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"Design and Synthesis of Some Novel Furfuryl/Furan amine-2, 4-Thiazolidinediones with Multifaceted Bioactivity: Antioxidant, Antibacterial, and Antifungal Agents"

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Abstract:

A novel series of thiazolidinediones was meticulously crafted, incorporating pharmacologically relevant elements such as substituted Furfuryl/*furan* amine groups. These elements were intricately linked to 5' (4'-sulphonylbenzylidene)]-2, 4-thiazolidinedione. The synthesis of these compounds involved a multi-step synthetic process, and their structural characterization was firmly established through comprehensive spectral analysis. To evaluate their potential, these newly synthesized compounds underwent rigorous testing for in vitro antioxidant, antibacterial activity and antifungal activity. The assessment encompassed antioxidant potential using DPPH assay, Antioxidant Assay- CUPRAC Assay, ABTS Radical Scavenging Ability, Reactive Nitrogen Oxide Scavenging Assay and Hydroxyl Free Radical Scavenging Assay. Among the synthesized compounds, Am3 and Am4 exhibited a noteworthy spectrum of antioxidant, antibacterial and antifungal activity a range of bacteria, including Gram-positive strains such as *B. subtilis* – MTCC 1133, as well as Gram-negative bacteria, exemplified by *E. coli* – MTCC452, *P. aeruginosa*- MTCC 3541 and fungus for instance *C. albicans* – MTCC 854.

Keywords: Furfuryl/furan amine-2, 4-Thiazolidinediones, Synthesis, Multifunctional Agents

Introduction: TZDs with various substitutions exhibit enhanced antioxidant properties. Methoxy groups improve antioxidant activity by donating electrons to the TZD ring, increasing its ability to neutralize reactive oxygen species (ROS) [1]. Ethoxy groups also contribute to antioxidant effects by increasing lipophilicity, which enhances interaction with lipid membranes and improves radical scavenging [2]. Bromine substitutions provide more complex effects due to their electron-withdrawing and steric properties, which influence antioxidant activity in a nuanced manner[3].Substituted TZDs show diverse antimicrobial activities. Methoxy groups enhance solubility and membrane penetration, making TZDs more effective against specific bacterial strains[4]. Ethoxy groups increase lipophilicity, potentially broadening the spectrum of antimicrobial activity[5]. Bromine-substituted TZDs offer unique mechanisms of action due to their electron-withdrawing effects and steric

interactions[6].The antifungal potential of substituted TZDs is also notable. Methoxy and ethoxy groups alter interactions with fungal cell membranes, affecting effectiveness[7]. Bromine substitutions further modify these interactions, contributing to a nuanced antifungal profile[8].Our research aims to investigate and develop more chemically versatile and diverse thiazolidinedione, incorporating essential elements of a suitable pharmacophore. This pharmacophore includes the components of a TZD-sulfonyl linker, as well as substituted Furfuryl/furan amine residues.

Materials and Methods:

The determination of melting points was conducted using the open cup capillary method. For thin-layer chromatography (TLC) analysis, glass plates coated with silica gel G60 were employed, and the spots were visualized under either ultraviolet light or through exposure to iodine vapors. Fourier-transform infrared (FT-IR) spectra were acquired using a Bruker spectrophotometer with KBr pellets as the medium. Proton nuclear magnetic resonance (1H NMR) spectra were obtained using the sophisticated multinuclear NMR Spectrometer Brucker Avance Neo 500, utilizing tetramethylsilane (TMS) as the internal standard, with dimethyl sulfoxide (DMSO) serving as the solvent. Mass spectra were recorded using the SYNAPT-XS#DBA064 Mass Spectrometer, TOF MS-ES 1.23e7, at Saif, Panjab University, Chandigarh.

Experimental:

Step I: Synthesis of 2, 4-thiazolidinedione

In 250ml three-necked flask was placed, a solution containing 56.6 g (0.6 M) of Chloroacetic ad in 60 ml of water and 45.6 g (0.6M) of thiourea dissolved in 60 ml of water. The mixture was stirred for 15 min to obtain a white precipitate, accompanied by considerable cooling. To the intents of the flask was then added slowly 60 ml con. Hydrochloric acid from a dropping funnel. The flask then connected with the reflux condenser and gentle heating was applied to affect complete dissolution, after which the reaction mixture was stirred and refluxed for 8-10 hr at 100-10°C on cooling the contents of flask solidified into a cluster of white needles. The product was filtered and washed with water to remove the traces of hydrochloric acid and dried. It was purified by recrystallization from ethyl alcohol. Yield 85%, m.p. 123-25°C.

Step II: Synthesis of 5-benzylidine-2, 4-thiazolidinedione [9, 10]

In a 250ml three-necked flask provided with a Dean-Stark apparatus, benzaldehyde (20 g. 0.188 mole) and 2, 4-thiazolidinedione (22 g. 0.188 mole) were together suspended in dry toluene. To this catalytic amount of piperidine (1ml) was added. The mixture was refluxed with stirring. After the complete removal of water and when the temperature crossed 110°C the reaction mixture was stirred for further 1 hr. on cooling, the product precipitated out from toluene. The compound was filtered and washed with cold, dry toluene and dry ethanol. Yield 89-93%, m.p.

Step III: Synthesis of 4'chlorosulphonylbenzylidene-2, 4-thiazolidinedione [9, 10]

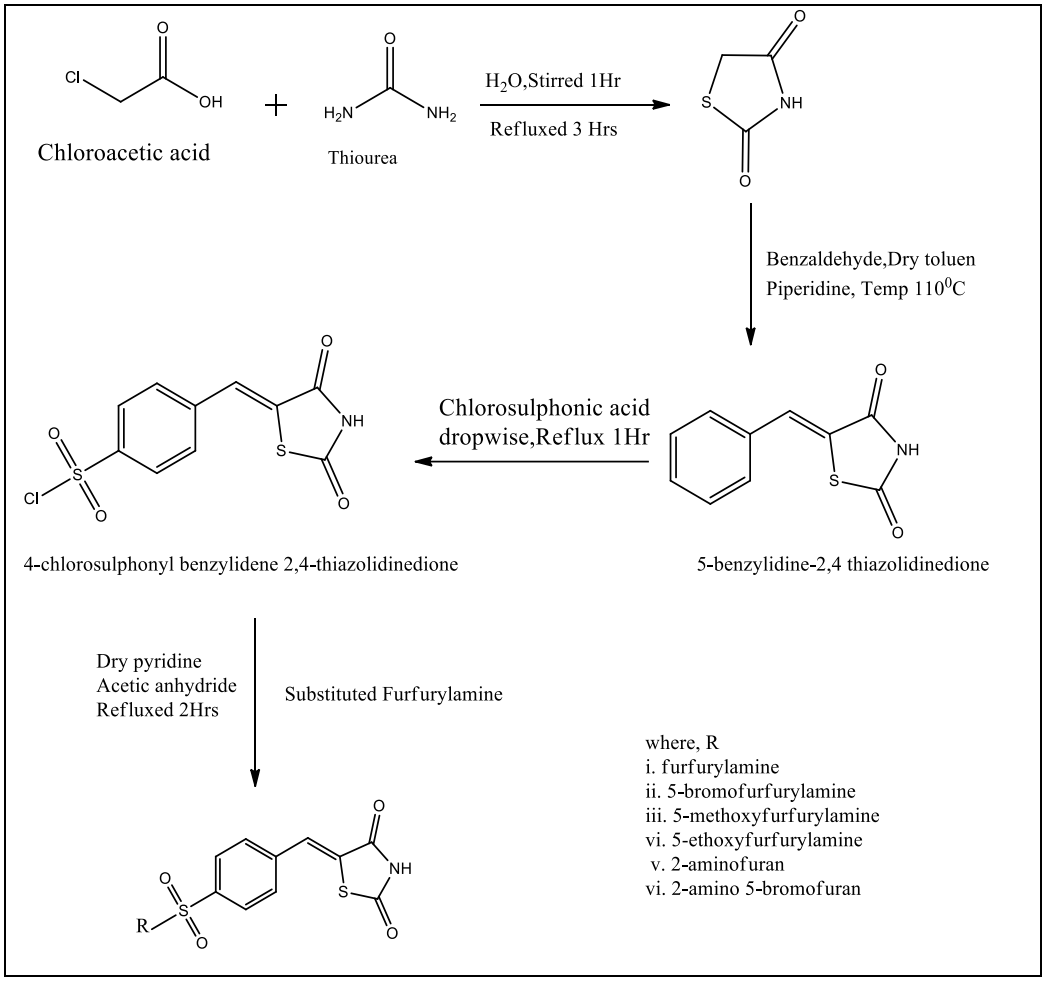
Benzylidine-2, 4-thiazolidinedione (8g, 0.0388 mole) was placed in a 100ml RBF equipped with a condenser and dropping funnel. Chlorosulphonic acid (18.08 g. 0.155 mole) was added at room temperature using dropping funnel. The reaction was found to be exothermic. After addition of Chlorosulphonic acid was over, the

mixture was refluxes for 1 hr on a water bath. The reaction mass was cooled and poured in a thin stream with stirring into crushed ice. It was filtered and dried. Yield 68%, m.p. 180-81°C.

Step IV: Synthesis of 2-amino [5' (4'-sulphonylbenzylidene)]-2, 4-thiazolidinedione

General Procedure for Synthesis: Nucleophilic displacement reaction with 5-(4-chlorosulphonyl benzylidene) thiazolidine-2, 4-dione using primary amine containing substituted furan and furfuryl rings (R) 5-[4-(furfuryl/furan) benzylidene] thiazolidine-2, 4-dione.[10,11]

Fig.1 Synthetic scheme: I



Synthesis of substituted 2-amino [5' (4'-sulphonylbenzylidene)]-2, 4-thiazolidinedione

Table 1: Physicochemical & TLC data of the synthesized compounds (scheme I).

Comp.code	Formula	Mol.Wt.	Melting pt.	R*f* value	% Yield
Am1	$C_{15}H_{12}N_2O_5S_2$	364	239-1	0.47	72
Am2	$C_{15}H_{11}BrN_2O_5S_2$	443	208-9	0.54	73
Am3	$C_{16}H_{14}N_2O_6S_2$	394	171-1	0.85	72
Am4	$C_{17}H_{16}N_2O_6S_2$	408	162-3	0.94	72
Am5	$C_{14}H_{10}N_2O_5S_2$	350	215-6	0.42	74
Am6	$C_{14}H_9BrN_2O_5S_2$	429	227-8	0.51	73

Am1: (Z) 5-[4-(2-aminomethylfuran) sulphonyl benzylidene] thiazolidine-2, 4-dione IR (KBr, cm-1) 3047.13 C-H (Aromatic) Stretching, 821.91 C-H Bending, 1333.24 S=O Asymmetric stretching, 1125.39 S=O Symmetric stretching, 3197.97 N-H (2^0 Amine), Stretching, 1493.08 N-H (2^0 Amine), Bending, 1331.57 C-N (2^0 Amine), Stretching, 1171.84 C=C, C-O (furan), Stretching, 1441.58C=O (furan), Stretching, 1284.90 C-O-C (Aromatic) Stretching.

Am2: (Z) 5-[4-(2-aminomethyl-5-bromofuran) sulphonyl benzylidene] thiazolidine-2, 4-dione IR (KBr, cm-1) 3426.80 N-H (TZD) Stretching, 1492.35 N-H (TZD)Bending, 1737.88, C=O Asymmetric 1682.96 Symmetric stretching, 680.68 C-S-C, 1606.25,1446.31 C=C (Aromatic) Stretching, 3031.96 C-H (Aromatic) Stretching, 855.71 C-H Bending, 1333.24, 1125.39 S=O Asymmetric, Symmetric stretching, 3125.49N-H (2^0 Amine), Stretching1492.35 N-H (2^0 Amine), Bending. M^+443.

Am3 :(Z)5-[4-(2-aminomethyl-5-methoxyfuran) sulphonyl benzylidene] thiazolidine-2, 4-dione IR (KBr, cm-1) 3324.81 N-H (TZD) Stretching, 1494.45 N-H (TZD)Bending, 1739.83, 1685.94 C=O Asymmetric, Symmetric stretching, 681.65 C-S-C, 1607.20,1448.36 C=C (Aromatic) Stretching, 3033.94 C-H (Aromatic) Stretching, 857.71 C-H Bending, 1335.23,1126.36 S=O Asymmetric, Symmetric stretching, 3127.46 N-H (2^0 Amine), Stretching, 1492.35 N-H (2^0 Amine), Bending, 1286.38 1336.27 C-N (2^0 Amine), Stretching, 1168.46 C=C, C-O (furan), Stretching, 1446.14 C=O (furan) Stretching, C-O-C(Aromatic).

Am4: (Z) 5-[4-(2-aminomethyl-5-ethoxyfuran) sulphonyl benzylidene] thiazolidine-2, 4-dione IR (KBr, cm-1) 3423.82 N-H (TZD) Stretching, 1483.40 N-H (TZD)Bending, 1738.80, 1684.95 C=O Asymmetric, Symmetric stretching, 682.671605.24,1447.34 C=C (Aromatic) Stretching, 3032.95 C-H (Aromatic) Stretching, 856.72 C-H Bending, 1334.26, 1127.37 S=O Asymmetric, Symmetric stretching, 3126.47 N-H (20 Amine), Stretching, 1493.36 N-H (20 Amine), Bending, 1332.20 C-N (20 Amine), Stretching, 1166.45 C=C, C-O (furan), Stretching, 1447.16 C=O (furan), Stretching, 1287.37 C-O-C(Aromatic).

Am6: (Z) 5-[4-(2-amino-5-bromofuran) sulphonyl benzylidene] thiazolidine-2, 4-dione IR (KBr, cm-1) 8.3 (S, 1H) NH (TZD), 7.95 (t, 1H) Benzylene, 4.0 (S, 1H) NH (Sulphonamide), 7.66 (d, 2H) 2 &6 benzene, 7.68 (d, 2H) 3 &5 benzene, 7.03 (t, 2H) Furan, 6.43 (t, 2H) Furan. M^+429.

Fig.2 General Structure of Thiazolidinedione (TZD)

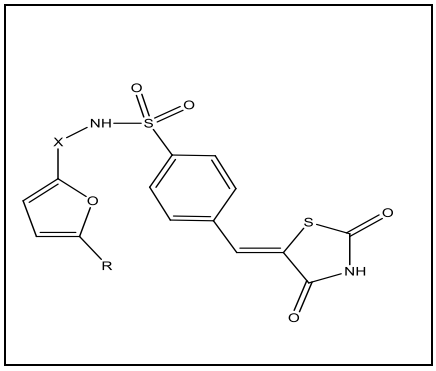
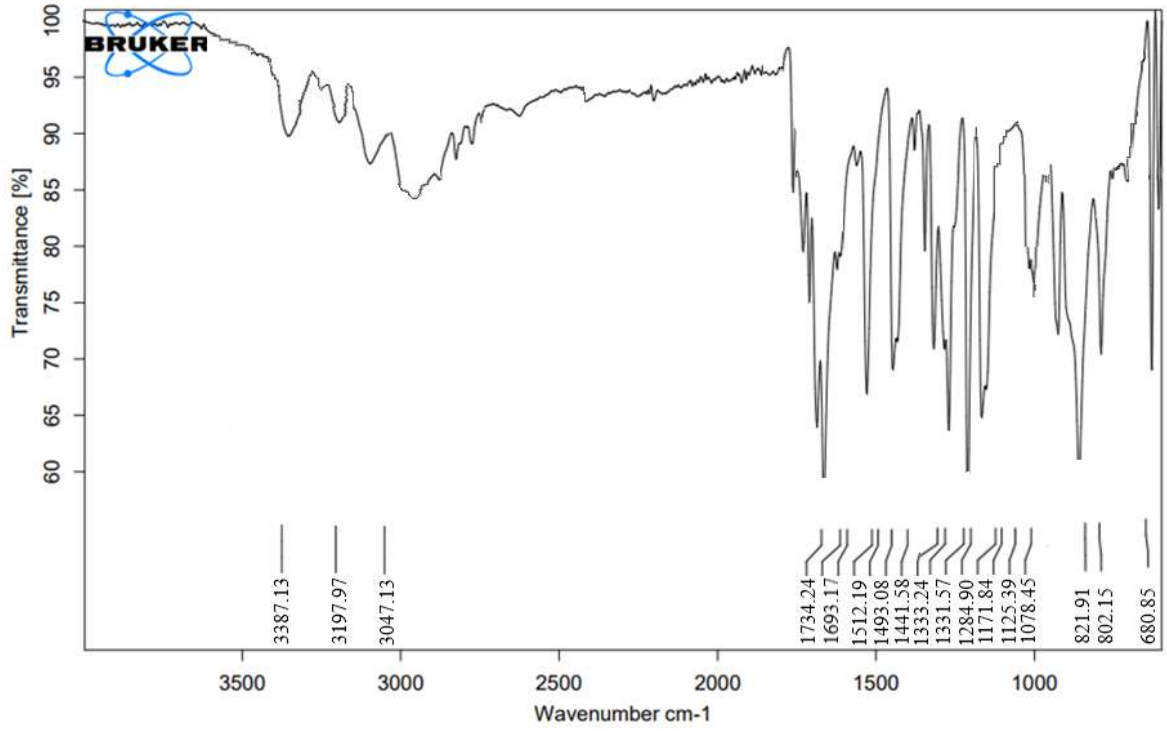


Table.1: Compounds for antioxidant, antibacterial and antifungal activities.

Compound Code	R	X
Am1	H	CH_2

Am2	Br	CH_2
Am3	OCH_3	CH_2
Am4	OC_2H_5	CH_2
Am5	H	N.A
Am6	Br	N.A

IR Spectrum of Compound Am1



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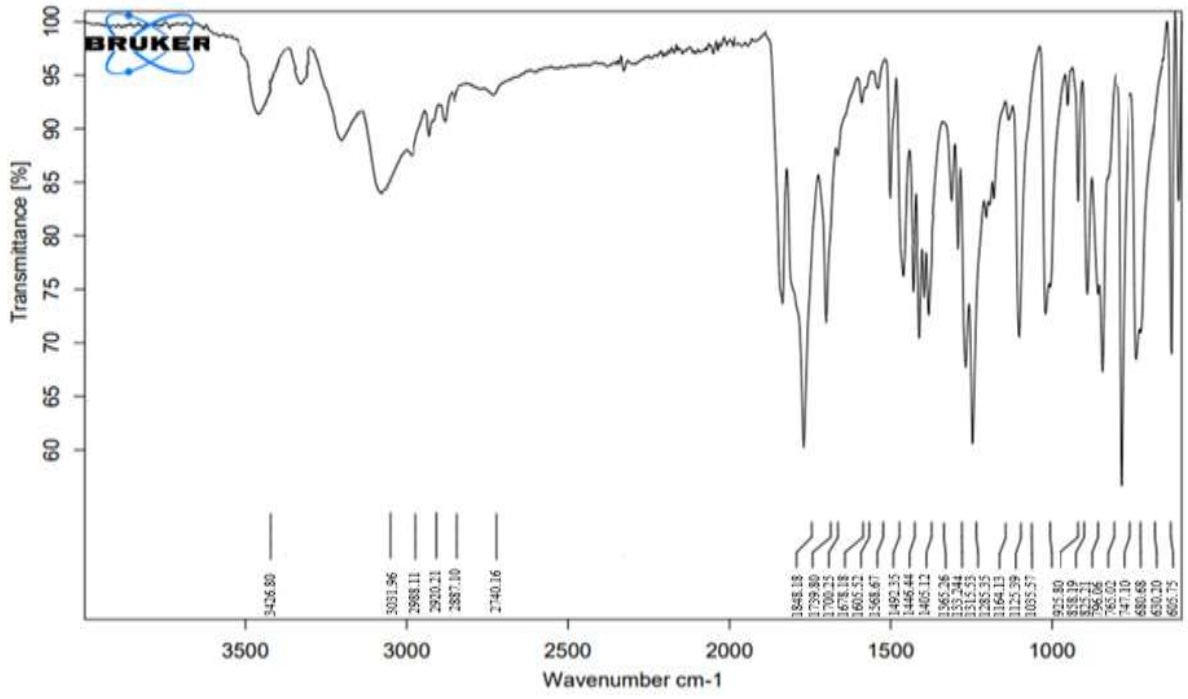
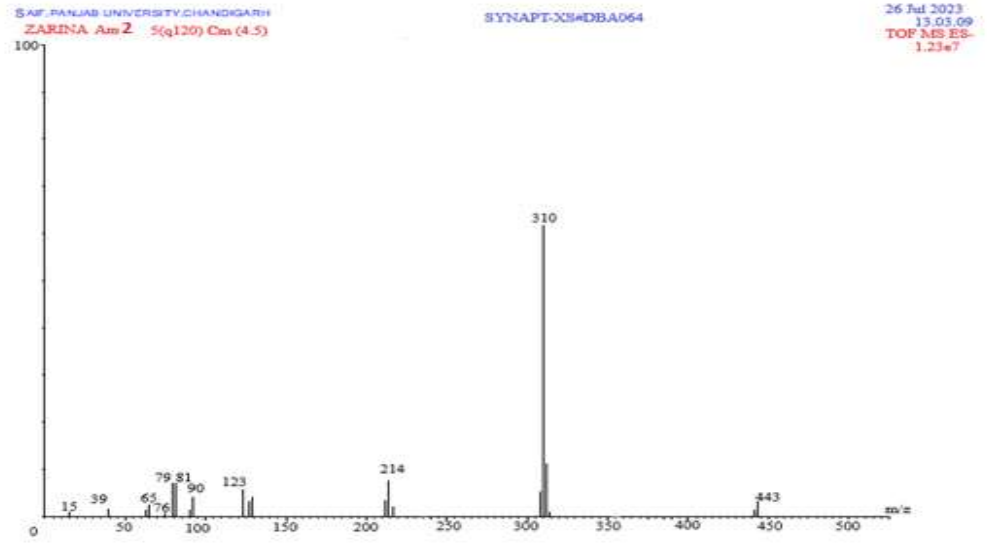
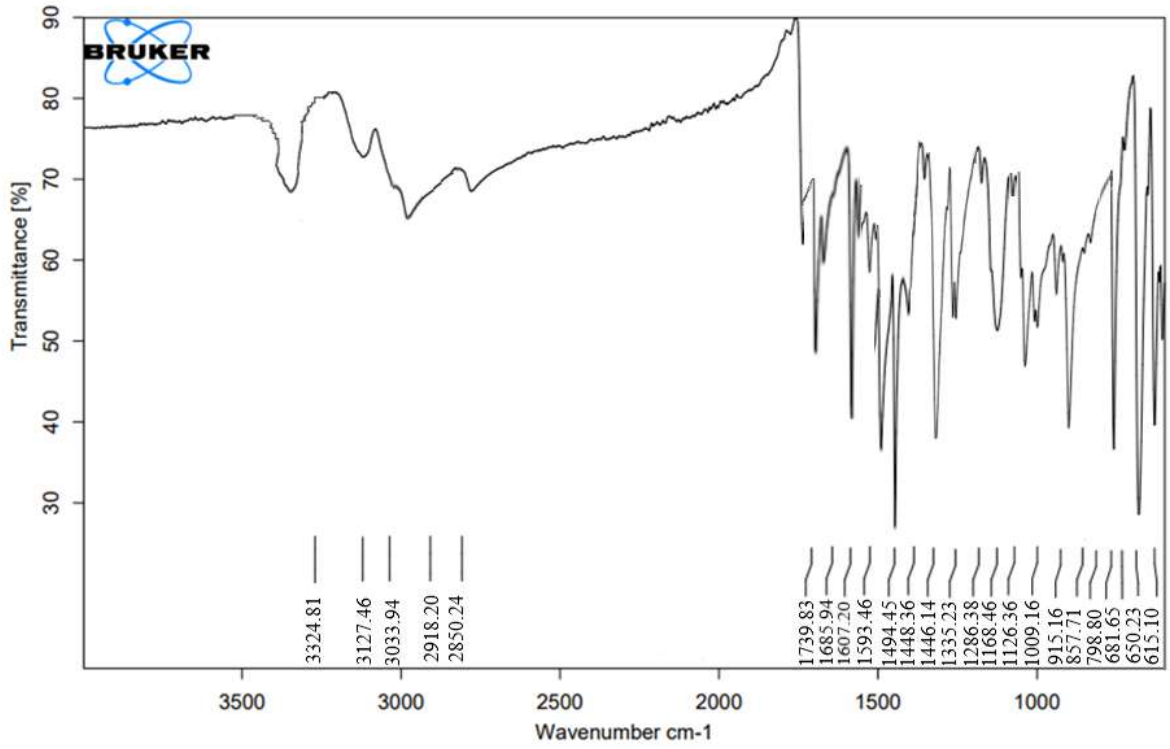
IR Spectrum of Compound Am2

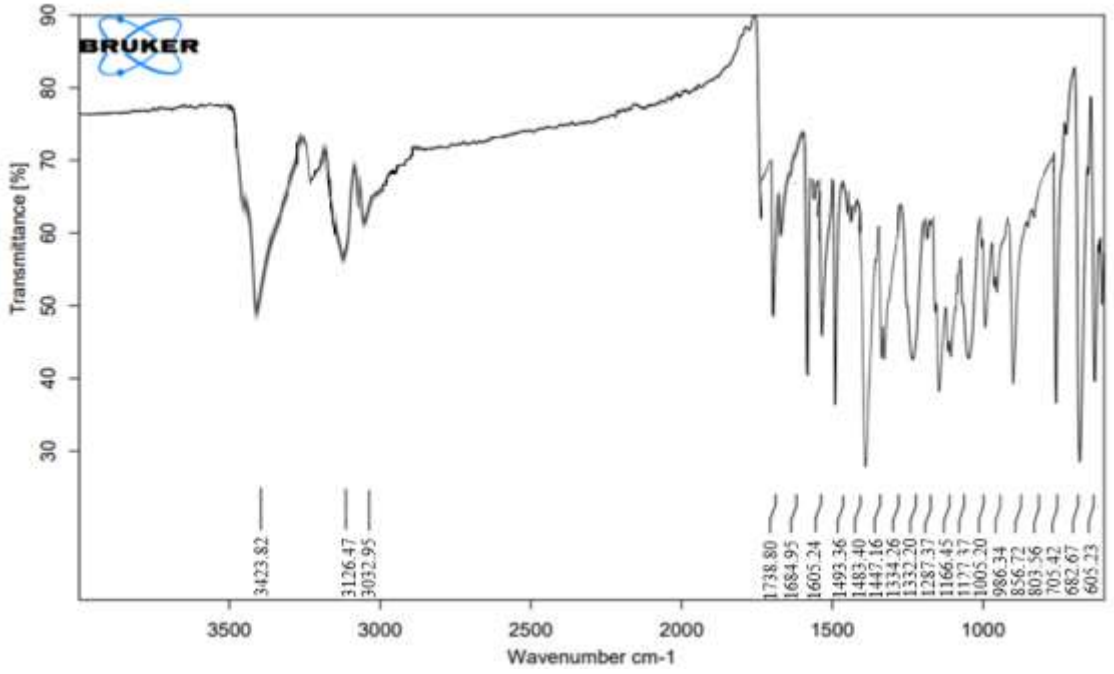




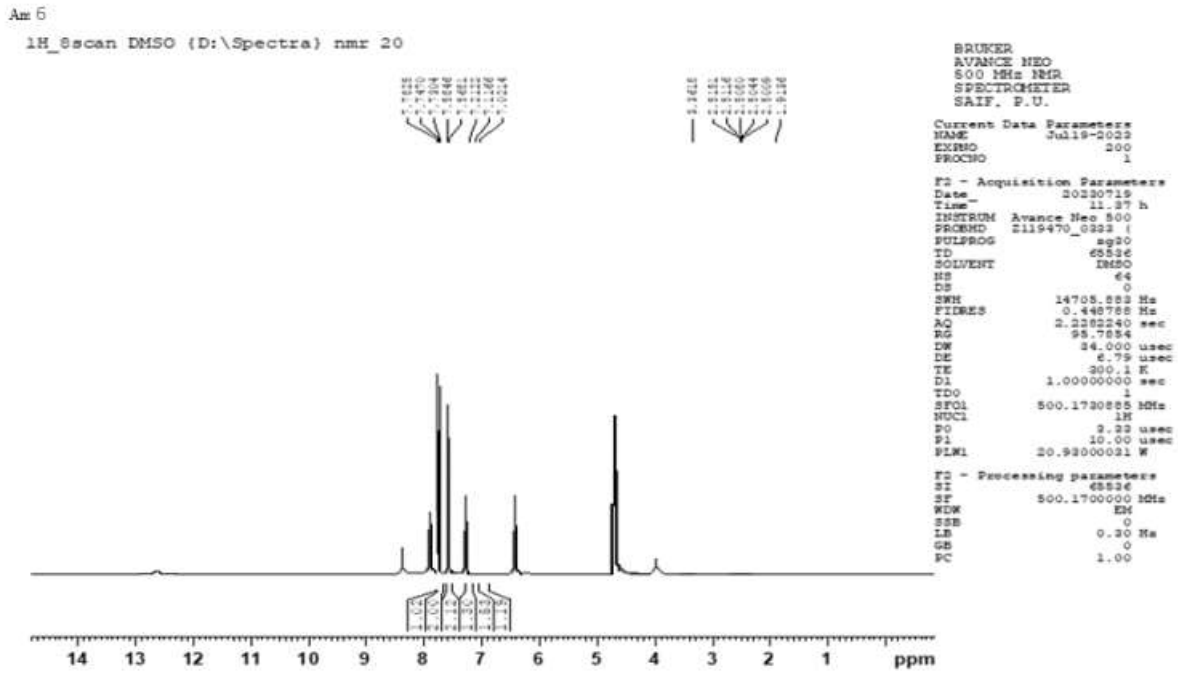
IR Spectrum of Compound Am3



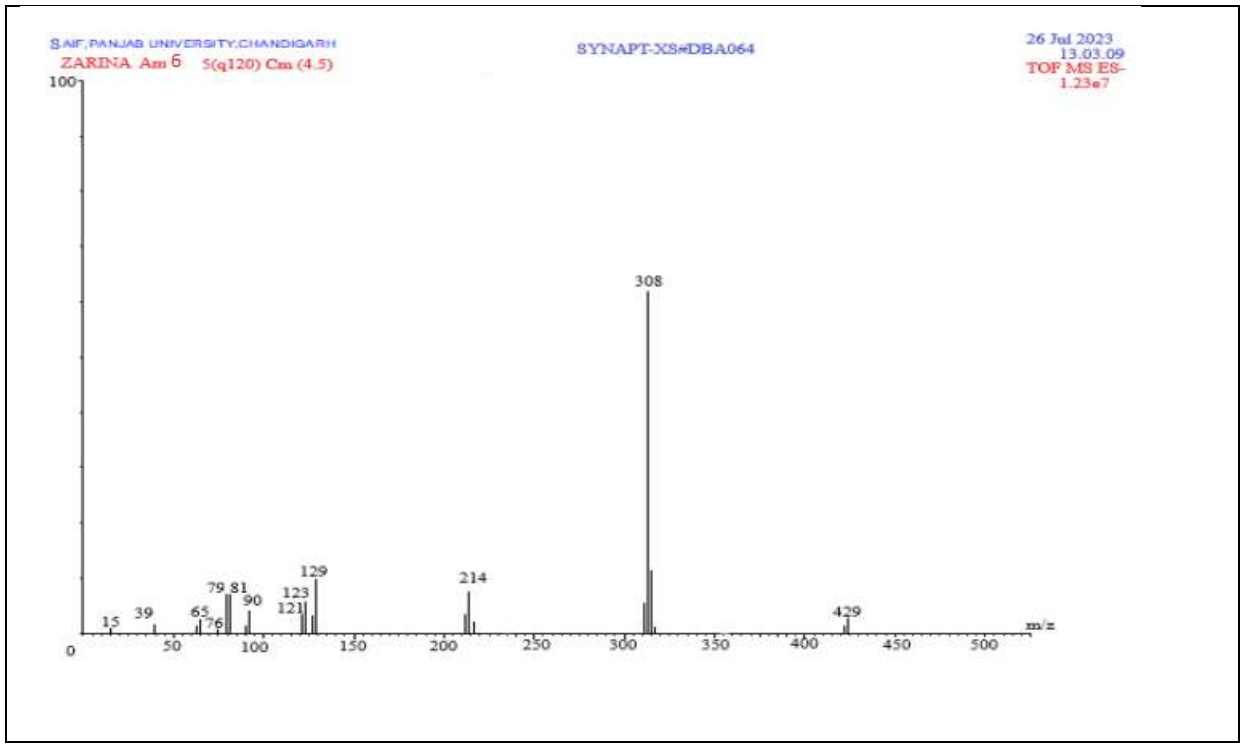
IR Spectrum of Compound Am4



Am6 Proton NMR (DMSO-d_6)



Am6 Mass Spectrum



Antioxidant Activity:

1. **DPPH Scavenging Assay:**

Methodology: 5µl of different stock of the test compound (0µg/ml-1000 µg/ml) was added to 0.1 ml of 0.1mM DPPH solution in a 96 well plate. The reaction was set in triplicate form and duplicates of blank was prepared containing 0.2 ml DMSO/Methanol and 5µl compound of different concentrations (0µg/ml-1000 µg/ml). The plate was incubated for 30 min in dark. At the end of the incubation, the decolorization was read 495 nm (**Table 1, 2**) using a micro plate reader (iMark, BioRad). Reaction mixture containing 20µl of deionized water was served as Control. The scavenging activity was presented as '% inhibition' with respect to control [11, 12]. IC-50 was calculated using Software Graph Pad Prism 6.

Calculations: DPPH Scavenging activity = $((Abs_{Control} - Abs_{Sample})/Abs_{Control}) \times 100$

Table 1: DPPH Scavenging Assay Standard (Ascorbic acid)

Sample Conc. (µg/ml)	Standard (Ascorbic acid)
0	-5.9E-15 ± 2.88
0.78	0.9066 ± 1.88
1.56	1.173 ± 0.43
3.125	8.48 ± 4.74
6.25	20.533 ± 2.15
12.5	39.04 ± 2.11
25	63.94 ± 1.86
50	90.98 ± 2.94

Absorbance at 495 nm using a micro plate reader (iMark, BioRad)

Table 2: DPPH Scavenging Assay Standard (Am1, Am2, Am3 and Am4)

Conc.	Am1	Am2	Am3	Am4
0	0 ± 0.55	0 ± 1.11	0 ± 8.23	0 ± 4.61
1	0.751 ± 1.59	0.30 ± 3.58	4.60 ± 1.02	0.61 ±1.09
10	7.919 ± 1.11	1.21 ± 1.300	7.83 ± 2.08	4.82 ± 4.59
50	12.43 ± 2.29	4.56 ± 0.91	18.78 ± 11.88	7.92 ± 1.45
100	16.69 ± 0.99	5.22 ± 0.94	28.82 ± 8.51	14.72 ± 1.73
250	19.89 ± 0.99	10.10 ± 1.54	33.33 ± 2.03	16.70 ± 3.60
500	58.91 ± 0.75	17.10 ± 1.29	43.28 ± 1.31	18.06 ± 3.82
1000	21.75 ± 16.72	31.16 ± 0.86	58.45 ± 1.81	20.66 ± 1.53

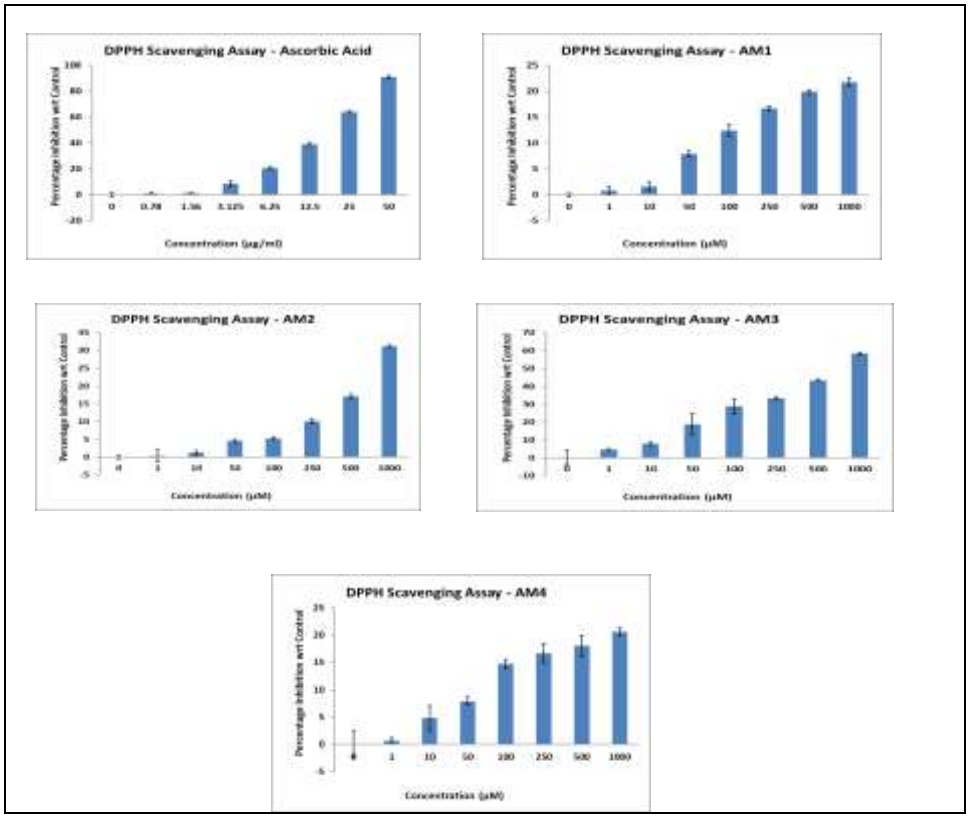
Absorbance at 495 nm using a micro plate reader (iMark, BioRad)

Table 3: IC50 value (µg/ml) Standard (Ascorbic acid) and test compounds

Sample code	IC50 value (µg/ml)
Ascorbic Acid	15.99
AM1	170.78
AM2	243.7
AM3	67.69
AM4	428.42

DPPH Scavenging Assay

Fig.3 DPPH Scavenging Assay (Ascorbic acid, Am1, Am2, Am3 and Am4)



2. ABTS Radical Scavenging Ability

Methodology: ABTS radicles were prepared by mixing APS (2.45 mM) and ABTS (7mM) solution, which was diluted 100X to prepare ABTS free radical reagent. Add 10µl of different stock of the standard (Ascorbic Acid, 1 mg/ml) and samples (0 to 1000µg/ml) to the 200µl of ABTS free radical reagent in 96 well plate and incubated at RT for 10 min in dark. After incubation measure absorbance of the decolorization at 750nm (**Table**

4, 5) using a microplate reader (iMark, BioRad). Results were presented with respect to negative control [13, 14]. IC-50 was calculated using Software Graph Pad Prism 6.

Table 4: ABTS Radical Scavenging Ability Standard (Ascorbic acid)

Sample Conc. (µg/ml)	Standard (Ascorbic acid)
0	0 ± 5.26
0.78	7.62 ± 0.51
1.56	22.32 ± 5.58
3.125	38.36 ± 6.86
6.25	67.11 ± 3.05
12.5	85.29 ± 4.43
25	91.17 ± 1.82
50	95.32 ± 6.48

Absorbance at 750 nm using a micro plate reader (iMark, BioRad)

Table 5: ABTS Radical Scavenging Ability Standard Compounds (Am1, Am2, Am3, Am4)

Conc.	Am1	Am2	Am3	Am4
0	1.39 ± 4.08	0 ± 2.42	8.44± 5.19	0 ± 4.94
1	1.38 ± 3.18	2.05 ± 5.02	1.07± 8.36	1.93 ± 6.97
10	3.63 ± 4.08	7.94 ± 2.88	2.15 ± 3.27	2.44 ± 4.99
50	12.70.75±18.78	16.61±3.27	8.16 ± 5.63	8.30 ± 2.31
100	17.16.14±16.03	20.29±2.47	16.02 ± 9.51	15.02 ± 2.09
250	18.55 ± 10.79	27.05±4.87	24.34 ± 3.07	22.28 ± 5.24
500	58.91 ± 10.79	37.50±2.73	27.58 ± 3.91	28.93 ± 0.50
1000	145.87± 14.61	114.55±21.94	30.50± 14.40	31.27 ± 5.92

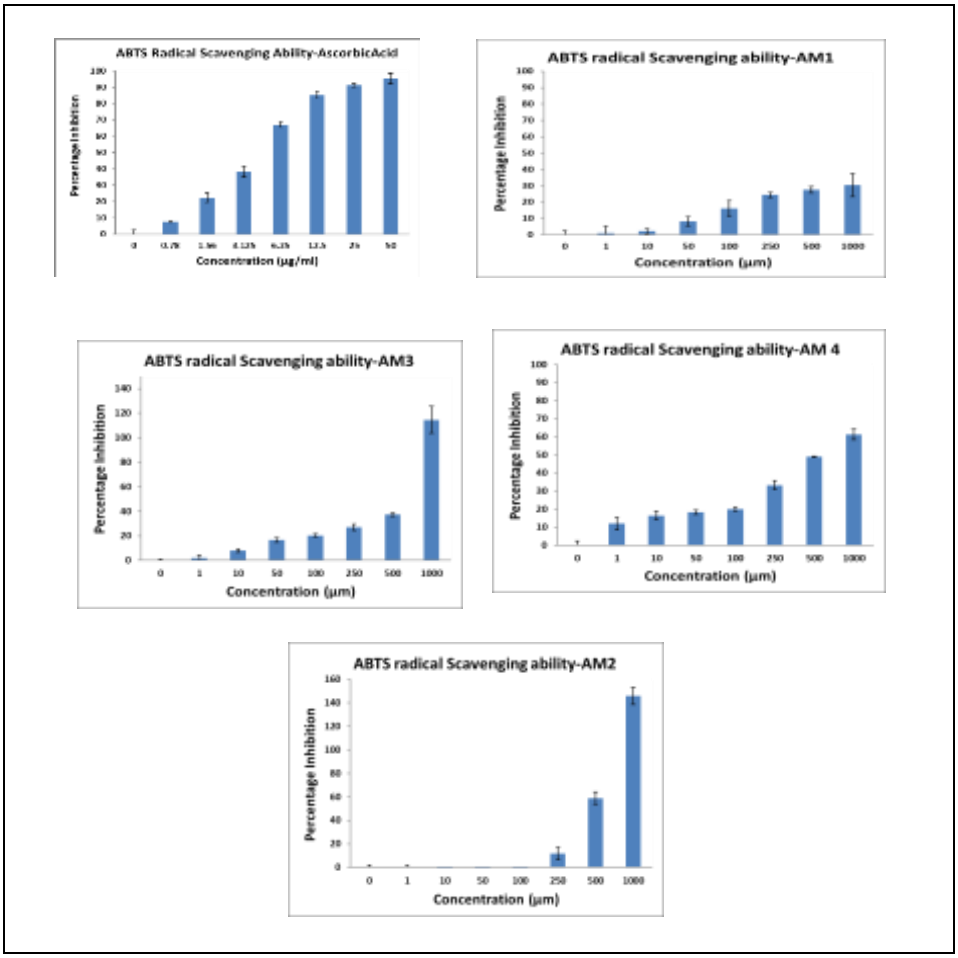
Absorbance at 750 nm using a micro plate reader (iMark, BioRad)

Table 6: IC50 value (µg/ml) Standard (Ascorbic acid) and test compounds

Sample code	IC50 value (µg/ml)
Ascorbic Acid	4.016
AM1	4213
AM2	~ 491.4
AM3	~ 513.7
AM4	615.8

ABTS radical Scavenging ability

Fig.4 ABTS Radical Scavenging Ability (Ascorbic acid, Am1, Am2, Am3 and Am4)



3. Total Antioxidant Assay-FRAP

Procedure: 10µl of different stock of the test compound (final concentration in range from 0,1,10,50,100,250,500,1000µg/ml) and standard (Ascorbic Acid (SRL, Cat no- 23006), 0,0.78,1.56,3.125,6.25,12.5,25 and 50 µg/ml) was added to 0.04 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 0.05 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] (SRL, Cat no- 15766) solution. The reaction mixture was vortexed well and then incubated at 50°C for 20 min using vortexed. At the end of the incubation, 0.5 ml of 10% trichloroacetic acid (SRL, Cat no-92390) was added to the mixture. Then 50µl of deionized water and 50µl of 0.1% ferric chloride (Fischer scientific – Cat no. 23585). The colored solution was read at 750 nm (**Table7, 8**) against the blank using microplate reader (iMark, BioRad) [15]. IC-50 was calculated by using software [11] Graph Pad prism 6.

Table 7: Total Antioxidant Assay-FRAP Standard (Ascorbic acid):

Sample Conc. (µg/ml)	Standard (Ascorbic acid)
0	0±4.65
0.78	-13.95±5.34
1.56	-8.37±11.90
3.125	41.86±29.47
6.25	86.04±30.09
12.5	195.34±38.73
25	487.44±63.93

50	765.58±58.87

Absorbance at 750 nm using a micro plate reader (iMark, BioRad)

Table 8: Total Antioxidant Assay-FRAP Compounds (Am1, Am2, Am3 and Am4):

Conc.	Am1	Am2	Am3	Am4
0	0±5.081007	7.11±8.37	0 ± 3.78	0 ± 3.65
1	7.78±6.41	-11.11 ±5.23	-9.57±5.79	-9.50±5.60
10	3.29±6.80	-3.35 ± 5.99	-7.44±5.79	-7.40±5.70
50	10.77±5.61	-2.84 ± 1.33	-7.18±4.78	-7.10±4.74
100	24.25±8.02	-25.58 ±4.00	0.53±2.60	0.50±2.55
250	61.07±10.64	119.12±44.04	1.06±1.50	1.03±1.40
500	100.59±18.4	-80.62±31.83	4.78±1.84	4.72±1.81
1000	169.46±28.5	-20.41±79.85	12.50±5.3	12.43±5.0

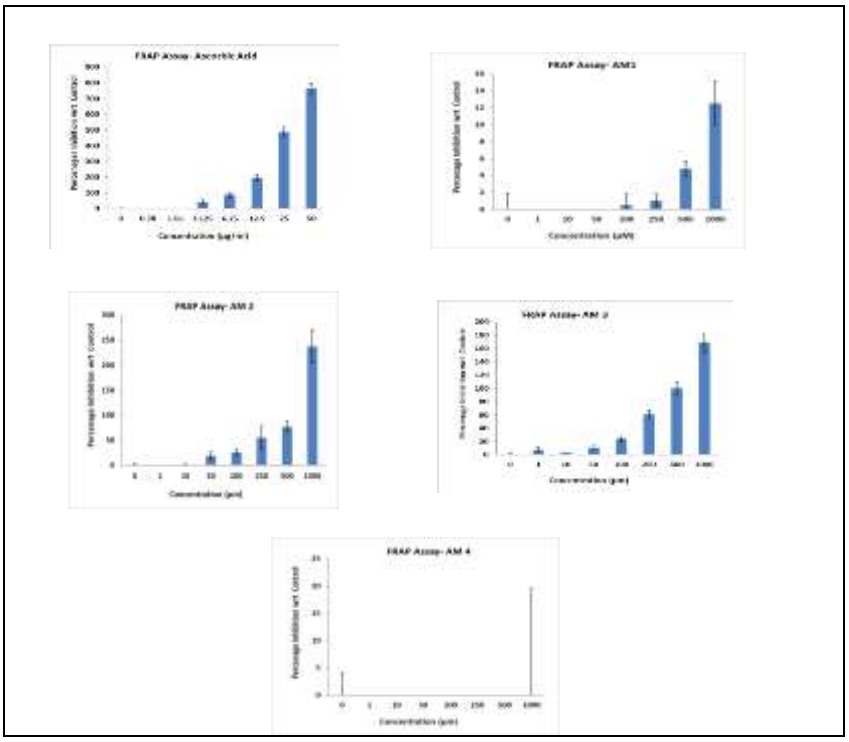
Absorbance at 750 nm using a micro plate reader (iMark, BioRad)

Table 9: IC50 value (µg/ml) Standard (Ascorbic acid) and test compounds

Sample code	IC50 value (µg/ml)
Ascorbic Acid	3.503
AM1	2983
AM2	206.4
AM3	176.8
AM4	Not covered

Total Antioxidant Assay-FRAP

Fig.5 Total Antioxidant Assay-FRAP (Ascorbic acid, Am1, Am2, Am3 and Am4)



4. **Total Antioxidant Assay- CUPRAC Assay**

Reagents

1. Solution A: CuCl2 solution, 10mM Cu (II), is prepared by dissolving 85mg CuCl2.2H2O in 50 ml water.
2. Solution B: Ammonium acetate (NH4Ac) buffer at pH ¼ 7.0, 1.0 M, is prepared by dissolving 3.85 g NH4Ac in 50 ml water.
3. Solution C: Neocuproine solution, 7.5 mM, is prepared daily by dissolving 78 mg Nc in 96% ethanol, and diluting to 50 mL with ethanol.
4. Standard: Trolox, 1mM, is prepared in 96% ethanol.

Reagent Mixture Preparation: Mix Solution A, B & C in equal proportion to make the desired quantity reagent mixture.

Methodology: Add 10µl of different stock of the test compound for samples (0µg/ml-1000 µg/ml) and for Standard (0- 1.25 %). Then add 200µl of reagent mixture in a 96 well plate. Prepare reaction mixture in triplicate form and prepare duplicates of blank containing 200µl Methanol and 10µl of compound of different concentrations for sample and standard and incubate for 30 minutes in dark. At the end of the incubation, absorbance of the decolorization was taken at 490 nm (**Table 10, 11**) using a microplate reader (iMark, BioRad). Reaction mixture containing 20µl of deionized water was served as Control. Scavenging activity was presented as '% inhibition' with respect to control[16, 17]. IC-50 was calculated by using Software Graph Pad Prism 6.[16,17]

Table 10: Total Antioxidant Assay- CUPRAC Assay Standard (Trolox)

Sample Conc. (µg/ml)	Standard (Trolox)
0	0 ± 10.34
0.78	-5.03± 3.71
1.56	3.59± 8.47
3.125	19.42± 2.34
6.25	28.77± 17.00
12.5	71.94± 9.50
25	121.58± 13.39
50	241.00± 36.01

Absorbance at 490 nm using a micro plate reader (iMark, BioRad)

Table 11: Total Antioxidant Assay- CUPRAC Assay Compounds (Am1, Am2, Am3, Am4)

Conc.	Am1	Am2	Am3	Am4
0	0 ±	0 ± 16.22	6.22 ± 5.92	0 ± 7.62
1	6.40 ± 4.23	11.73 ± 4.62	19.76 ± 8.25	9.23 ± 7.63
10	10.29 ± 7.01	17.15 ± 6.89	22.11 ± 8.50	22.69 ± 5.47
50	4.06 ± 9.49	30.69 ± 3.85	25.64 ± 5.62	28.75 ± 5.61

100	25.43 ± 9.34	40.18 ± 8.02	32.94 ± 7.30	36.14 ± 2.02
250	33.00 ± 3.37	52.59 ± 16.25	32.47 ± 7.42	2.67 ± 4.44
500	40.77 ± 6.72	65.23 ± 10.29	36.47 ± 4.13	42.48 ± 6.59
1000	52.96± 20.42	91.64 ± 9.39	40.94 ± 5.76	48.81 ± 12.44

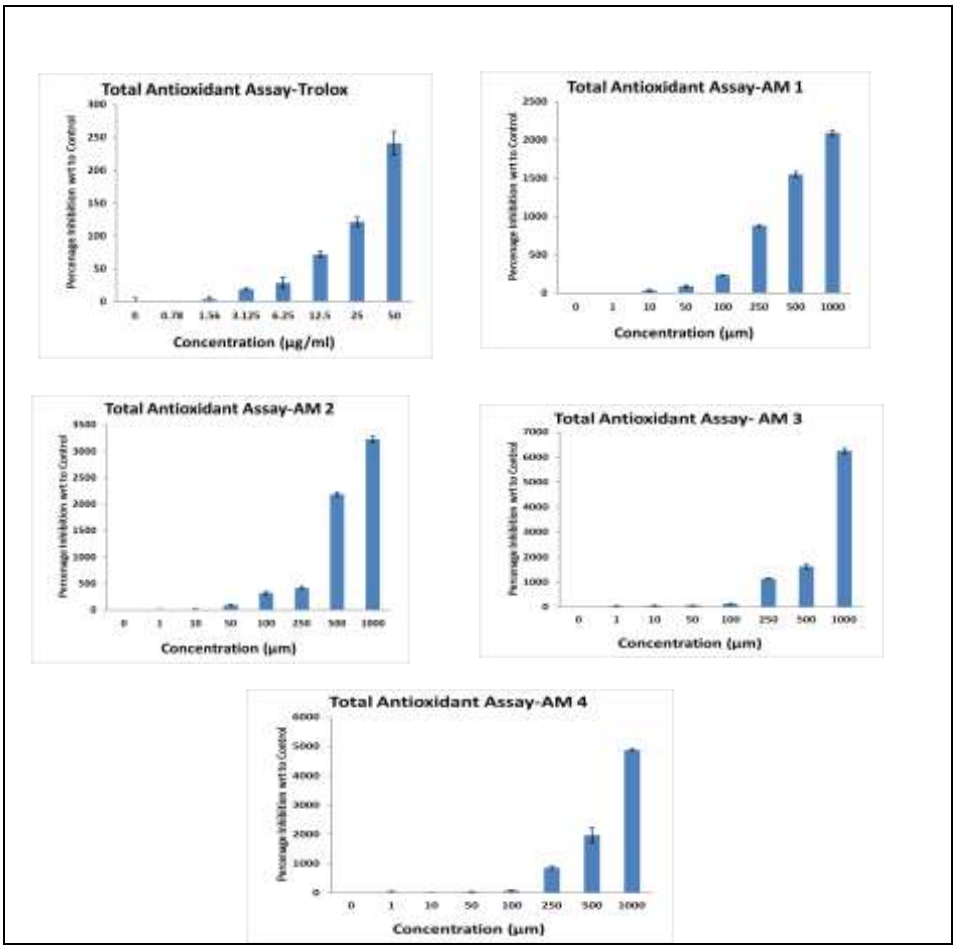
Absorbance at 490 nm using a micro plate reader (iMark, BioRad)

Table 12: IC50 value (µg/ml) Standard (Trolox) and test compounds

Sample code	IC50 value (µg/ml)
Trolox	8.464
AM1	13.13
AM2	16.37
AM3	11.69
AM4	12.37

Total Antioxidant Assay- CUPRAC

Fig.6 Total Antioxidant Assay- CUPRAC Assay (Trolox, Am1, Am2, Am3 and Am4)



5. Super Oxide Anion Radical Scavenging Assay:

Requirements: Sodium Phosphate Buffer (Rankem, Cat no. - S0240), Riboflavin (SRL-Chem – 34392), Gallic Acid (Standard) (SRL-Cat no.-5995-86-8). Reaction Mixture: Potassium pyrophosphate buffer ((Sigma -Adrich-322431), PMS (HiMedia -MB -206), NBT (SRL-Chem-11207), and GSH (SD-fine – F14A/2214/0304/62)

Methodology: Different concentrations of extract and standard (as per mentioned in excel sheet) were added with riboflavin solution and this mixture was incubated for 30 minutes under light at room temperature. After incubation, reaction mixture was added to above incubated mixture and mixed thoroughly. After that, absorbance (**Table 13, 14**) was taken using Elisa plate reader (iMark, Biorad, USA) at 560 nm[18]. IC-50 was calculated using Software Graph Pad Prism 6.

Table 13: Super Oxide Anion Radical Scavenging Assay Standard (Gallic acid)

Sample Conc. (µg/ml)	**Standard (Gallic acid)**
0	0 ± 0.9
0.78	40.14 ± 3.97
1.56	58.24 ± 4.15
3.125	60.27 ± 12.19
6.25	68.95 ± 9.82
12.5	78.36 ± 6.83
25	86.82 ± 4.35
50	97.86 ± 8.27

Absorbance at 560 nm using Elisa plate reader (iMark, Biorad, USA)

Table 14: Super Oxide Anion Radical Scavenging Assay Standard (Am1, Am2, Am3 and Am4)

Conc.	Am1	Am2	Am3	Am4
0	0 ± 0.54	0 ± 0.82	0 ± 0.25	0 ± 0.23
1	48.76 ± 8.34	50.24 ± 6.43	40.62 ± 4.26	38.83 ± 4.62
10	50.15 ± 8.72	55.77 ± 6.22	42.26 ± 7.24	39.15 ± 5.83
50	53.70 ± 5.75	60.73 ± 5.65	48.06 ± 9.40	40.75 ± 3.80
100	63.98 ± 7.34	59.18 ± 3.05	50.48 ± 8.54	44.22 ± 8.05
250	80.44 ± 6.50	62.67 ± 5.48	53.05 ± 3.46	50.64 ± 17.25
500	90.47 ± 4.17	64.50 ± 6.70	55.78 ± 6.84	52.27 ± 13.27
1000	98.95 ± 5.81	66.91 ± 14.43	57.92 ± 20.45	57.64 ± 9.45

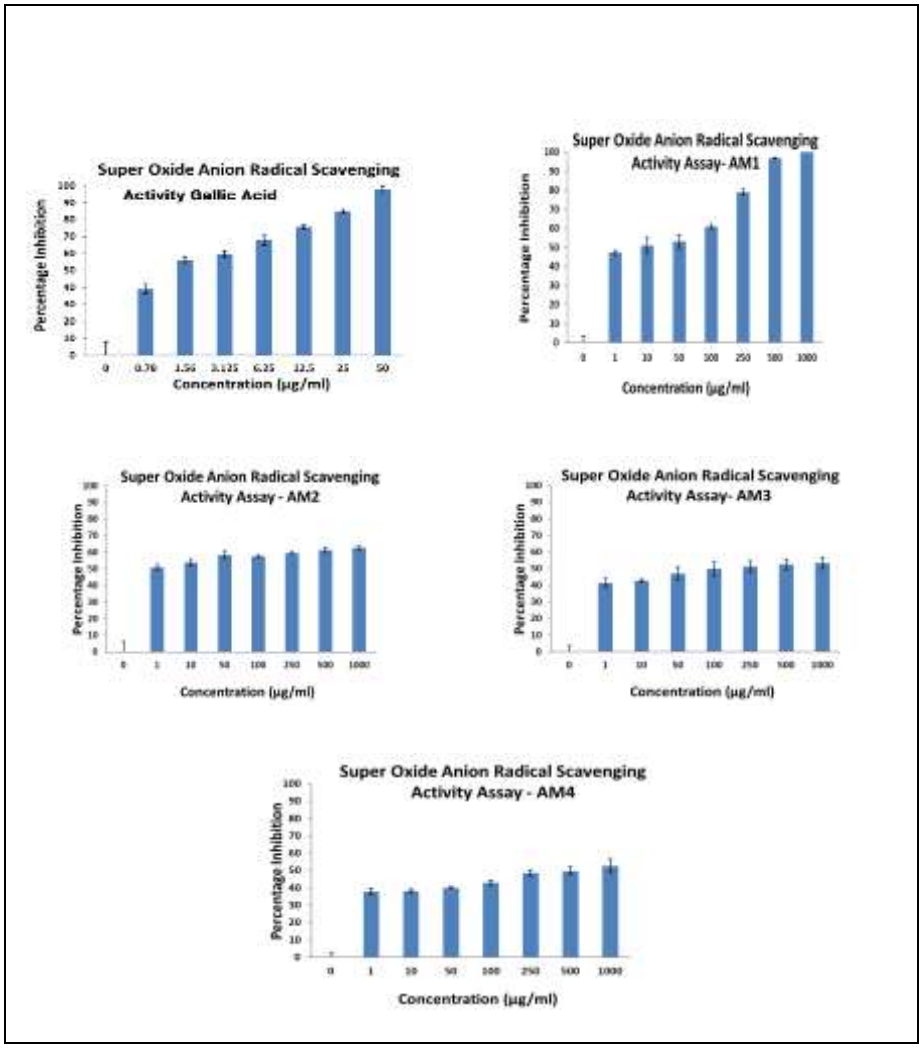
Absorbance at 560 nm using Elisa plate reader (iMark, Biorad, USA)

Table 15: IC50 value (µg/ml) Standard (Gallic Acid) and test compounds

Sample ID	***IC*$_{50}$ Value (µg/ml)**
Gallic Acid	1.489
AM1	5.505
AM2	2.744
AM3	1.64
AM4	7.795

Super Oxide Anion Radical Scavenging Assay

Fig.7 Super Oxide Anion Radical Scavenging Assay (Gallic acid, Am1, Am2, Am3 and Am4)



6. Reactive Nitrogen Oxide Scavenging Assay

Methodology: A reaction mixture was prepared containing 50 µL of 10 mM sodium nitroprusside (Fisher Scientific, Cat no.-27864), 40 µL of distilled water and 10 µl sample/ standard (Gallic Acid – SRL, Cat no.-13142)/ blank. This mixture was pre-incubated at room temperature for 15 minutes in the presence of light. After incubation 100 µl of Griess reagent was added in the test and control wells. And again, incubated for 5-10 mins at room temperature for chromophore development and stabilization. Absorbance was measured at 540 nm and 660 nm (Table 16, 17) using a microplate reader (iMark, BioRad) [19]. IC-50 was calculated using Software Graph Pad Prism 6.

Table 16: Reactive Nitrogen Oxide Scavenging Assay Standard (Gallic acid):

Sample Conc. (µg/ml)	Standard (Gallic acid)
0	3.55 ± 9.21
0.78	9.31 ± 3.40
1.56	14.73 ± 6.13
3.125	20.79 ± 6.25
6.25	23.40 ± 8.21

12.5	30.10 ± 5.516
25	50.70 ± 12.93
50	73.15 ± 3.55

Absorbance at 540 nm and 660 nm using a microplate reader (iMark, BioRad)

Table 17: Reactive Nitrogen Oxide Scavenging Assay Compounds (Am1, Am2, Am3, Am4):

Conc.	Am1	Am2	Am3	Am4
0	-3.60 ± 5.81	0 ± 27.34	5.55 ± 3.24	-5.70 ± 4.09
1	0.34 ± 2.22	4.074 ± 2.55	-2.70 ± 8.04	-1.69 ± 6.67
10	0.87 ± 3.95	9.59 ± 3.05	-1.85 ± 6.20	-2.62 ± 4.50
50	4.06 ± 3.64	12.68 ± 5.62	-1.20 ± 6.11	-2.05 ± 4.61
100	15.21 ± 5.48	13.20 ± 2.34	0.45 ± 7.72	1.02 ± 4.57
250	44.25 ± 2.09	14.38 ± 0.49	3.50 ± 7.48	2.67 ± 4.44
500	48.19 ± 5.31	18.61 ± 1.39	5.95 ± 7.61	7.30 ± 7.39
1000	52.96 ± 4.83	26.04 ± 2.08	11.10 ± 5.13	21.28 ± 12.44

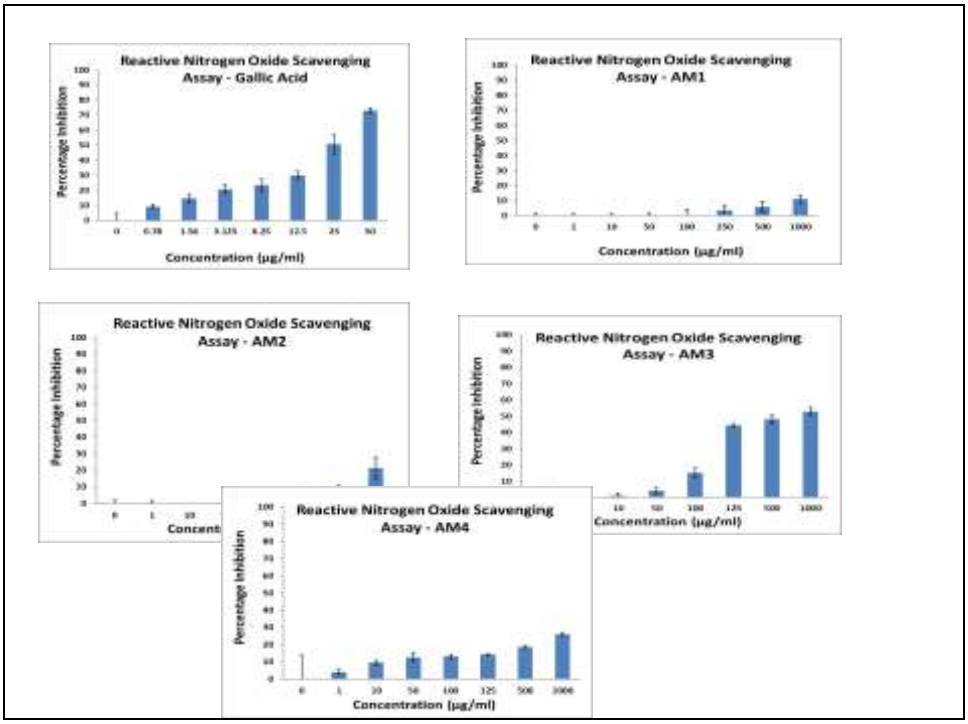
Absorbance at 540 nm and 660 nm using a microplate reader (iMark, BioRad)

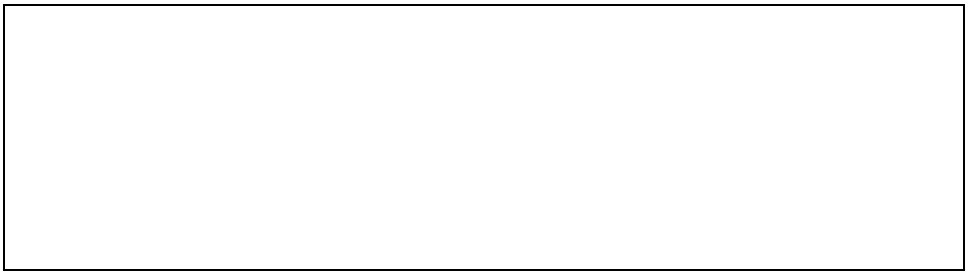
Table 18: IC50 value (µg/ml) Standard (Gallic Acid) and test compounds

Sample code	IC50 value (µg/ml)
Gallic Acid	22.2
AM1	6367
AM2	2112
AM3	606.7
AM4	6899

Reactive Nitrogen Oxide Scavenging Assay

Fig.8 Reactive Nitrogen Oxide Scavenging Assay (Gallic acid, Am1, Am2, Am3 and Am4)





7. Hydroxyl Free Radical Scavenging Assay

Methodology: 66µl Reagent Mixture (10µl EDTA (0.5M), 24.14 mg Deoxyribose, 88µl FeCl3 (10mg/ml), 28 µl H_2O_2 (6%), water upto 33 ml), 10µl of plant extract (1-1000µg/ml), 24µl of phosphate buffer (50 mM, pH 7.4) and 10µl of ascorbic acid were added in the wells of 96 well plate in sequence and the mixture was incubated at 37°C for 1hr. Gallic Acid (as per mentioned in the excel sheet) was used as standard. After incubation 50µl of 10% TCA and 50µl of 1% TBA were added to each well. A pink chromogen was developed. After that absorbance was taken at 540 nm wavelength (**Table 19, 20**) using microplate reader (iMark, BioRad) [20, 21]. Scavenging activity was calculated by the following formula…

$$\frac{A(control)-A(sample)}{A(control)} x\,100$$

A (control): Absorbance of the control and A (Sample): Absorbance of the extracts/standard.

Table 19: Hydroxyl Radical Scavenging Assay Standard (Gallic acid)

Sample Conc. (µg/ml)	Standard (Gallic acid)
0	0 ± 13.61
0.78	5.75 ± 5.58
1.56	15.54 ± 3.81
3.125	23.99 ± 10.04
6.25	28.98 ± 8.00
12.5	45.10 ± 6.04
25	54.70 ± 4.29
50	70.82 ± 9.26

Absorbance at 540 nm using a microplate reader (iMark, BioRad)

Table 20: Hydroxyl Radical Scavenging Assay Compounds (Am1, Am2, Am3 and Am4)

Conc.	Am1	Am2	Am3	Am4
0	0 ± 17.55	0 ± 16.22	6.22 ± 5.92	0 ± 7.62
1	6.40 ± 4.23	11.73 ± 4.62	19.76 ± 8.25	9.23 ± 7.63
10	10.29 ± 7.01	17.15 ± 6.89	22.11 ± 8.50	22.69 ± 5.47
50	4.06 ± 9.49	30.69 ± 3.85	25.64 ± 5.62	28.75 ± 5.61
100	25.43 ± 9.34	40.18 ± 8.02	32.94 ± 7.30	36.14 ± 2.02
250	33.00 ± 3.37	52.59 ± 16.25	32.47 ± 7.42	2.67 ± 4.44
500	40.77 ± 6.72	65.23 ± 10.29	36.47 ± 4.13	42.48 ± 6.59
1000	52.96± 20.42	91.64 ± 9.39	40.94 ± 5.76	48.81 ± 12.44

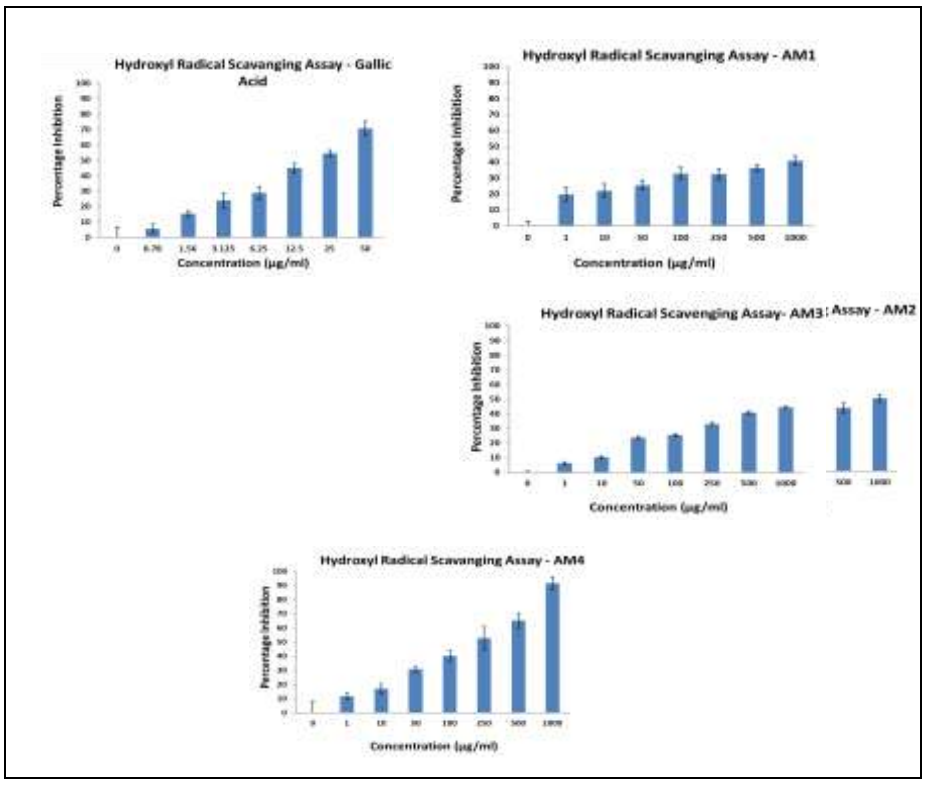
Absorbance at 540 nm using a microplate reader (iMark, BioRad)

Table 21 IC50 value (µg/ml) Standard (Gallic Acid) and test compounds

Sample code	IC50 value(µg/ml)
Gallic Acid	17.3
AM1	15024
AM2	1143
AM3	155.3
AM4	1570

Hydroxyl Radical Scavenging Assay

Fig.8 Hydroxyl Radical Scavenging Assay (Gallic acid, Am1, Am2, Am3 and Am4)



8. Minimum Inhibitory Concentration Activity – B. subtilis

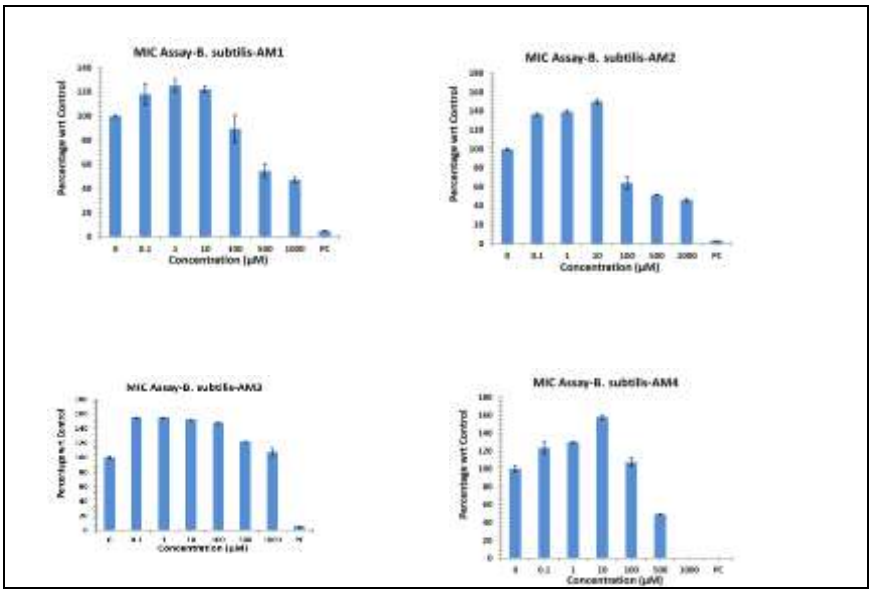
Experiments: 0.5 Mc farland Standard dilution of microbes to be used for the study. 100 µl diluted log cultures of bacteria (*B. subtilis* – MTCC 1133) was added to the micro centrifuge tube and added with 5 µl of prepared treatment dilutions of different concentrations (Mentioned in excel sheet) to the defined tubes and incubated for the 24 hours. After Incubation all content was transferred to the 96 well plate and turbidity reading was taken by Elisa Plate Reader (iMark Biorad) at 630 nm. Ciprofloxacin (SRL Chem- 78079) (10µg) was used as Positive Control.[22,23,24]

Table 22: IC50 Value (µM) and MIC (µM) – B. subtilis

Sample Code	IC50 Value(µM)	MIC (µM)
AM1	763.5 ± 0.216	120
AM2	578.6 ± 0.420	50
AM3	298.6 ± 0.564	100
AM4	Not converged	Above Maximum Dose Limit

Turbidity at 630 nm using Elisa Plate Reader (iMark Biorad)

Fig.9 Minimum Inhibitory Concentration Activity – B. subtilis (Am1, Am2, Am3 and Am4)



9.* Minimum Inhibitory Concentration Activity – *E. coli

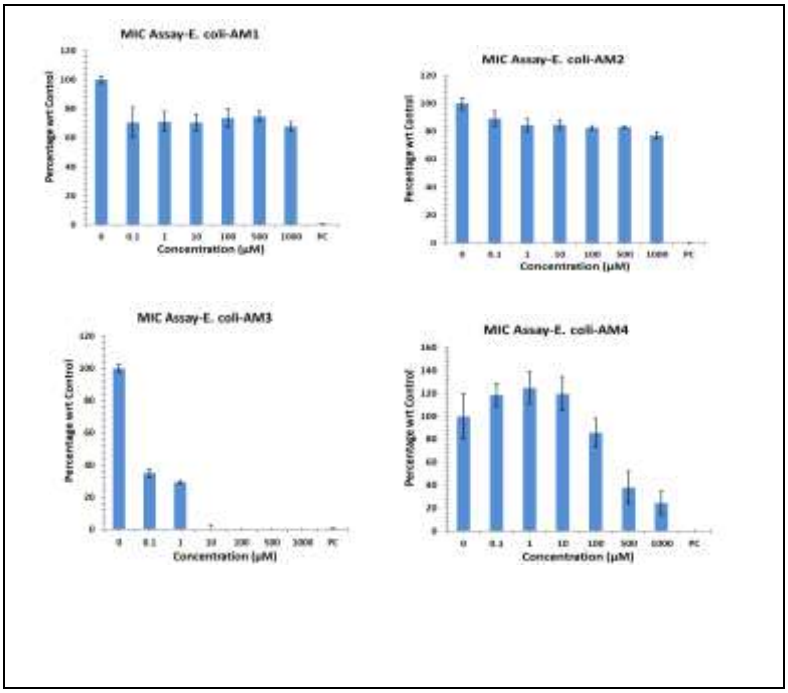
Experiments: 0.5 Mc farland Standard dilution of microbes to be used for the study. 100 µl diluted log cultures of bacteria (*E. coli* – MTCC452) was added to the micro centrifuge tube and added with 5 µl of prepared treatment dilutions of different concentrations (0,0.1,1,10,100,500,1000) to the defined tubes and incubated for the 24 hours. After Incubation all content was transferred to the 96 well plate and turbidity reading was taken by Elisa Plate Reader (iMark Biorad) at 630 nm. Ciprofloxacin (10µg) was used as Positive Control [25,26, 27]

Table 23: Minimum Inhibitory Concentration Activity – E-coli

Conc. (µM)	Am1	Am2	Am3	Am4
0	100 ± 5.42	100± 8.38	100±4.897	100±38.9716
0.1	70.60±20.85	89.26±10.91	35.10±5.070	118.45±20.529
1	71.15±13.95	84.45±9.44	29.15±2.176	124.89±28.126
10	70.51±11.55	84.75±7.27	-0.63±6.709	119.74±29.323
100	73.57 ± 13.0	82.59±3.47	-8.43±6.238	85.83±24.724
500	74.78 ± 7.47	83.19±1.52	-2.55±3.382	37.76±28.425
1000	67.70 ±6.61	77.04±5.16	-1.72±1.523	24.46±20.397
PC	0.51±0.38	0.40±0.25	0.60±0.218	-5.36±3.599
IC50	1575± 0.348	2755± 0.230	0.06863±0.196	374.4 ± 0.214
MIC	0.08	1	0.01	100

Turbidity at 630 nm using Elisa Plate Reader (iMark Biorad)

Fig.10 Minimum Inhibitory Concentration Activity – E-coli (Am1, Am2, Am3 and Am4)



10. **Minimum Inhibitory Concentration Activity – P. aeruginosa**

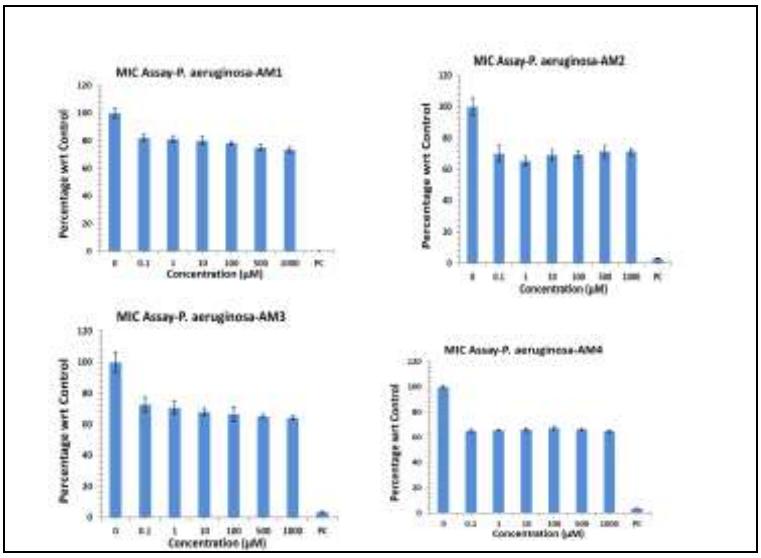
Experiments: 0.5 Mc farland Standard dilution of microbes to be used for the study. 100 µl diluted log cultures of bacteria (*P. aeruginosa*- MTCC 3541) was added to the micro centrifuge tube and added with 5 µl of prepared treatment dilutions of different concentrations (Mentioned in excel sheet) to the defined tubes and incubated for the 24 hours. After Incubation all content was transferred to the 96 well plate and turbidity reading was taken by Elisa Plate Reader (iMark Biorad) at 630 nm. Ciprofloxacin (10µg) was used as Positive Control [28,29,30]

Table 24: Minimum Inhibitory Concentration Activity – P. aeruginosa

Conc.(µM)	Am1	Am2	Am3	Am4
0	100±7.379	100± 8.38	100±4.897	100±38.9716
0.1	82.12±5.157	89.26±10.91	35.10±5.070	118.45±20.529
1	81.09±5.157	84.45±9.44	29.15±2.176	124.89±28.126
10	80.11±5.795	84.75±7.27	-0.63±6.709	119.74±29.323
100	78.20±2.651	82.59±3.47	-8.43±6.238	85.83±24.724
500	75.00±4.282	83.19±1.52	-2.55±3.382	37.76±28.425
1000	73.55±3.392	77.04±5.16	-1.72±1.523	24.46±20.397
PC	0.15±0.616	0.40±0.25	0.60±0.218	-5.36±3.599
IC50	1958± 0.260	1574 ± 0.388	1096 ± 0.332	1152 ± 0.377
MIC	0.1	1	0.01	100

Turbidity at 630 nm using Elisa Plate Reader (iMark Biorad)

Fig.11 Minimum Inhibitory Concentration Activity – *P. aeruginosa* (Am1, Am2, Am3 and Am4)



Anti-fungal activity assay:

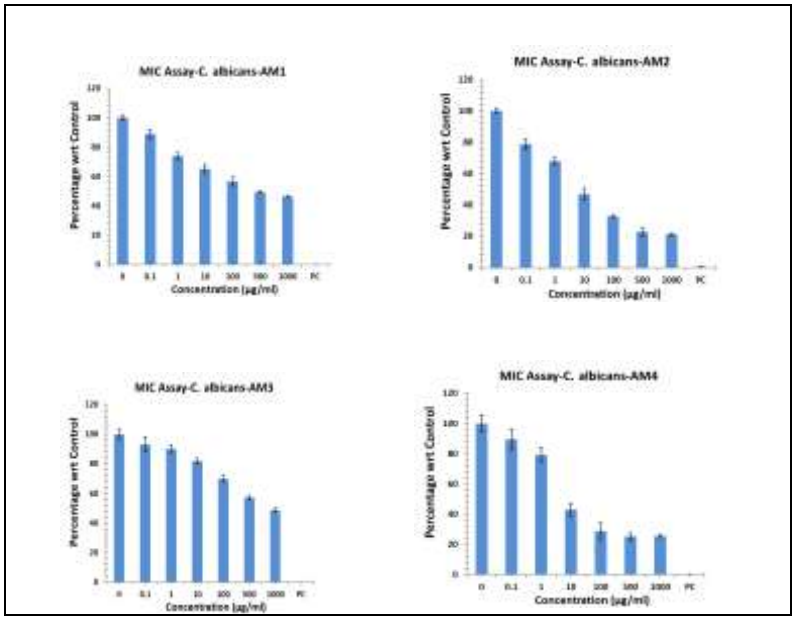
Experiments: 0.5 Mc farland Standard dilution of microbes to be used for the study. 100 µl diluted log cultures of test organism (*C. albicans* – MTCC 854) was added to the micro centrifuge tube and added with 5 µl of prepared treatment dilutions of different concentrations (Mentioned in excel sheet) to the defined tubes and incubated for the 24 hours. After Incubation all content was transferred to the 96 well plate and turbidity reading was taken by Elisa Plate Reader (iMark Biorad) at 630 nm. Amphotericin B (50 µg) was used as Positive Control 31,32

Table 25: IC50 Value (µg/ml) Minimum Inhibitory Concentration Activity – C. albicans

Sample Code	MIC (µg/ml)	IC50 Value (µg/ml)
AM1	Approx 0.3	412.5 ± 0.281
AM2	Approx 0.08	9.181 ± 0.339
AM3	Approx 10	658.4 ± 0.151
AM4	Approx 0.08	9.11 ± 0.298

Turbidity at 630 nm using Elisa Plate Reader (iMark Biorad)

Fig.12 Minimum Inhibitory Concentration Activity – *C. albicans* (Am1, Am2, Am3 and Am4)



11. ADMET and toxicity Prediction of Synthesized Compounds

Online tools such as PreADMET, Molinspiration, and Protox-II were used for predicting ADMET properties. PreADMET (https://preadmet.webservice.bmdrc.org/) and Protox-II (https://tox.charite.de/protox3/).The computer system used had an Intel® Core™ i5 9th Gen CPU, 4GB RAM, and a 500GB hard disk, running on Windows 10.

Table 26: ADME Predictions of Synthesized compounds.

Comp. Code	GI	BBB	Bioavailability Score	Skin Permeation Log Kp (cm/s)	Lipophilicity Log P	Synthetic accessibility	Molar Refractivity	TPSA
AM1	Low	No	0.55	-7.27	1.24	3.40	91.78	139.16
AM2	Low	No	0.55	-7.03	1.66	3.43	99.48	139.16
AM3	Low	No	0.55	-7.24	1.86	3.59	98.27	148.39
AM4	Low	No	0.55	-7.07	2.03	3.70	103.08	148.39
AM5	Low	No	0.55	-6.93	1.46	3.33	88.51	139.16
AM6	Low	No	0.55	-6.93	1.82	3.36	96.21	139.16

Online tools PreADMET, Molinspiration

Table 27: Protox-II -prediction of toxicity of synthesized compounds.

Comp. code	Predicted LD50 (mg/kg)	Predicted Accuracy	Hepatotoxicity	Carcinogenicity	Mutagenicity	Immunotoxicity	Cytotoxicity
AM1	1400	54.26	Inactive	Inactive	Inactive	Inactive	Inactive
AM2	1400	23.00	Inactive	Inactive	Inactive	Inactive	Inactive
AM3	350	23.00	Inactive	Inactive	Inactive	Inactive	Inactive
AM4	350	23.00	Inactive	Inactive	Inactive	Inactive	Inactive
AM5	1400	54.26	Active	Inactive	Inactive	Inactive	Inactive
AM6	1400	54.26	Active	Inactive	Inactive	Inactive	Inactive

Online tool Protox-II -prediction of toxicity

Discussion:

Antioxidant Activity Assays DPPH Scavenging Assay (Table 3) Ascorbic Acid shows the highest scavenging activity with an IC50 of 15.99 µg/ml. AM3 follows at 67.69 µg/ml. AM1, AM2, and AM4 show decreasing effectiveness with IC50 values of 170.78, 243.7, and 428.42 µg/ml, respectively. ABTS Radical Scavenging (Table 6) Ascorbic Acid is most effective with an IC50 of 4.016 µg/ml. AM2 and AM3 have moderate activity (197.4 and 205.9 µg/ml). AM4 is weaker (247.4 µg/ml). AM1 shows negligible activity. Total Antioxidant Assay-FRAP (Table 9) Ascorbic Acid is most potent (IC50 = 3.503 µg/ml). AM3 and AM2 follow with IC50 values of 176.8 and 206.4 µg/ml, respectively. AM1 is least effective (2983 µg/ml).CUPRAC Assay (Table 12) Trolox has an IC50 of 8.464 µg/ml. AM3 shows the lowest IC50 (0.68 µg/ml), indicating strong activity. AM1 and AM2 show moderate activity (5.29 and 6.59 µg/ml). AM4 is weakest (23.89 µg/ml).Superoxide Anion Scavenging (Table 15) Gallic Acid is most effective (IC50 = 1.489 µg/ml). AM3 follows closely (1.64 µg/ml). AM2 and AM1 have moderate and lower activity (2.744 and 5.505 µg/ml). AM4 is least effective (7.795 µg/ml).Reactive Nitrogen Oxide Scavenging (Table 18) Gallic Acid is most effective (IC50 = 22.2 µg/ml). AM3, AM2, AM1, and AM4 follow with IC50 values of 606.7, 2112, 6367, and 6899 µg/ml, respectively. Hydroxyl Radical Scavenging (Table 21) Gallic Acid is most effective (IC50 = 17.3 µg/ml). AM3 shows good activity (155.3 µg/ml). AM4 and AM2 have IC50 values of 1570 and 1143 µg/ml, respectively. AM1 is least effective (15,024 µg/ml).Antimicrobial Activity Results Bacillus subtilis (Table 22) AM3 is the most potent with an IC_{50} of 298.6 µM and a MIC of 100 µM. AM2 follows with an IC_{50} of 578.6 µM and a MIC of 50 µM. AM1 has an IC_{50} of 763.5 µM and a MIC of 120 µM. AM4 exceeds the maximum dose limit. Escherichia coli (Table 23) AM3 is most effective, with a MIC of 0.01 µM and an IC_{50} of 0.06863 µM. AM1 has a MIC of 0.08 µM and an IC_{50} of 1575 µM. AM2's MIC is 1 µM, with an IC_{50} of 2755 µM. AM4 is least effective, with a MIC of 100 µM and an IC_{50} of 374.4 µM .Pseudomonas aeruginosa (Table 24) AM3 has the lowest IC_{50} (1096 µM) and a MIC of 0.01 µM, indicating high effectiveness. AM1 and AM2 have MICs of 0.1 µM and 1 µM, respectively. AM4 is least effective with a MIC of 100 µM. Candida albicans (Table 25) AM2 and AM4 are most effective with MICs of 0.08 µg/ml and IC_{50} values around 9 µg/ml. AM1 has an IC_{50} of 412.5 µg/ml. AM3 is least effective with an IC_{50} of 658.4 µg/ml. Other Findings (Table 26) All compounds show low GI absorption and do not cross the BBB, with a bioavailability score of 0.55 and negative Log Kp values indicating low skin permeation. Synthetic accessibility scores range from 3.33 to 3.70, suggesting moderate ease of synthesis. Molar Refractivity and TPSA values indicate moderate polarity. Toxicity Predictions (Table 27) AM1 and AM2 have an LD50 of 1400 mg/kg, while AM3 and AM4 have an LD50 of 350 mg/kg, suggesting higher potential toxicity for AM3 and AM4. All compounds are predicted to be inactive in hepatotoxicity, carcinogenicity, mutagenicity, Immunotoxicity, and cytotoxicity. AM1 has a higher predicted accuracy (54.26%) compared to the others (23.00%).

Conclusion:

All the new synthesized compounds were tested for their in vitro antioxidant activity using DPPH assay, Antioxidant Assay- CUPRAC Assay, ABTS Radical Scavenging Ability, Reactive Nitrogen Oxide Scavenging Assay and Hydroxyl Free Radical Scavenging Assay and antibacterial activity against the Gram-positive viz. Bacillus subtilis. Gram-negative viz. *E.coli, Pseudomonas aeruginosa* and *C.albican* organism using broth dilution method. The compound Am3 containing methoxy furfurylamine showed good spectrum of activity. All

compounds were less active than the standard drug. The synthesized compounds are predicted to have low gastrointestinal absorption and limited skin permeability, with varying lipophilicity and moderate synthetic accessibility. They are also expected to have limited central nervous system penetration and moderate polar surface areas. According to Protox-II predictions, all compounds (AM1, AM2, AM3, and AM4) are expected to have high LD50 values, indicating low acute toxicity. Additionally, they are predicted to be inactive across various toxicity categories, suggesting they are not likely to exhibit toxicity related to hepatotoxicity, carcinogenicity, mutagenicity, immunotoxicity, or cytotoxicity

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Conflict of Interest:

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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