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Design, development and antimicrobial screening of hybrids of alkyl/arylthioureas and 6-methoxy-2aminobenzothiazole

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Abstract
In quest for biologically more potent compounds, we envisioned synthesizing series of hybrids of alkyl/aryl urea and 6-methoxy-2-aminobenzothiazole by the treatment of 6-nethoxy-2-aminobenzothiazole with phenylchloroformate using anhydrous pyridine in dry THF at room temperature (RT) initially to obtain corresponding isocyanates. The resultant isocyanate is further refluxed for 10-12 hrs with monoalkyl/aryl urea derivatives in presence of sodium hydride (NaH) in dry THF. The synthesized biuret compounds were characterized by ^{1}HNMR and R_f values, and subsequently evaluated for antibacterial activity against Staphylococcus aureus, Escherichia coli, Klebsiella pnemoniae, and Pseudomonas aeruginosa, as well as antifungal activity against Aspergillus Niger, Aspergillus flavus, and *Fusarium monoliforme*. Among the synthesized compounds, some of the biurets demonstrated significant activity, while rest of the biuret compounds exhibited moderate activity.

Keywords: Antimicrobial resistance, hybrids, 6-methoxy-2-aminobenzothiazole, zone of inhibition, isocyanates and Tetrahydrofuran (THF).

Introduction
Nowadays the aim of the researchers is to develop new motifs which are structurally modified from the basic structure and that can effectively inhibit the growth of microorganisms. Therefore, the basic pharmacophoric unit structure is altered by linking various functional groups like amides, imides and large alkyl groups which will bring different mode of action that could be beneficial for the treatment of microbial pathogens.
2-aminobenzothiazole compounds found to possess pharmacological activities such as antimicrobial [1], anti-inflammatory [2-3], antitumor [4-5], antihelmintic [6] anti-tubercular activity [7], antileishmanial activity [8] and anticonvulsant activity [9].
Thiourea and its derivatives such as thioureides possess interesting biological properties such as herbicidal, fungicidal [10], antiHIV [11] an impressive number of currently used drugs can be regarded as thiourea derivatives for example, thyreostatic: Carbimazole, propylthiouracil, methylthiouracil, and ultrashortnarcotic: Thiamylal and antibacterial [12-14], insecticide [15] and rodenticide [16].
In this connection, series of biurets were synthesized from urea on reaction 2-aminobenzothiazole with monoalkyl/arylthiourea in sequential steps as shown in scheme-1 and screened for the antimicrobial activity.

Experimental work
Materials and Methods
All chemicals ammonia, monomethylamine, benzyl amine, ethylamine, propyl amine, butyl amine, cyclohexylamine, TEA, DCM and other chemicals were purchased from s, d-fine chemicals, Merck, India. Methyl, ethyl, propyl urea and thiourea were procured from sigma Aldrich. All the solvents used for the synthesis and analysis were of analytical grade. TLC was carried out on percolated silica gel plates prepared in laboratory using silica gel. ^{1}H NMR spectra were obtained on a 400 MHz Bruker FT-NMR spectrometer instrument using DMSO as solvent and TMS as an internal standard. Elemental analysis was obtained by using VARIO EL III CHNS Elementary.

General procedure for the preparation of 6-Substituted-1, 3-benzothiazol-2-amine [17]

p-nitroaniline (0.95g, 0.01 M) and potassium thiocyanate (0.97g, 0.01 M) were mixed in 20 mL of glacial acetic acid and then cooled and stirred. Bromine (4.95g, 0.01 M) was added from a dropping funnel at a controlled rate to prevent the temperature from rising above 0 °C. After the addition of bromine, the solution was stirred for an additional 2 hours at 0 °C. The resulting orange precipitate was allowed to settle overnight, then water (6 mL) was added and the slurry was heated at 85 °C on a steam bath and filtered while hot. The orange residue was then treated with 10 mL of glacial acetic acid, heated to 85 °C, and filtered while hot. The combined filtrate was cooled and neutralized with concentrated ammonia solution to pH-6, resulting in the appearance of a dark yellow precipitate, which was recrystallized from benzene to obtain the 6-substituted-1, 3-benzothiazol-2-amine.

6-Methoxy-2-aminobenzothiazole (2)

Yield-78%; M. P. 260-262°C; IR (KBR): γ_{max} cm^{-1} 3357, 3325, 1620; ^1H NMR ($CDCl_3$) δ 3.79 (s, 3H, Ar), 7.35 (s, 1H, Ar), 5.45(s, 2H, NH_2).

General procedure for synthesis of the mono substituted Alkyl/Aryl thioureas derivatives [18]

Silica gel-supported ammonium thiocyanate was prepared as follows. To the solution of ammonium thiocyanate (20 mmol, 1.52 g) in dry acetonitrile (50 mL) a Silica gel (18.48 g) was added, and the mixture was stirred at room temperature for 30 min. The acetonitrile was removed using rotovapor. The resulting reagent was dried in vacuum (15 mmHg) at room temperature for 3hr.

Typical procedure for N-substituted-N[1]-benzoylthioureas

To the slurry of NH_4SCN/SiO_2 (20mmol, 20g) in 1, 2-dichloroethane (100 mL), benzoyl chloride (1.7g, 10mmol) was added and the mixture was stirred at 25^{0}C for 2 hr and then an amine (20 mmol) was added to it and stirred for an additional 1hr. The reaction mixture was filtered to remove the supported reagent, and the filtrate was washed with 5% HCl, brine, and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to leave the crude product, which was purified by recrystallization (methanol). Finally, hydrazine hydrate (10 mmol) was added and the resulting mixture was stirred for 1 h. The products were extracted with 1, 2-dichloroethane followed by isolation of *N*-phenylthiourea (89%) by flash column chromatography. N-Benzylthiourea was obtained in 90% yield by this method.

General procedure for synthesis of hybrids of alkyl/aryl urea and 6-methoxy-2-aminobenzothiazole [19]

Solution of 6-methoxy-2-aminobenzothiazole (0.01mmol) and pyridine (2.47mmol) in dry THF (10 mL) stirred at 0^0 C in an ice bath. The mixture was stirred for 0.5 h. phenyl chloroform ate (0.015mmol) was added drop wise at such a rate to keep the temperature below 10^{0}C. The reaction was stirred at room temperature for 5-6hr and filtered. The white to light yellow solid was collected and washed with DCM to obtain crude benzothiazol-2-yl-carbamate (80-90%).

A mixture of mono *N*-substituted urea and thioureas (0.013mmol) and sodium hydride (5mmol) stirred for 30 mins and then a solution of crude benzothiazol-2-yl-carbamate (0.01mmol) in dry THF was added. The mixture was refluxed for 10-12hr before cooling to r.t. and concentration to about 1/3 of the initial volume on rotavator. Hexane was added to the residue and the obtained precipitate was collected by filtration under reduced pressure to yield the crude product. When necessary, the isolated material was purified chromatography on silica gel with $CHCl_3$-EtOAc as the eluent.

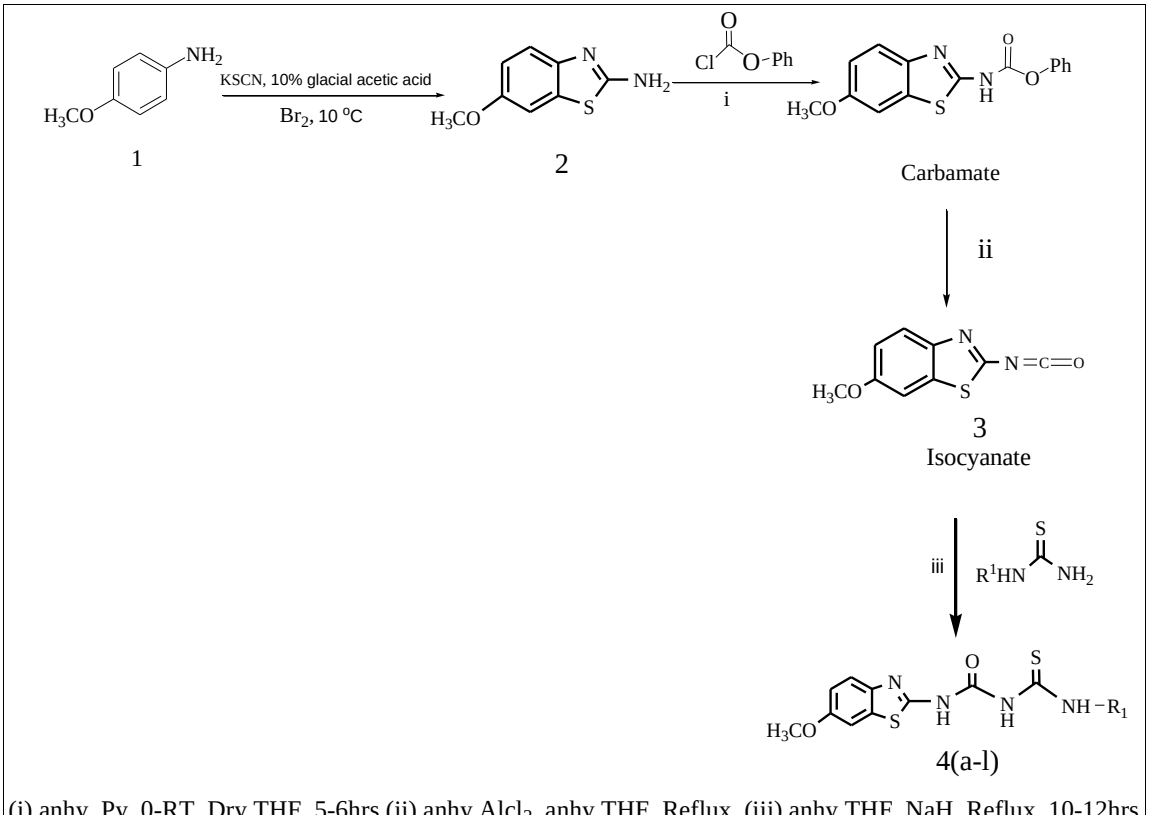
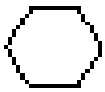
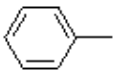
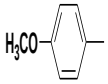
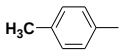
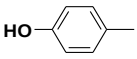
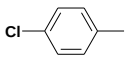
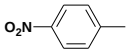


Fig 1: Overview of glaucoma surgical options

Table 1: Physical characterization data of hybrids of alkyl / arylthiourea and 6-methoxy-2-aminobenzothiazole.

Entry	R	Yield (%)	Molecular formula	Elemental analysis (%) Calculated (found)				^{1}HNMR (DMSO, δ ppm)
				C	H	N	S	
4a	CH_3-	85	$C_{10}H_{12}N_4OS_2$	44.76 (45.05)	4.51 (4.55)	20.88 (21.15)	23.90 (24.10)	7.39-8.35(m, 4H, ArH-Bz), 6.4(2H, NHCO), 6.6(1H, NH, imide), 2.3(d, 2H, CH_2, NHCSNH), 2.41(t, 3H, CH_3). IR (KBr, cm^{-1}): 3412(N-H), 3155(C-H), 1751(N=C-N), 1577(C=C), 1110(C=S), 1199(C-N), 1730 (C=O).
4b	CH_3CH_2-	86	$C_{11}H_{14}N_4OS_2$	46.79 (46.81)	5.00 (5.35)	19.84 (20.15)	22.71 (23.12)	7.45-8.45(m, 4H, ArH-Bz), 6.51(2H, NHCO), 6.67(1H, NH, imide), 2.5(d, 2H, αCH_2, NHCSNH), 3.6(q, 2H, αCH_2), 1.1(t, 3H, βCH_3). IR (KBr, cm^{-1}): 3415(N-H), 3160(C-H), 1760(N=C-N), 1580(C=C), 1205(C-N), 1120(C=S), 1738 (C=O).
4c	$CH_3(CH_2)_2$-	90	$C_{12}H_{16}N_4OS_2$	48.62 (49.35)	5.44 (5.69)	18.90 (19.15)	21.64 (22.10)	7.4-8.4(m, 4H, ArH-Bz), 6.57(2H, NHCO), 6.75(IH, NH, imide), 2.55(2H, αCH_2, NHCSNH), 3.6(q, 2H, β CH_2, 1.7(t, 2H, βCH_2), 1.15(t, 3H, βCH_3). IR (KBr, cm^{-1}): 3420(N-H), 3162(C-H), 1757(N=C-N), 1555(C=C), 1202(C-N), m1115(C=S), 1720(C=O).
4d	$CH_3(CH_2)_3$-	88	$C_{13}H_{18}N_4OS_2$	50.30 (50.37)	5.84 (5.95)	18.05 (18.20)	20.66 (20.71)	7.4-8.35(m, 4H, ArH-Bz), 6.5(2H, NHCO), 6.65(s, 1H, NH, imide), 2.45 (2H, αCH_2, NHCSNH), 3.55(m, 2H, αCH_2), 1.65(m, 2H, βCH_2), 1.4 (m, 2H, γCH_2), 1.05(t, 3H, δCH_3).IR (KBr, cm^{-1}): 3430(N-H), 3165(C-H), 1761(N=C-N),1566(C=C),1210(C-N), 1105(C=S), 1720(C=O).
4e	$(CH_3)_3C$-	91	$C_{13}H_{18}N_4OS_2$	50.30 (50.45)	5.84 (5.99)	18.05 (18.20)	20.66 (21.15)	7.45-8.45(m, 4H, ArH-Bz), 6.55(2H, NHCO), 6.71(s, 1H, NH, imide), 2.4 (2H, αCH_2, NHCSNH), 1.15(s, 9H, CH_3).IR (KBr, cm^{-1}): 3425(N-H), 3160(C-H),1750(N=C-N), 1560(C=C), 1210(C-N), 1127(C=S), 1719 (C=O).
4f		82	$C_{15}H_{20}N_4OS_2$	53.54 (53.65)	5.99 (6.15)	16.65 (16.79)	19.06 (19.75)	7.41-8.30(m, 4H, ArH-Bz), 6.35(2H, NHCO), 6.42(s, 1H, NH imide), 2.7 (2H, αCH_2, NHCSNH), 3.52(m, 1H, CH, Cyclohexane), 1.45-1.82(m, 10H, CH_2 Cyclohexane). IR (KBr, cm^{-1}): 3432(N-H), 3155(C-H), 1751(N=C-N), 1555(C=C), 1995(C-N), 1190(C-C), 1490(C=C), 1116(C=S), 1741C=O).
4g		85	$C_{15}H_{14}N_4OS_2$	54.52 (54.72)	4.27 (4.51)	16.96 (17.21)	19.41 (19.85)	7.25-8.35(m, 4H, ArH-Bz), 5.1(s, 1H, NHCO), 6.5(s, 1H, NHCONH, zmide), 4.3(s, 1H, NHCSNH), 6.7(m, 2H, αCHNCS, ArH), 6.65-7.2(m, 5H, ArH). IR (KBr, cm^{-1}): 3432(N-H), 3155(C-H), 1751(N=C-N), 1555(C=C), 1995(C-N).
4h		90	$C_{16}H_{16}N_4O_2S_2$	53.31 (53.45)	4.47 (4.51)	15.54 (15.65)	17.79 (18.15)	7.35-8.55(m, 4H, ArH-Bz), 5.55(s, 1H, NHCO), 6.1(s, 1H, NHCONH, imide), 4.9(s, 1H, NHCSNH), 6.61-6.9(m, 4H, ArH), 3.69(s, 3H, OCH_3). IR (KBr, cm^{-1}): 3390(N-H), 3150(C-H), 1755(N=C-N), 1550(C=C), 1200(C-N), 1100(C=S), 1718 (C=O).
4i		92	$C_{16}H_{16}N_4OS_2$	55.79 (55.81)	4.68 (4.76)	16.27 (16.55)	18.62 (18.75)	7.3-8.32(m, 4H, ArH-Bz), 5.53(s, 1H, NHCO),6.15(s, 1H, NHCONH, imide), 4.5(s, 1H, NHCSNH), 6.55-6.93(m, 2H, ArH), 2.4(s, 3H, CH_3, $ArCH_3$). IR (KBr, cm^{-1}): 3455 (N-H), 3150 (C-H), 1753(N=C-N), 1552(C=C), 1209(C-N), 1129(C=S), 1727 (C=O).
4j		90	$C_{15}H_{14}N_4O_2S_2$	52.01 (51.89)	4.07 (4.15)	16.17 (16.31)	18.51 (18.65)	7.45-8.3(m, 4H, ArH-Bz), 5.55(s, 1H, NHCO), 6.16(s, 1H, NHCONH, imide), 4.5(s, 1H, NHCSNH), 6.41-6.9(m, 4H, αCHNCO), 4.9(m, 1H, Ar-OH). IR (KBr, cm^{-1}): 3427 (N-H), 3152 (C-H), 1757 (N=C-N), 1525 (C=C), 1210 (C-N), 2900 (O-H), 1110(C=S), 1726(C=O).
4k		93	$C_{15}H_{14}ClN_4OS_2$	49.38 (49.39)	3.59 (3.65)	15.36 (15.37)	17.58 (17.29)	7.45-8.2(m, 4H, ArH-Bz), 5.45(s, 1H, NHCO), 6.1(s, 1H, NHCONH), 4.5(d, 1H, NHCSNH), 6.5-7.1(m, 4H, ArH). IR (KBr, cm^{-1}): 3430 (N-H), 3148 (C-H), 1735 (N=C-N), 1556(C=C),1995 (C-N), 1130(C=S), 1743(C=O).
4l		80	$C_{16}H1_5N_5O_4S_2$	47.40 (47.12)	3.73 (3.55)	17.27 (17.29)	15.82 (15.85)	7.15-8.3(m, 4H, ArH-Bz), 5.15(s, 1H, NHCO), 6.05(s, 1H, NHCONH) 4.1(d, 1H, NHCSNH), 6.35-8.1(m, 4H, ArH). IR (KBr, cm^{-1}): 3478 (N-H), 3161 (C-H), 1765 (N=C-N), 1557(C=C), 1235 (C-N), 1122(C=S), 1714(C=O).

Antibacterial assay

The antibacterial assay was carried out against gram +ve and gram -ve bacteria by following the procedure of Kato K *et al.* [20] with slight modifications.

General method for antibacterial assay

Antibacterial assays were conducted *in vitro* using the agar well diffusion method against Staphylococcus aureus, Escherichia coli, Klebesiella pnemoniae, and Pseudomonas auregenosa. The bacterial strains were cultivated in Muller-Hinton broth, and the inocculum concentration was adjusted using the mid-logarithmic phase method (OD 600=0.5). The molten media was prepared by adding Muller-Hinton agar in sterile distilled water and autoclaved for 1 hour. After solidifying, the media was scooped out at the center using a sterilized cup-borer, and inocculum were spread over the media. Stock solution of compounds (10 µg/well) was added to the well made in the petriplate and kept for 3-4 days at 37 °C. All the synthesized compounds were tested in triplicate; Streptomycin was used as positive control and water as negative control. The zone of inhibition was measured in mm and presented in Table-respectively.

Antifungal activity

The antifungal activities of the synthesized compounds were evaluated by following the procedure of Kato *et al.*,[21] with slight modifications.

General method of antifungal assay

In vitro antifungal tests were conducted on Aspergillus Niger, Aspergillus flavus, and *Fusarium monoliforme* using the agar well diffusion method. The fungal strains were cultivated on PDA media with a pH of 7.4 for six days at 25°C. Spores were collected in sterilized normal saline (0.9% NaCl in distilled water) and adjusted to a concentration of 1 x 106/ml using a Haemocytometer. Autoclaved molten media (20mL) was poured into each 90 mm sterilized petri dish and allowed to solidify. To assess the growth response of the fungi, 0.4 mL of the synthesized compounds (10 µg/mL) was added to each plate and evenly spread over the agar. A 10 µl spore

suspension was placed in a small depression at the center of the plate and then incubated for 6 days at 25 °C. After the incubation period, the plates were examined and compared to their respective controls. The control plates contained only distilled water, representing 100% fungal growth (no inhibition). The fungicidal activity of the synthesized compounds was determined by comparing the zone of fungal growth in treated plates to that of control plates in millimeters, and the results are presented in the table.

Table 2: Antibacterial activity of hybrids of alkyl/aryl urea and 6-methoxy-2-aminobenzothiazole.

Compounds [a]	Inhibitory Zone (diameter) mm [b]			
	Staphylococcus aureus	***Escherichia coli***	***Pseudomonas auregenosa***	***Klebesiella pneumoniae***
4a	06	07	06	05
4b	07	07	05	06
4c	08	07	06	07
4d	08	08	07	08
4e	06	07	07	07
4f	06	07	06	07
4g	06	06	07	06
4h	07	07	08	08
4i	07	06	05	06
4j	07	08	07	07
4k	07	08	07	06
4l	10	09	08	08
Streptomycin	13	11	10	11

[a] Concentration of compounds and reference drug: 10 µg/well.
[b] Values are mean of three determinations, the ranges of which are less than 5% of the mean in all cases.

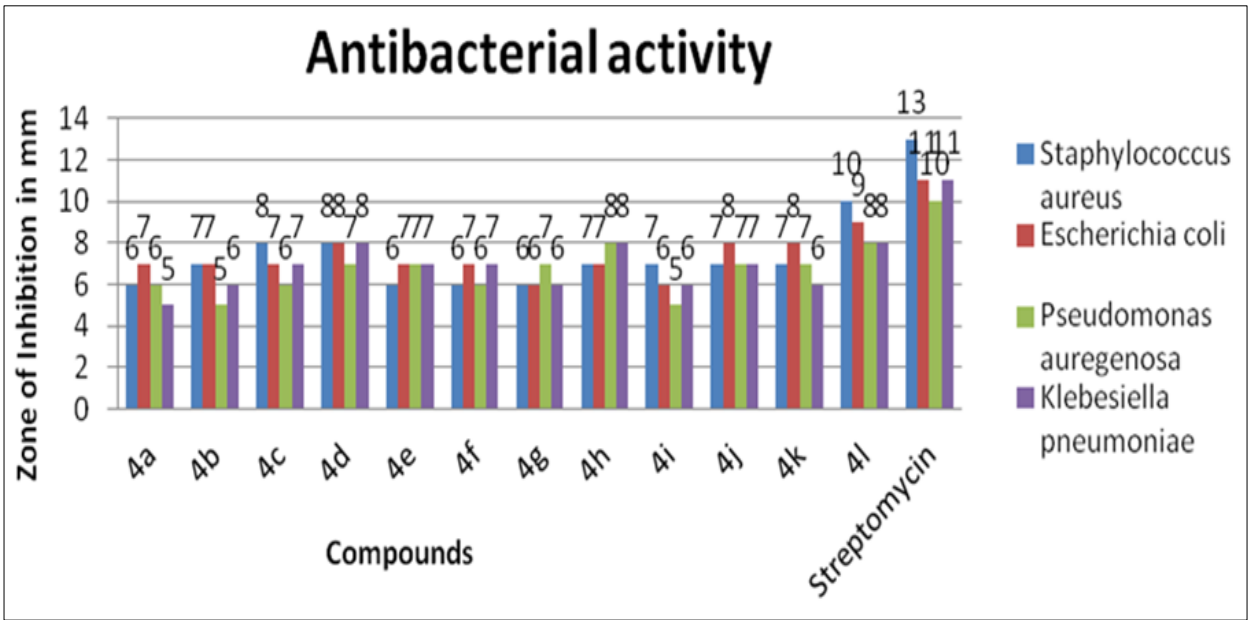


Fig 2: Assessment of Corneal Endothelium

Table 3: Antifungal activity of hybrids of alkyl/aryl urea and 6-methoxy-2-aminobenzothiazole.

Compounds [a]	Inhibitory Zone (diameter) mm [b]		
	Fusarium monoliforme	***Aspergillus niger***	***Aspergillus flavus***
4a	06	06	05
4b	06	06	05
4c	06	07	06
4d	07	08	07
4e	06	05	06
4f	06	06	06
4g	06	07	06
4h	08	07	08
4i	07	07	06
4j	08	07	07
4k	08	07	08
4l	09	07	08
Bavistin	11	09	11

[a] Concentration of compounds and reference drug: 10 µg/mL
[b] Values are mean of three determinations, the ranges of which are less than 5% of the mean in all cases.

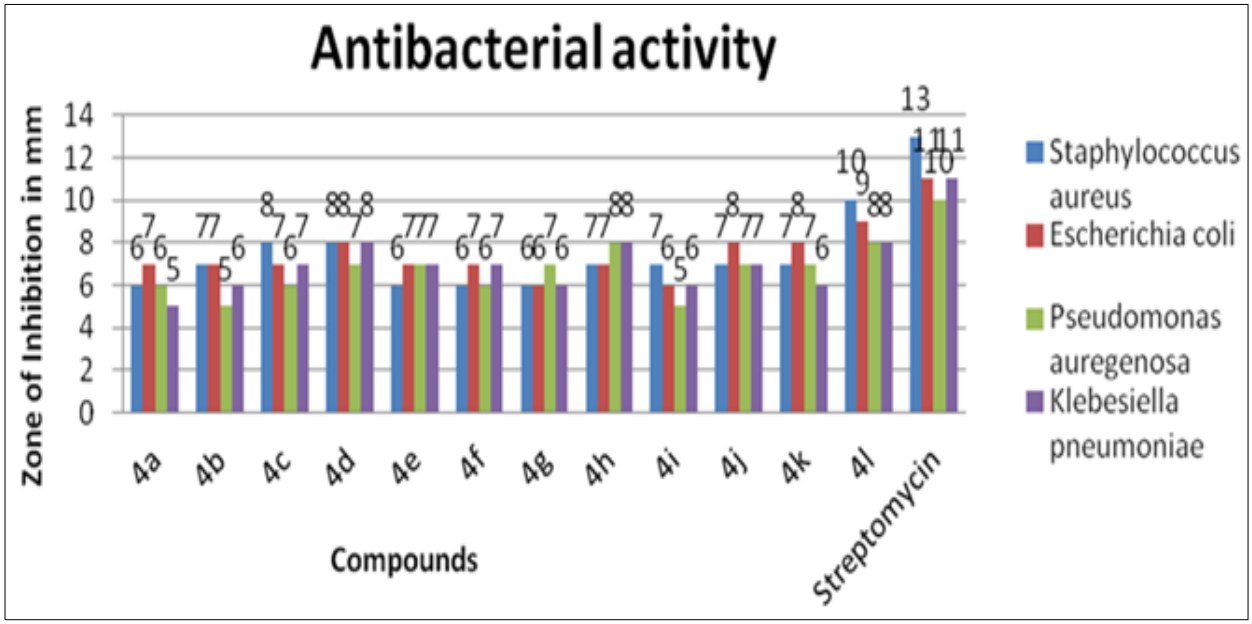


Fig 3: Etiology of endothelial cell dysfunction

Results and Discussion

We have developed a novel category of hybrids of alkyl/aryl urea and 6-methoxy-2-aminobenzothiazole.

The resulting compound was identified through TLC, elemental analysis, and 1H NMR. These synthesized compounds were tested for antimicrobial and antioxidant properties**.**

Structural activity relationship of synthesized compounds.

Antibacterial activity

All synthesized compounds were tested against gram-positive and gram-negative bacteria strains including Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Escherichia coli. Streptomycin served as the positive control, while DMSO was used as the negative control. The concentration remained consistent for both the test compounds and the standard. Compounds 4c, 4d, and 4l, containing electron-releasing or electron-withdrawing groups in the benzotriazole moiety or substituted urea/thiourea, exhibited superior activity compared to other compounds. Factors contributing to the enhanced antibacterial activity include the presence of electron-releasing groups such as OCH_3, OH, CH_3, and electron-withdrawing groups like Cl and NO_2, which also enhance antifungal activity. These groups facilitate better interaction/penetration with the cell membrane of microorganisms, leading to their inactivation.

Antifungal activity

All Compounds synthesized were tested against fungal strains such as *Aspergillus Niger, Aspergillus flavus and Fusarium monoliforme.* Nysatin was used as positive control and DMSO as a negative control. Among all the synthesized compounds, compounds 4h, 4j, 4k and 4l with electron releasing group and electron withdrawing groups showed better activity over the other compounds, the other compounds in all the series showed mild to moderate antifungal activity. Here also the factors explained under antibacterial activity equally holds well.

Conclusion

In summary we have reported an efficient synthesis of hybrids of alkyl/aryl urea and 6-methoxy-2-aminobenzothiazole starting from 2-amino-6-methoxybenzothiazole on reaction with alyl/arylthioureas in presence of phenylchloroformate using anhydrous pyridine in dry THF at room temperature (RT) in subsequent steps. The results of this study reveal that hybrids of alkyl/aryl urea and 6-methoxy-2-aminobenzothiazole can be used explored as antimicrobial molecules.

Acknowledgement

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Conflict of Interest

The authors declare no conflict of interest.

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