect cells from the deleterious effects of ROS. Nrf2 is a broadly expressed transcription factor and a key regulator of antioxidant-responsive genes and phase II detoxifying enzymes. Under basal conditions, Nrf2 is bound to its inhibitor, Kelch-like ECH-associated protein 1 (Keap1), which promotes its proteasomal degradation. Stress-induced modification of cysteine residues in Keap1 causes the release of Nrf2, which translocates from the cytosoplasm into the cell nucleus, where it binds to AREs in genes that encode antioxidant enzymes that protect the cell from the deleterious effects of ROS [6].

In general, pathogenic microorganisms utilize a limited number of mechanisms involving acetylation of Histone and non-histone proteins, including modulation of the activity and expression of HAT and HDAC enzymes thorough diverse bacterial effectors as well as the production of metabolites regulating the so-called "acetylome". The regulation of Anti-microbial response by HDAC inhibitors is pictorized under Fig. 3. HDACi impair the phagocytosis and the killing of bacteria by phagocytes remains unknown. To more deeply characterize the influence of HDACi on innate immune responses, we investigated whether HDACi have an impact on key antibacterial defense mechanisms of macrophages [7].

Fundamentally, HDAC inhibitors can be non-selective HDAC inhibitors can be non-selective HDAC inhibitors (pan-HDAC inhibitors) [8, 9].

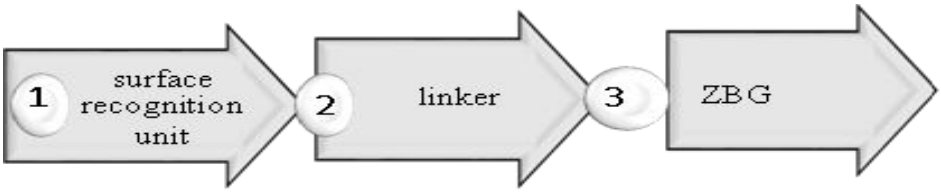


Fig. 1: Structural requirements for HDAC inhibitor

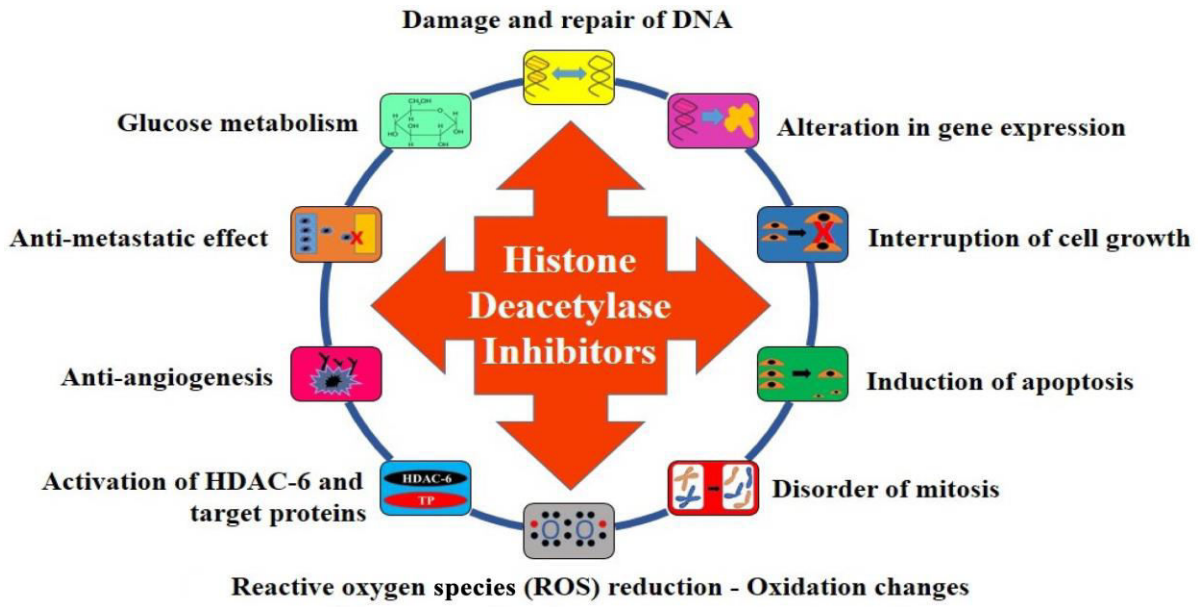


Fig. 2: Mechanism of Actions of HDAC inhibitor

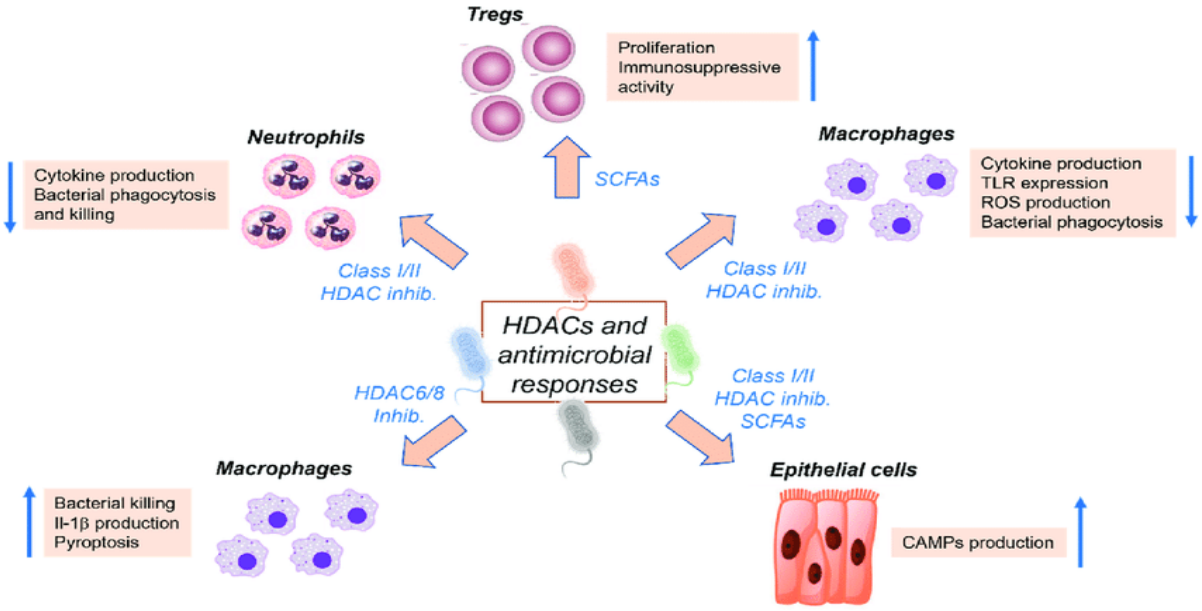


Fig. 3: Regulation of Anti-microbial responses of HDAC inhibitor

2. MATERIAL AND METHODS

2.1. Synthesis of novel 4, 6-diphenylpyrimidine substituted benzamide derivatives

Scheme for the synthesis of 4, 6-diphenylpyrimidine substituted benzamide derivatives is as follows:

Where,

i: Acetic anhydride, Reflux 2hours.

ii: Methanol, Acidic conditions, Reflux 2hours.

iii: Ethanol, 40% NaOH, 0°C, Stirring.

iv: Guanidine, DMF, Reflux 7 hours, 50-60°C.

v: HATU, DMF, Dipea.

vi: DCM, TBD, Ethanol.

1: Homophthalic anhydride,

2: 2, 2-methoxy (2-oxoethyl benzoic acid),

3a-3f: Substituted chalcone,

4a-4f: 4, 6-substituted diphenyl-2-amino-pyrimidine,

5a-5f: [2-(4, 6-Diphenyl-pyrimidin-2-ylcarbamoyl)-phenyl] acetic acid methyl ester,

6a-6f: 2-(2-((2-aminophenyl) amino)-2-oxoethyl)-N-(4, 6-diphenylpyrimidin-2-yl) benzamide.

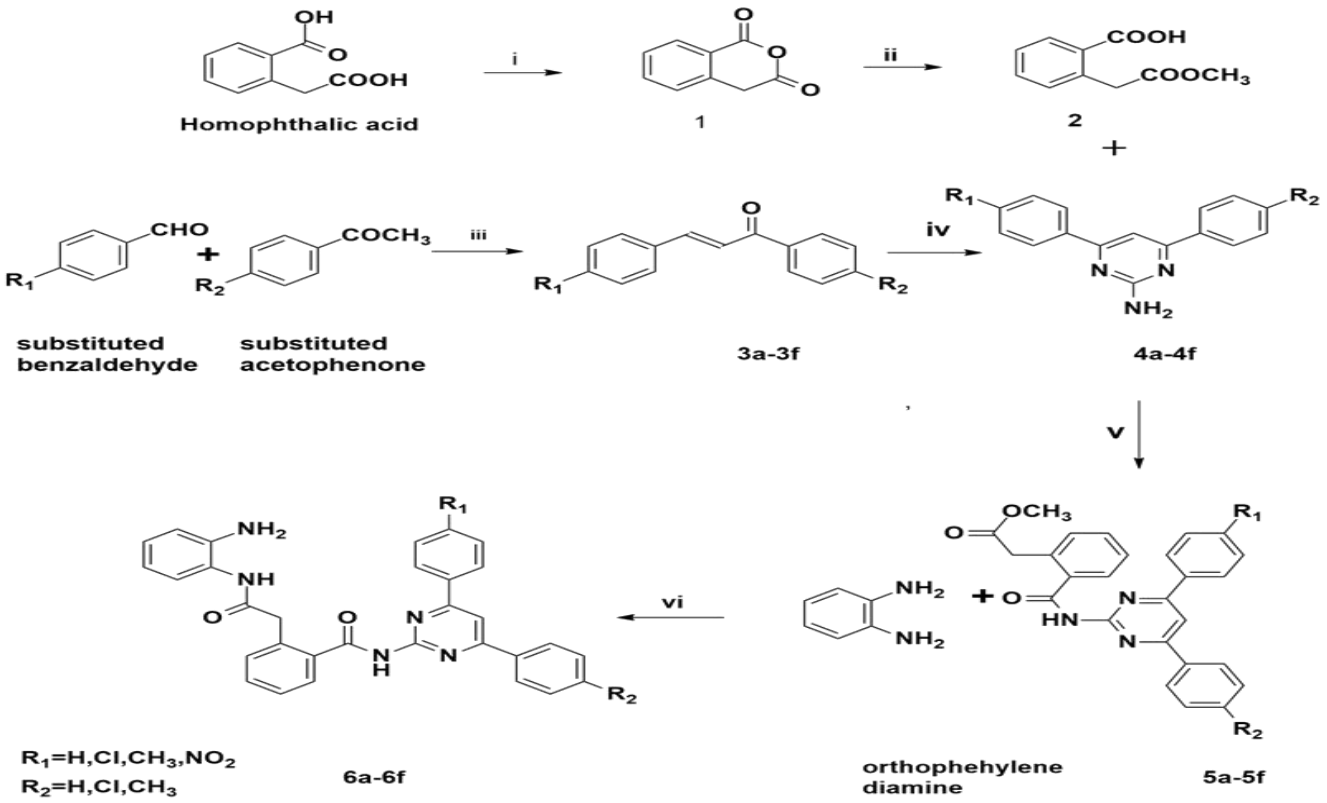


Fig. 4: Scheme synthesis of 4, 6-diphenylpyrimidine substituted benzamide derivatives

2.1.1. Synthesis of Homophthalic Anhydride from Homophthalic Acid

A mixture of 0.33 mole of dry homophthalic acid and 0.33 mol of acetic anhydride was added in a 200-ml round-bottomed flask fitted to a reflux condenser by a ground-glass joint and refluxed for 2 hours. The mixture was cooled to about 10°C for 30 minutes, and the solid anhydride was collected on a Buchner funnel with the aid of suction. It was washed with 10 ml of glacial acetic acid and pressed as much so that solvent as possible is removed by suction. The product spreaded out on a porous plate for several hours to obtain homophthalic anhydride. The solvent system used for TLC is Hexane: Ethyl acetate: Methanol (2:1:1).

2.1.2. Synthesis of 2, 2(methoxy-2oxoethyl Benzoic Acid) from Homophthalic Anhydride

Homophthalic anhydride 37mmol was refluxed in methanol (50 mL) for 2hours at 70°C. Then solvent was concentrated in vacuum to give 2, 2(methoxy-2-oxoethyl Benzoic Acid) the pure pale yellow crystalline compound. The solvent system used for TLC is Hexane: Ethylaceteate: Methanol (2:1:1).

2.1.3. Synthesis of Chalcone from Aromatic Aldehyde and Aromatic Ketone

The substituted chalcones was prepared by reacting an equimolar quantity of 0.01mol of aromatic aldehyde and 0.01mol of acetophenone in absolute ethanol then 40% NaOH solution was added to the reaction mixture drop wise with stirring maintaining the temperature about 0-2°C. After completion of the reaction the solid was separated out and was poured into ice, the precipitate was filtered and re-crystallized with ethanol. This lead to the formation of substituted chalcones *i.e.* a, b unsaturated ketones which were carried out by the Claisen-Schmidt condensation reaction. The solvent system used for TLC is Hexane: Ethylaceteate (1:1).

2.1.4. Synthesis of 4, 6-Diphenyl-2-Amino Pyrimidine through Condensation of Chalcone with Guanidine

Substituted pyrimidines was prepared by refluxing an equimolar quantity of substituted 0.01mol of chalcones with 0.01mol of guanidine in the presence of dimethylformamide (DMF) at 50-60°C for 6-7 h, the reaction mixture was cooled and poured into crushed ice, kept overnight for complete precipitation. The product was filtered and re-crystallized with methanol

and later was washed with petroleum ether. The solvent system used for TLC is Hexane: Ethylaceteate (2:1).

2.1.5. Coupling of 2, 2(mehtoxy-2-oxoethyl Benzoic Acid) with 4, 6-diphenyl-2- amino pyrimidine:

In 50ml conical flask with magnetic spin 1mmol of 2-(2-Methoxy-2- oxoethyl) benzoic acid and DMF at 0°C then add 3mmol of DIPEA were stirred for 30 minutes and then 1.5mmol of HATU reagent and 1mmol of 2-amino diphenyl-pyrimidine were added followed by stirring at room temperature for 4 hours. The reaction was monitored by through TLC. The reaction mixture was then diluted with water and extracted with ethyl acetate (50ml) and dried with anhydrous sodium sulphate, filtered and concentrated the solvent in vacuum to get final product and then purified through column chromatography.

2.1.6. Reaction of the Intermediate Ester with O-Phenylene-diamine to get 2-(2-((2- (4,6-aminophenyl)amino)-2-oxoethyl)-N--diphenyl-pyrimidin-2-yl)benzamide

To the obtained 1mmol of above product 1mmol of *O*-Phenylene-diamine and 2mmol of TBD at 0°C under nitrogen atmosphere were added. After addition, reaction mixture was brought to room temperature then extracted the product with ethyl acetate and purified with column chromatography.

In-Silico studies were already done in previous work by molecular docking and the synthesized novel 4, 6-diphenylpyrimidine substituted benzamide were evaluated for anti-oxidant and anti-microbial activity [5].

2.2. Molecular Docking Studies of synthesized compounds:

Molecular docking studies were performed on the active site of the five subtypes of HDAC enzyme (HDAC-2, HDAC-6, HDAC-7, HDAC-8, and HDAC-10). All the designed compounds showed good binding energies with the target protein as compared to standard (Vorinostat). The compound 6b (-9.30 k.cal/mol) showed exceptionally good binding energy with HDAC-10 which is more than that of a standard drug. Compound 6b has high selectivity towards HDAC-10. So, compound 6b may inhibit Cervical Cancer cells and Neuroblastoma cells.

Pharmacokinetic parameters were predicted. Compound 6e has good pharmacokinetic properties. The designed compounds are non-mutagens except compound 6d in Ames test carcinogenicity test in mouse and carcinogenicity test in rat found that all the compounds showing positive carcinogenic toxicity on the mouse except compound 6f whereas in rat all the compounds reported negative except 6d showing positive results. The results of the designed molecules were comparable with the standard drug SAHA (Vorinostat). So, the designed and synthesized compounds have the drug likeliness and are suggested as potential anticancer agents. The 4, 6-diphenyl-pyrimidine substituted benzamide has potential HDAC inhibition activity [5, 11].

2.3. Anti-Microbial activity evaluation of synthesized novel 4, 6-Diphenyl Pyrimidine substituted benzamide derivatives

The official Indian Pharmacopoeial method *i.e.,* cylinder-plate method (Method A) depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish or plate to an extent such that growth of the added microorganism is prevented entirely in a zone around the cylinder containing a solution of the antibiotic.

The synthesized compounds (6a-6f) were screened by cup plate method for their antimicrobial activity (anti-bacterial and anti-fungal) [10].

2.3.1. Test Organism and Sub-culturing:

The anti-bacterial activity was evaluated against two gram positive bacteria i.e., *Staphylococcus aureus (ATC* 29737) and *Bacillus cereus* (ATCC11778) and against two gram negative bacteria i.e., *Pseudomonas aeruginosa* (ATCC25619) and *Escherichia coli* (ATCC35218). Anti-fungal activity was evaluated against two fungal strains of *Candida albicans* (ATCC10231) and *Aspergillus niger* (ATCC6275). The strains were procured from Microbiology R and D Laboratory. For sub-culture one day prior to the testing, inoculations of the above bacterial cultures were made in the nutrient agar and incubated at 37°C for 18-24 hours.

2.3.2. Preparation of Plates

Petri dish was filled to a depth of 4-5 mm with a nutrient agar medium (Mueller Hiton Agar medium for Anti-bacterial activity and Sabraoud Dextrose Agar medium for Anti-fungal activity) that was previously inoculated with suitable inoculums of suitable test organism, and then allowed to solidify. Small sterile borer of uniform size was placed approximately at 10

cm height, having an internal diameter of approximately 6-8 mm and made of stainless steel. Each plate was divided in to four equal portions along the diameter. To each portion one cylindrical cavity was made in medium with the help of sterile borer. Three cavities for test compounds and one cavity for the standard.

2.3.3. Anti-bacterial activity

For anti-bacterial activity the standard drug, Chloramphenicol was used. The standard and test compounds (6a-6f) were prepared in different concentrations using dimethyl sulfoxide (DMSO) as solvent. Standard and test solutions were prepared by dissolving 5 mg of substance each in 5 ml of DMSO at a concentration of 1000 μg/ml. Volumes of 0.05 ml and 0.1 ml of each compound were used for testing. Using a standard procedure the sub-culture, base layer medium, agar medium and peptone water were prepared. Mueller - Hinton Agar medium was prepared as per standard procedure for anti-bacterial activity evaluation. Base layer medium was prepared with Muller-Hinton agar. Muller Hinton agar medium constitutes of Beef infusion - 300.0 g, Casein acid hydrolysates - 17.5 g, Starch- 1.5 g, and Agar-17.0 g per liter of water. Then the final pH adjusted to 7.3 ± 0.2 at 25°C. 0.1 mL solutions of each test compound (0.05 ml and 0.1 ml) were added separately in the cups and petri dishes. As a standard drug for both the type of strain, Chloramphenicol was selected, and aliquots of concentration (1000 μg/ml-0.05mL and 0.1 mL) were prepared in water, separately. Parallely, in order to maintain the control group, 0.1 ml of DMSO was added. The petri dishes were incubated at 37°C for 24 hours. Diameter of the zone of inhibition was measured and the average diameter for each sample was calculated. The diameter obtained by the test sample was compared with that produced by standard. Zones of inhibition produced by each compound were measured (in mm) and the results of antibacterial studies are presented in Table 1.

2.3.4. Anti-fungal activity

For anti-fungal activity Fluconazole was opted as standard drug. The standard and test compounds (6a-6f) were prepared in different concentrations using dimethyl sulfoxide (DMSO) as solvent. Standard and test solutions were prepared by dissolving 5 mg of substance each in 5 ml of DMSO at a concentration of 1000 μg/ml. Volumes of 0.05 ml and 0.1 ml of each compound were used for testing. The cup plate method using Sabraoud Dextrose Agar medium was used for screening of antifungal activity. Sabraoud Dextrose Agar medium constitutes of Peptone - 10g, Dextrose - 40g, Agar – per litre of water. The nutrient broth medium and other subculture were prepared as per standard procedure. The solutions of each test compound (0.05 ml and 0.1 ml) were added separately in the cups and petri dishes. As a standard drug for both the type of strain, Fluconazole (1000 μg/ml-0.05mL and 0.1 mL) was used in the antifungal activity evaluation. Parallely, in order to maintain the control group, 0.1 ml of DMSO was added. The petri dishes were incubated at 37°C for 24 hours. Diameter of the zone of inhibition was measured and the average diameter for each sample was calculated. The diameter obtained by the test sample was compared with that produced by standard. Zones of inhibition produced by each compound were measured (in mm) and the results of antibacterial studies are presented in Table 2.

2.4. Anti-oxidant activity evaluation of synthesized novel 4, 6-Diphenyl Pyrimidine Substituted benzamide derivatives

2.4.1. FRAP Assay

FRAP (Ferric Reducing Antioxidant Power) assay involved the mechanism of reducing power and is an electron transfer method for estimation of total anti-oxidant activity. The principle involved is antioxidant reaction with a Fe (III) complex and end point determination by colorimetry. This method measures the ability of antioxidant to reduce ferric iron. It is based on the reduction of the complex of ferric iron and 2, 3, 5-triphenyl- 1, 3, 4- triaza-2-azoniacyclopenta-1, 4-diene chloride (TPTZ) to the ferrous form at low pH. A blue colored ferrous-tripyridyl triazine complex is formed this reduction is monitored by measuring the change in absorption at 593 nm, using a diode-array spectrophotometer [11].The FRAP reagent was freshly prepared by mixing 2,4,6-tripyridyltriazine (10 mM, 1.0 mL) and ferric chloride (20 mM, 1.0 mL) in acetate buffer (0.25 M, 10 mL, pH 3.6). Samples solutions dissolved in solvent (Compounds 6a, 6b, 6c, 6d, 6e and 6f) of different concentrations 50 μL were added to the FRAP reagent (3.0 mL). The tests were carried out in triplicates. After a period of 8 min incubation at room temperature, the absorbance was measured at 593 nm. A calibration curve of ascorbic acid was developed to quantitatively determine the antioxidant capacity of the

samples (compounds 6a to 6f) expressed as mmol ascorbic acid equivalent per gram of dry extract. A calibration curve of ascorbic acid was developed. The antioxidant content of the compounds was expressed as mmol ascorbic acid equivalent per gram of dry extract.

2.4.2. DPPH Assay

[2, 2-di (4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) involves antioxidant reaction with an organic radical and end point determination by colorimetry. This assay measures by spectrophotometer the ability of antioxidants to reduce 2, 2- diphenylpicrylhydrazyl (DPPH), another radical not commonly found in biological systems.

The compounds (6a to 6f) were dissolved in methanol to obtain the concentration to obtain a stock solution with a concentration of 25 mg/mL. From the stock solution different volumes were taken and diluted with DMSO in order to obtain 5 different concentrations. The sample solution (400 µL) was added to 2.5 mL methanolic solution of DPPH radical (0.1 mM) BHT (dibutyl hydroxy toluene) was used as a positive standard. The contents of the tube were mixed and rested for 30 min in dark. The absorbance was then measured at 517 nm against a blank (methanol) using a UV-VIS spectrophotometer. Inhibition of free radical scavenging activity was calculated using the equation:

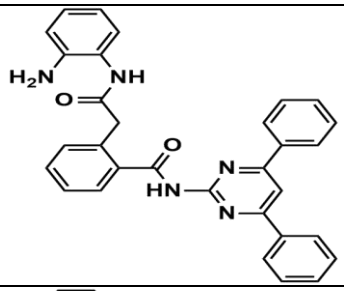
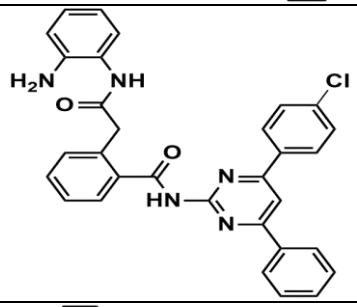
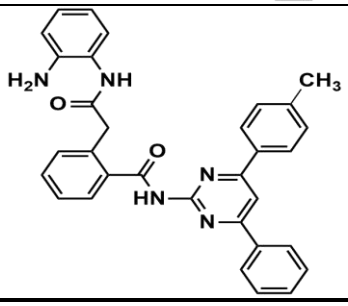
% Inhibition = 100 × (absorbance of the control-absorbance of the sample)/absorbance of the control. IC_{50} value (µg/mL) is the effective concentration at which DPPH radicals are scavenged by 50%.

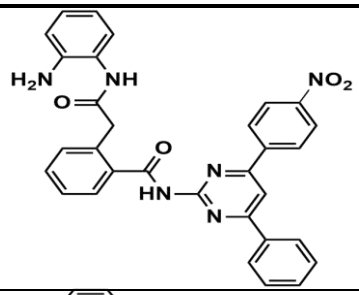
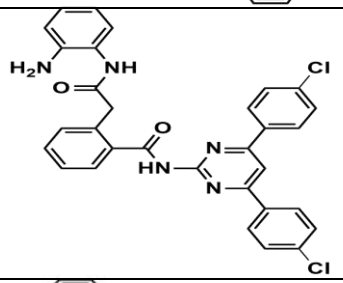
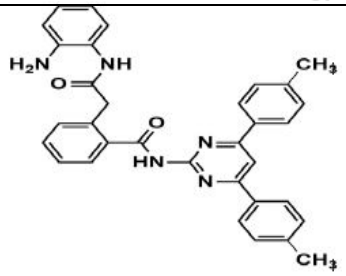
3. RESULTS AND DISCUSSION

3.1. Structures of novel synthesized 4, 6-diphenylpyrimidine substituted benzamide derivatives and Characterization of Compounds:

In Table 1 the structures, IUPAC name and Molecular weight are tabulated.

Table 1: Structures and chemical names of compounds

S. No.	*Compound*	*Structure*	*IUPAC Name*	*M.Wt.*
1.	6a		2-(2-((2-aminophenyl)amino)-2-oxoethyl)-N-(4,6-diphenylpyrimidin-2-yl)benzamide	499.56
2.	6b		2-(2-((2-aminophenyl)amino)-2-oxoethyl)-N- (4-(4-chlorophenyl)-6-phenylpyrimidin-2-yl)benzamide	534.01
3.	6c		2-(2-((2-aminophenyl)amino)-2-oxoethyl)-N-(4-phenyl-6-(*p*-tolyl)pyrimidin-2-yl)benzamide	513.59

4.	6d		2-(2-((2-aminophenyl)amino)-2-oxoethyl)-N-(4-(4-nitrophenyl)-6-phenylpyrimidin-2-yl)benzamide	544.56
5.	6e		2-(2-((2-aminophenyl)amino)-2-oxoethyl)-N-(4,6-bis(4-chlorophenyl)pyrimidin-2-yl)benzamide	568.42
6.	6f		2-(2-((2-aminophenyl)amino)-2-oxoethyl)-N-(4,6-di-*p*-tolylpyrimidin-2-yl)benzamide	527.62

3.2. Spectral data

The FTIR and NMR data obtained is mentioned below for the compounds 6a to 6f.

3.2.1. Compound 6a

Yield= 75 %; IR (KBr, cm^{-1}): 3010 (C-H Str. Aro.), 1550 (C-H Bend. Aro.), 1250 (C-N Str. Aro,), 3499 (N-H Str.Ali.), 1799 (C=O Str.Ali.), 2800 (C-H Str. Ali.), 1252(C=N Str.Aro.) 1245 (C-N Str. Aro.); NMR Spectra (δ ppm): 6.7-7.0 (4 H Ar-CH), 9.4-9.8 (4 H Ar-CH), 7.3-8.5 (10H Ar-CH), 0.8-1.2 (3H, Ar-NH), 1.2 (1H NH), 1.4 (1H, CH_2).

3.2.2. Compound 6b

Yield= 74 %; IR (KBr, cm^{-1}): 2999 (C-H Str. Aro.), 1555 (C-H Bend. Aro.), 1349 (C-N Str. Aro,), 3300 (N-H Str.Ali.), 1700 (C=O Str.Ali.), 2720 (C-H Str. Ali.), 1322(C=N Str.Aro.) 1150 (C-N Str. Aro.), 650 (C-Cl Str.Aro.); NMR Spectra (ppm): 6.2-6.5 (4 H Ar-CH), 9.0-9.1 (4 H Ar-CH), 6.5-6.8 (9 H Ar-CH), 0.5-0.6 (3H, Ar-NH), 1.0 (1H NH), 0.8 (1H, CH_2).

3.2.3. Compound 6c

Yield= 65 %; IR (KBr, cm^{-1}): 2887 (C-H Str. Aro.), 1700 (C-H Bend. Aro.), 1250 (C-N Str. Aro,), 3400 (N-H Str.Ali.), 1669 (C=O Str.Ali.), 2992 (C-H Str. Ali.), 1250 (C=N Str.Aro.) 1010 (C-N Str. Aro.), 1350 (C-H methyl Str. Ali) NMR Spectra (ppm): 6.9-7.3 (4 H Ar-CH), 9.6-10.0 (4 H Ar-CH), 7.2-8.2 (9 H Ar-CH), 0.9-1.3 (3H, Ar-NH), 1.4 (1H NH), 1.6 (1H, CH_2), 0.5 (1H CH_3)

3.2.4. Compound 6d

Yield= 73 %; IR (KBr, cm^{-1}): 2787 (C-H Str. Aro.), 1759 (C-H Bend. Aro.), 1150 (C-N Str. Aro,), 3320 (N-H Str.Ali.), 1589 (C=O Str.Ali.), 2925 (C-H Str. Ali.), 1230 (C=N Str.Aro.) 1022 (C-N Str. Aro.), 1500 (N-O Nitro Str. Ali.) methyl Str. Ali.); NMR Spectra (ppm): 6.6-6.9 (4 H Ar-CH), 8.9-9.0 (4 H Ar-CH), 7.1-7.3 (9 H Ar-CH), 0.6-0.9 (3H, Ar-NH), 1.0 (1H NH), 1.2 (1H, CH_2).

3.2.5. Compound 6e

Yield= 74 %; IR (KBr, cm^{-1}): 1300 (C-H Str. Aro.), 1255 (C-H Bend. Aro.), 1259 (C-N Str. Aro,), 2933 (N-H Str.Ali.), 1549 (C=O Str.Ali.), 2620 (C-H Str. Ali.), 1282(C=N Str.Aro.) 1050 (C-N Str. Aro.), 750 (C-Cl Str.Aro.); NMR Spectra (ppm): 5.9-6.2 (4 H Ar-CH), 8.8-9.0 (4 H Ar-CH), 6.4-6.6 (8 H Ar-CH), 0.4-0.6 (3H, Ar-NH), 0.9 (1H NH), 0.7 (1H, CH_2).

3.2.6. Compound 6f

Yield= 69 %; IR (KBr, cm^{-1}): 2858 (C-H Str. Aro.), 1659 (C-H Bend. Aro.), 1150 (C-N Str. Aro,), 3320

(N-H Str.Ali.), 1759 (C=O Str.Ali.), 2672 (C-H Str. Ali.), 1130 (C=N Str.Aro.) 1012 (C-N Str. Aro.), 1470 (C-H methyl Str. Ali) NMR Spectra (ppm): 7.0-7.2 (4 H Ar-CH), 9.7-9.8 (4 H Ar-CH), 7.4-7.9 (9 H Ar-CH), 1.2-1.5 (3H, Ar-NH), 1.6 (1H NH), 1.8 (1H, CH_2), 0.9 (1H CH_3).

3.3. Anti-microbial Activity

3.3.1. Anti-Bacterial Activity

DMSO was used as control and no sign of zone of inhibition were observed. After the performance of test, as per the mentioned procedure the average zone of inhibition (in mm) against two *gram positive* bacteria and *gram negative* bacteria is tabulated under Table 2.

3.3.2. Anti-Fungal Activity

After the performance of test, as per the mentioned procedure the average zone of inhibition (in mm) against two *fungi strains* is tabulated under Table 3.

3.4. Anti-oxidant activity

3.4.1. FRAP Assay

After the performance of test, as per the mentioned procedure, the absorbance were measured for triplicate samples of each concentration and mean values were considered to plot the graph. The calibration graphs were plotted for the standard and compounds 6a to 6f and are represented under Fig. 5 to 11. The FRAP Assay values were calculated and graph was for the compounds 6a to 6f as represented under Fig. 12.

Table 2: Results for Antibacterial activity of 4, 6-diphenylpyrimidine substituted benzamide derivatives

Compound	Average Zone of Inhibition (in mm)							
	Gram Positive Bacteria				*Gram Negative Bacteria*			
	Staphylococcus aureus		*Bacillus cereus*		*Pseudomonas aeruginosa*		*Escherichia coli*	
	0.05 mL	**0.1 mL**	**0.05 mL**	**0.1 mL**	**0.05 mL**	**0.1 mL**	**0.05 mL**	**0.1 mL**
6a	11	16	15	19	10	18	09	16
6b	09	15	16	20	07	12	11	17
6c	10	12	14	16	12	19	06	15
6d	12	16	20	26	14	24	12	19
6e	11	17	18	22	09	15	09	15
6f	08	12	12	16	06	11	09	14
Chloramphenicol (1000 μg/ml)	19	28	22	31	16	29	18	29

× *indicates No Zone of Inhibition*

Table 3: Results for Antifungal activity of 4, 6-diphenylpyrimidine substituted benzamide derivatives

Compound	Average Zone of Inhibition (in mm)			
	Fungi			
	Candida albicans		*Aspergillus niger*	
	0.05 mL	**0.1 mL**	**0.05 mL**	**0.1 mL**
6a	09	12	08	11
6b	17	29	15	18
6c	11	16	10	14
6d	07	11	06	10
6e	16	29	13	19
6f	08	10	07	12
Fluconazole (1000 μg/ml)	24	36	22	29

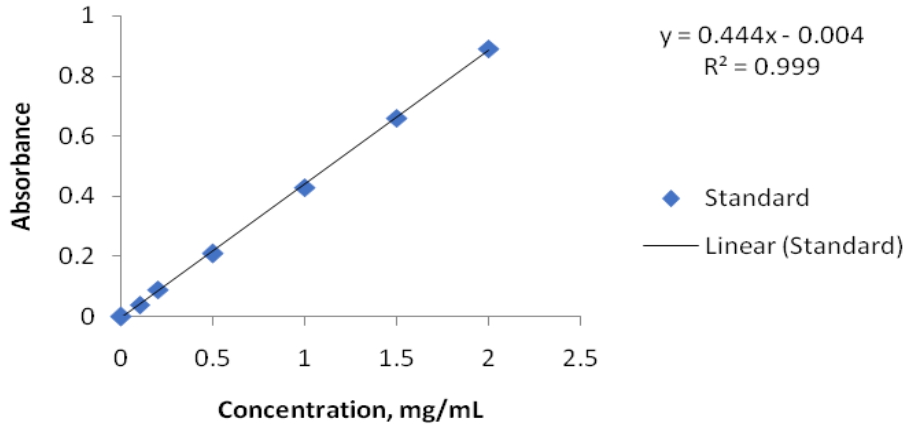


Fig. 5: Antioxidant activity-calibration curve of standard FRAP assay

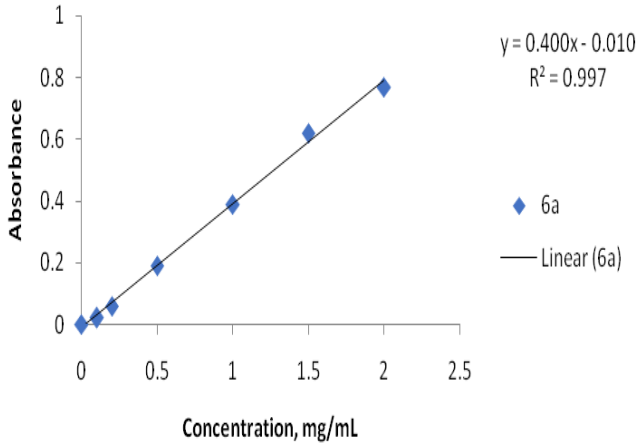


Fig. 6: Antioxidant activity-calibration curve of compound 6a

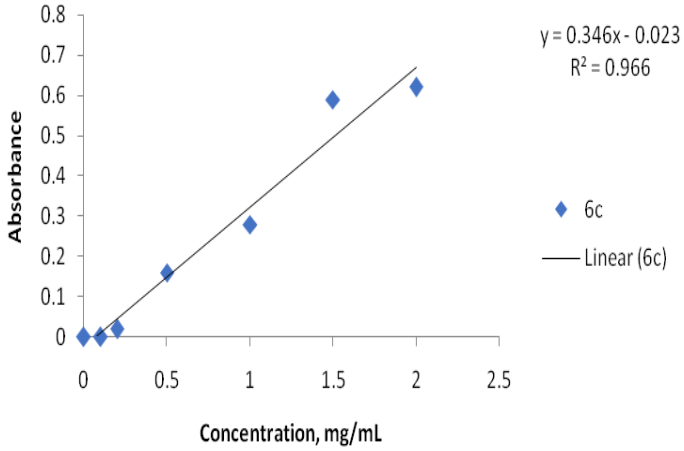


Fig. 8: Antioxidant activity-calibration curve of compound 6c

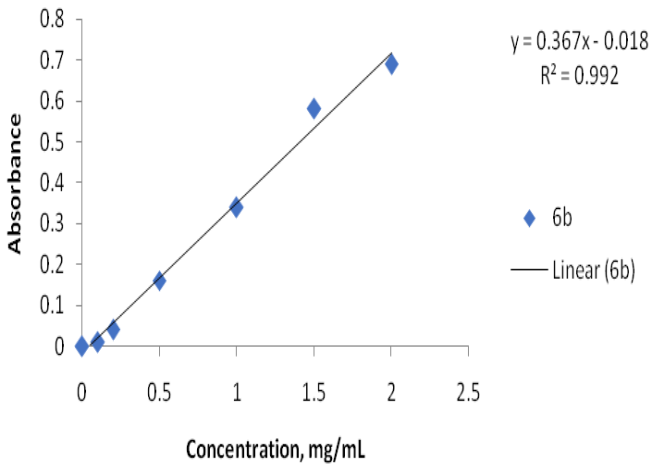


Fig. 7: Antioxidant activity-calibration curve of compound 6b

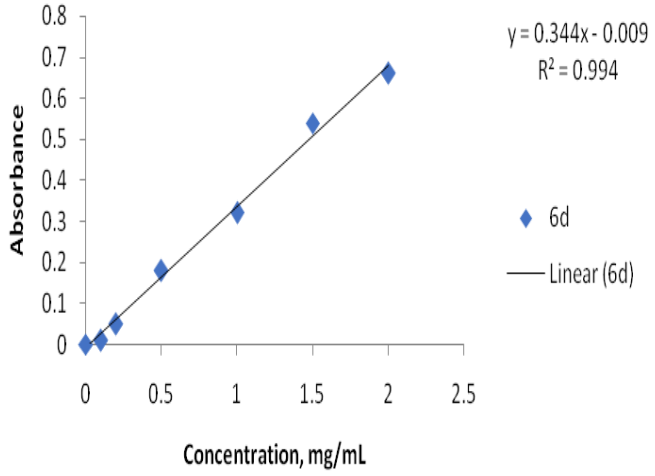


Fig. 9: Antioxidant activity-calibration curve of compound 6d

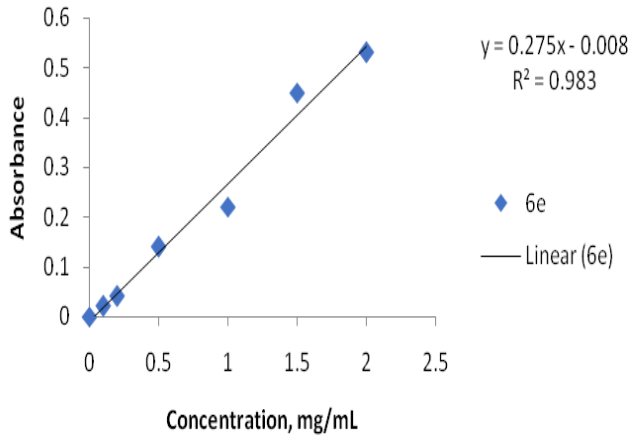


Fig. 10: Antioxidant activity-calibration curve of compound 6e

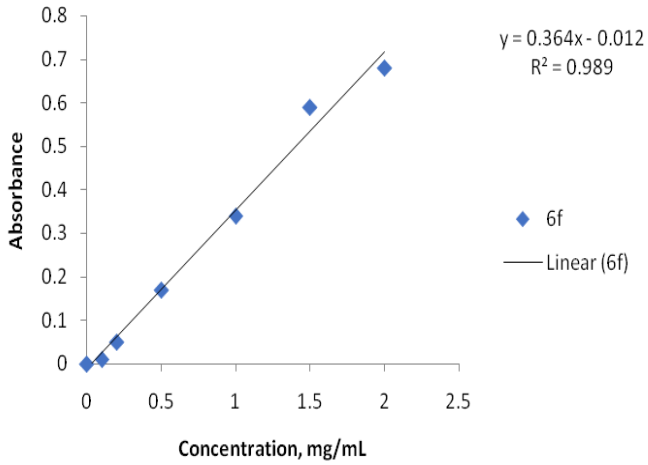


Fig. 11: Antioxidant activity-calibration curve of compound 6f

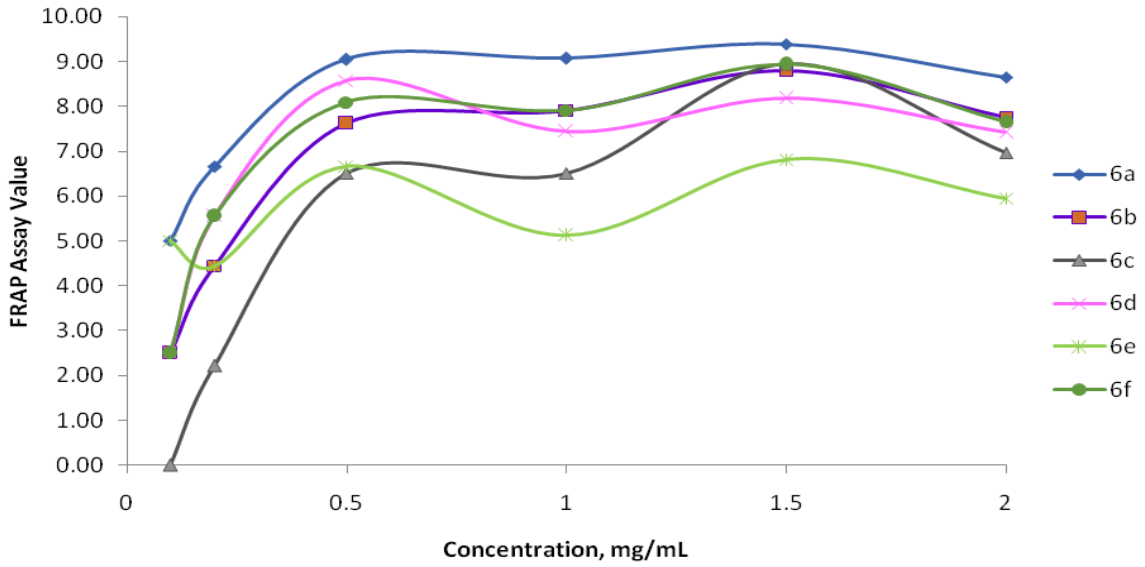


Fig. 12: Antioxidant activity-FRAP assay values of compounds 6a to 6f

3.4.2. DPPH Assay

After the performance of test, as per the mentioned procedure the % DPPH scavenging activity is tabulated under Table 4. A graph is plotted for the standard and compounds 6a to 6f and is represented between concentrations versus % DPPH scavenging activity as shown under Fig. 13.

In this study the structure of the synthesized compounds were elucidated by means of IR,1HNMR as mentioned. All the compounds were evaluated for antibacterial and antifungal activity by cup-plate method.

As concerns the anti-bacterial potency of the compounds against two strains of gram positive bacteria(*Staphylococcus aureus*, Bacillus cereus) and two strains gram negative bacteria (*Pseudomonas aeruginosa, Escherichia coli)* were evaluated. It was found that the compounds 6a to 6f exhibited mild to moderate anti-bacterial activity as shown in table 2. The compounds 6a, 6d and 6e were found to be exhibit more activity than 6b, 6c and 6f. The compounds were found to be more effective towards gram positive bacteria Bacillus cereus than *Staphylococcus aureus* as shown in table 2. The compounds were found to be equipotent towards both gram negative bacteria *Pseudomonas aeruginosa and Escherichia coli*. The anti-fungal data reveals that the compounds have shown mild to moderate anti-fungal

activity as compared to the standard Fluconazole. The compounds 6b and 6e were found to be potent compared to 6a, 6c, 6d and 6f as shown in Table 3.

Table 4: Results for DPPH radical scavenging activity (inhibition rate - %) of compounds (6a to 6f) and standard BHT at 30 min

Compound	DPPH Scavenging Activity (%) at different concentrations						IC_{50} value
	C1 (50µg/ml)	C2 (100µg/ml)	C3 (150µg/ml)	C4 (200µg/ml)	C5 (250µg/ml)	C6 (500µg/ml)	
6a	8	17	25	34	42	84	297
6b	7	16	24	32	40	79	315
6c	6	13	20	25	32	50	480
6d	9	18	27	36	45	80	317
6e	7	15	23	30	38	75	333
6f	9	15	22	29	36	77	331
Standard BHT	12	22	36	46	55	98	-

**C1 to C6 is concentrations of samples*

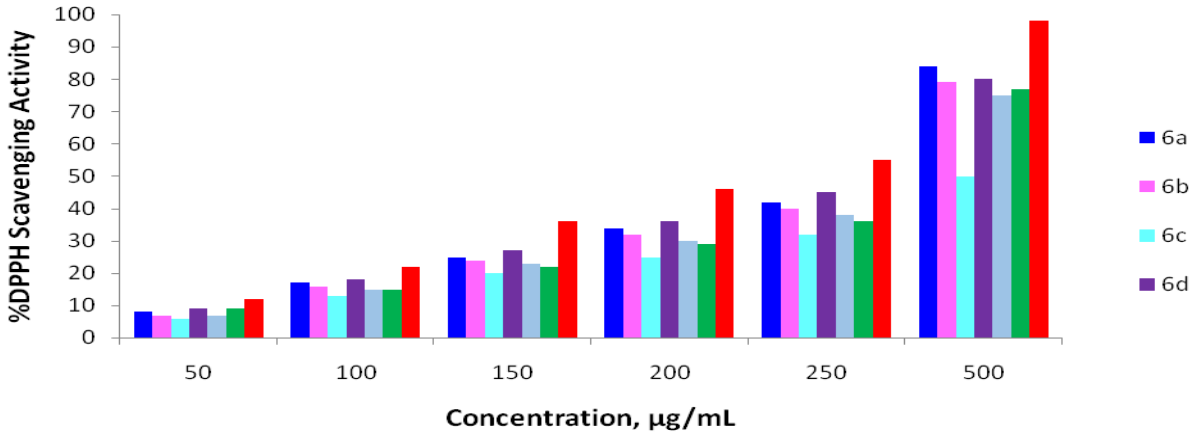


Fig. 13: DPPH radical scavenging activities of *compounds* and Standard at various concentrations. Values are means ± SE of three experiments

The anti-oxidant activities were evaluated by DPPH and FRAP assay. Based on this, the compounds 6a to 6f showed mild anti-oxidant activity compared to the standards. By FRAP assay, the absorbance of six compounds were measured and a graph was plotted between concentration versus Absorbance. The y-intercept and correlation coefficients of six compounds and the standard BHT were displayed under calibration curves as shown under Fig. 5 to 11. FRAP assay values were also calculated and a graph was plotted between concentration versus FRAP Assay values as shown under Fig. 12. Based on obtained data the compounds 6a to 6f showed mild anti-oxidant activity than the standard Ascorbic acid. By DPPH radical assay DPPH scavenging activity assay in percentage at different concentrations were noted and the graph was plotted between concentrations versus % DPPH scavenging activity as shown under Fig. 13. IC_{50} values were calculated based on obtained data for compounds 6a to 6f and tabulated under Table 4.

Based on the obtained data it was found that the synthesized compounds 6a to 6f exhibited mild to moderate anti-microbial and anti-oxidant activities.

4. CONCLUSION

The study revealed the fact that the six novel 4, 6-diphenylpyrimidine substituted benzamide derivatives compounds 6a to 6f synthesized exhibited significant anti-microbial and anti-oxidant activities. The synthesized compounds were characterized. Their structures were confirmed by FTIR and NMR. The results of this investigation revealed that the compounds

6a to 6f possess differentiating mild antimicrobial activity against selected bacterial and fungal strains. The differentiating activities against variety of microorganisms of these compounds encourage developing a novel broad spectrum anti-microbial formulation in future. By FRAP assay and DPPH assay it was found that the compounds 6a to 6f exhibited mild anti-oxidant activity. The findings suggested that the compounds 6a to 6f have excellent scope for further development as commercial anti-microbial and anti-oxidant agent. Further experiments were needed to elucidate their mechanism of action.

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Conflicts of interest

We declare no conflicts of interest.

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