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A Comparative Analysis of the Biocidal Activity of Benzothiazole Incorporated Aromatics

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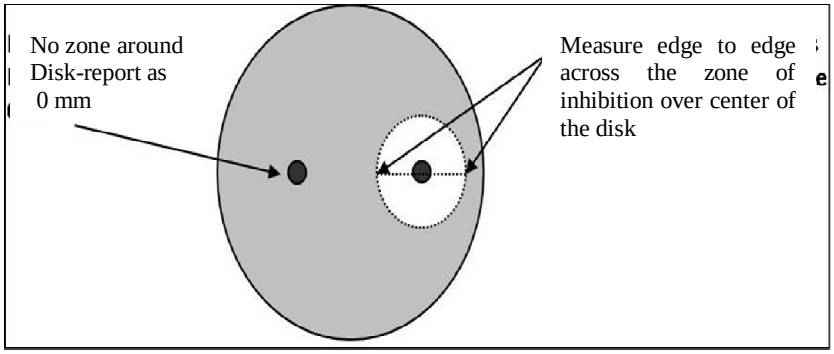
ABSTRACT

Heterocyclic chemistry deals with the innovation of inexhaustible source of co-ordination complexes and pharmaceuticals that are found to be promising for future researchers. In the current scenario, compounds are more promising as they are improved versions of previously reported aromatics. But all that matters is the coordinating behavior between the ligands and the metal ion because of which the prominent activities are pronounced. To study the same, in this thematic issue we synthesize heterocyclic complexes of N/S donor ligands (prominently a Benzothiazole moiety). Aromatic heterocycles that bear Nitrogen, Sulphur and Thiazole moiety as a core structure shows biologically interesting activities. The myriad spectrum of antimicrobial and biocidal properties of this trio - combination encourages the chemists to synthesize the novel versions of these therapeutic agents as they exhibits a significant wide range of anti-tumor, antimicrobial, anti-diabetic, anti-inflammatory, anticonvulsant, antiviral, antioxidant, anti-tubercular, anti-malarial, anti-asthmatic, anti-helmintic, photosensitizing, anti-diabetic, diuretic, analgesic and other activities. In this thematic issue, we have synthesized the vacillating versions of Benzothiazole moiety by variation with respect to fatty acid used. Their antimicrobial activities were evaluated and analyzed.

Graphical Abstract:



Schematic representation for measuring the Zone of Inhibition

Keywords: Benzothiazole, Heterocycles, Aromatics, Antimicrobial, Biocidal.

INTRODUCTION

An antimicrobial is any agent that destroys microorganisms or suppresses their multiplication or growth. Their unique capacity makes them for the control of large variety of pathogenic microorganisms which are causing deadly infections. They can be grouped as antibacterial when used against bacteria and antifungal when used against fungi. Agents that kill microbes are called micro-biocide, while those that merely inhibit their growth are called biostatic". During the last decade, the incidence microbial infections caused by multidrug resistant strains have increased alarmingly. So, innovations, inventions and improvements in these "wonder drugs" are solely crucial since after their usage threat of contiguous microbes as significantly reduced. One of the major applications of them is medical testing as antibacterial and antitumor agents aiming toward the discovery of an effective and safe therapeutic regime [**1**]. Transition metal ion chemistry has a great interdisciplinary relevance in present era. The amended and upgraded syntheticals' are no doubt a trigger to control the harmful effects of bacteria, fungi and viruses [**2**]. It definitely creates zeal to ameliorate versions [**3**].

Special emphasis is laid on the preparation of binuclear macro cyclic complexes of azoles ring derivatives with transition metal ions (special emphasis on copper), which possess beneficial properties like antibacterial, anti-malarial, antifungal, antibacterial, anti-cancerous, anti-allergic, anticonvulsant, antibiotic [**4**]. Along with these properties the complexes are also excellent dyes for fibers. They are used as direct dyes and coloring to plastics, varnishes, rubber and paper. In polar and non polar solvents they share a remarkable interest and application like foaming, wetting emulsification and lubrication due to surface active properties and solute solvent interactions [**5**]. Hence, they share an important account in industries, modern engineering and pharmacy. All the aforementioned activities become significant and pronounced only when chelation between ligand and metal ion accomplished. This stimulates to motif novel complexes of biological interest related to circadian events. Biotic plushing compounds are found most decisive classes of materials in reference to microbial threats [**6**]. Enormously ascending microbial infectious diseases and resistant pathogens puts on a stipulation to develop newfangled, commanding, secured and improved variety of antimicrobial agents [**7**]. So, synthesis of chemicals with lower toxicity is the foremost task for the current chemistry for a promising future [8]. For the same much more discovery and improvement is needed to upgrade this field of research. Binuclear aromatic heterocycles share an important place in this regards [**9**]. Further, it is evidenced that complexation of transition metal ions with nitrogen and sulphur donor ligands increases the efficiency of biocidal activity [**10, 11**].

Generally, in vitro antimicrobial susceptibility testing methods are divided mainly into Diffusion and Dilution methods- Among the above Diffusion methods are important techniques typically used for antimicrobial susceptibility testing. Stokes and Kirby-Bauer method is foremost recommended by the NCCLS [**12**].

Stokes-Kirby Method: The Stokes Kirby Bauer test, known as the disc-diffusion method, is the most widely used antibacterial susceptibility test to determine the sensitivity or resistance of a pathogenic aerobic or facultative anaerobic bacterium to various antimicrobial compounds [**13**]. This method relies on the inhibition of bacterial growth measured under standard conditions. The presence or absence of growth around the antimicrobial disc is an indirect measure of the ability of the antibiotic to inhibit the organism. Concentration of a particular antimicrobial is placed on the medium. The organism will grow on the agar plate while the antimicrobial works' to inhibit the growth. If the organism is susceptible to a specific antimicrobial drug, there will be no growth around the disc containing the antibiotic. Thus, a 'zone of inhibition' can be observed and measured to determine the susceptibility to an antimicrobial for that particular organism. In this method a known quantity of bacteria is grown on agar plates in the presence of thin wafers containing relevant standard antibiotics. If the bacteria are susceptible to a particular antimicrobial, an area of clearing surrounds the wafer where bacteria are not capable of growing (called a zone of inhibition). Also, the rates of

antimicrobial diffusion are determined and these values are used to estimate the bacteria's sensitivity to that particular antimicrobial agent [**14**].

In general, larger zones correlate with smaller concentration of test compounds for a specific microorganism. This information can be used to choose appropriate antimicrobials to combat a particular infection. During the last decades, several experimental procedures were developed for Antimicrobial Susceptibility Testing (AST) by CLSI (Clinical and Laboratory Standards Institute) that created standards to perform ASTS. These methods are extensively being used to determine the molecular potency against microbes [**15**].

MATERIALS AND METHODS

Chemicals: All chemicals used were LR/AR grade.

Synthesis of 2-amino 6- bromo Benzothiazole: 2-amino 6-bromo Benzothiazole was synthesized using Thiocyanogenation method. In the Thiocyanogenation method 12.3 gms p-chloro aniline/13.8 p-methyl aniline (0.1 mole) was treated with a mixture of 7.6 gms ammonium thiocynate and 80 mL glacial acetic acid in a 250 mL three necked round bottom flask, with stirrer, dropping funnel and reflux condenser at room temperature for one and half hour. The thiocyanogen of substituted anilines takes place in the presence of thiocyanogen gas, which is generated in situ by the reaction of cupric chloride and ammonium thiocynate. After cooling, in the reaction mixture 100 mL of concentrated HCl (6 N) is added. Heat again for half an hour, then cool it and saturated solution of sodium carbonate (Na_2CO_3) is added to neutralize it, till the solid was formed. The precipitate was filtered washed with cold water, dried and recrystallised with ethanol.

Synthesis of Copper Surfactants: Copper Stearate was prepared by mixing one gm of into 25 mL ethyl alcohol, shake the mixture in hot water bath and then add one drop of phenolphthalein. A saturated solution of KOH in another beaker was prepared then it was added into Stearic acid solution drop by drop until the light pink color appears. Now again in another beaker prepare a saturated solution of $CuSO_4$ (about 2-3 gms in 5 mL H_2O) and mix it into above solution with stirring till the blue colored soap is formed. Filtered and washed with warm water and 10% ethyl alcohol then dried and recrystallised with hot benzene.

Preparation of Complexes: The complexes of Copper Stearate and Benzothiazole were prepared by adding (0.001 mole) of Stearic acid with 0.002 moles of benzothiazoles in 25-30 mL ethyl alcohol and the mixtures were refluxed for about two hours with constant stirring. After cooling the precipitate were filtered, dried and recrystallized with hot benzene.

The formation of complexes was confirmed by using IR, NMR techniques and elemental analysis. Melting points were determined on Toshniwal apparatus and were uncorrected. The purity of compounds was checked on thin layers of silica gel. IR spectra (KBr) were recorded on FT IR spectrophotometer model 8400 S Shimadzu as nujol mull using KBr pellets in the range of 4000- 400 cm^{-1} and ^1H NMR was recorded in DMSO-d^6 using Bruker DPX-300 spectrophotometers using TMS as internal reference.

Antimicrobial Susceptibility Testing:

Purposes of Stokes-Kirby Disc Diffusion Method: The Stokes Kirby Disc Diffusion Method is a laboratory technique used for several purposes, including:

1. **Determining antimicrobial susceptibility:** The primary purpose of the Stokes Disc Diffusion Method is to determine the antimicrobial susceptibility of a microorganism to various antibiotics or

other antimicrobial agents. The test helps to identify which antibiotics or agents are effective in treating an infection caused by the microbe.

2. **Quality control of antibiotics:** The Stokes Disc Diffusion Method is also used to ensure the quality and potency of antibiotics. The test is performed on antibiotic products to determine their efficacy against specific microorganisms.

3. **Surveillance of antibiotic resistance:** The test is used to monitor the emergence of antibiotic resistance in microbial populations. The results of the test can help to identify trends in antibiotic resistance and guide appropriate treatment strategies.

4. **Research:** The Stokes Disc Diffusion Method is used in research studies to investigate the mechanisms of antibiotic resistance and to evaluate the effectiveness of new antimicrobial agents. Overall, the Stokes Disc Diffusion Method is a valuable tool for determining the susceptibility or resistance of microorganisms to various antimicrobial agents. This information can be used to guide appropriate treatment strategies and to monitor the emergence of antibiotic resistance.

Procedure of Stokes Kirby Disc Diffusion method:

1. Colonies should be well-isolated and with the same morphological form of both control and test strains. Transfer the colonies to tubes that contain up to 5mL of tryptic soy broth.
2. The broth should be incubated at 37°C until it attains the turbidity of 0.5 McFarland standards and it generally takes between 2 and 6 hours.
3. Change the turbidity level of the broth that is growing culture by using sterile saline, or tryptic soybean broth if the turbidity is high, but it is low. Further, let it incubate until you achieve an optically similar turbidity to that from that of the 0.5 McFarland standards. This standard corresponds to a suspension that contains between 1 and 2 x 1 108 CFU/mL of bacteria under study.
4. For this procedure to be done correctly you must either read the inoculums using a McFarland densitometer , or visual method, if you choose to do it sufficient light is required to see the inoculums tube as well as its 0.5 McFarland standard against a card that has white background and contrasting black lines, also known as Wickerham card.
5. Inject sterile cotton swabs of each of the suspensions that have been adjusted (within 15 minutes of adjusting the volume of turbidity).
6. The swabs should be rotated multiple times before pressing them on the surface of the tube, above the fluid level. This will eliminate any excess inoculums from the swabs.
7. Dry the inoculation plates by letting the lid opened so that there are no droplets of water over the surfaces.
8. Place the control culture on two bands on each of the plates, leaving one central band that is not inoculated the aid of sterilized wipes.
9. Place the test organism in the middle of the area without touching any side, and this can be done with the conventional Stokes disc diffusion technique, whereas the modified Stokes disc diffusion technique is the reverses of steps 9 and 10
10. Place the discs of antimicrobials using forceps along the line between the control and test organisms. Press gently to ensure that they are in touch with medium. Be aware that there must be a an absolute minimum distance of 2 centimeters between the two disks. The plates should be incubated overnight aerobically at 35 to 37 ℃.

Determine the inhibition zones radius starting from to the edges of the disc until its edge, as illustrated in the below image figure 1.

- **Sensitive (S):** The size of the zone of the strain being tested is greater in comparison to that of the control strain. If the measurement of the bacterium is less than the control strain, the difference must not exceed 3 millimeters.

- **Intermediate (I):** The Zone size of the strain test is greater than 2 millimeters, but less that the test strain by more than 3 millimeters.

- **Resistant (R):** The area dimension of the test strain is not greater than 2 millimeters.

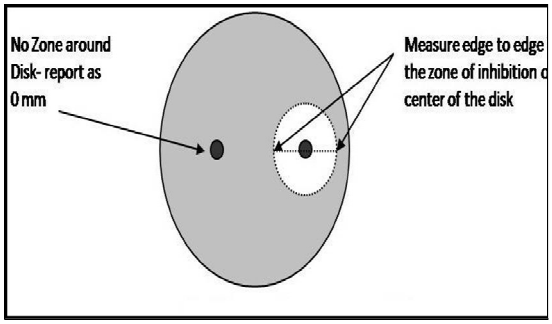


Figure 1. Measuring The Zone of Inhibition.

Note:

- DMSO (negative control) show slight activity against test organism.
- Streptomycin is for possible control in antibacterial [5 mg (w/v)].
- Sample [CS (BTA) Br (complex of copper stereate with 2-amino 6-bromo benzothiazole).

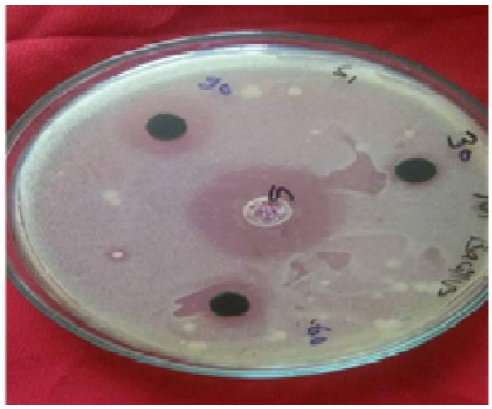


Figure 2. Antibacterial Sensitivity of Synthesized complex against the bacteria *Bacillus Subtilis.*

Figure 3. Antibacterial Sensitivity of Synthesized complex against the bacteria Escherichia *E.coli.*

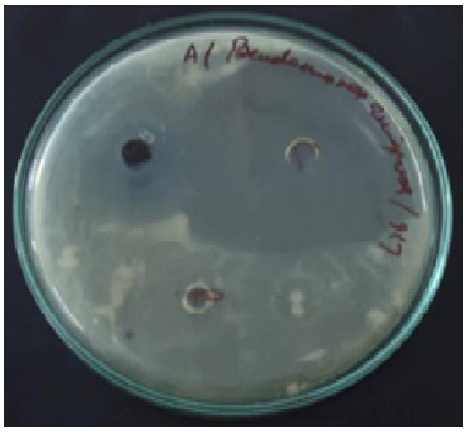


Figure 4. Antibacterial Sensitivity of Synthesized complex against the bacteria *Pseudomonas Aeruginosa.*

Figure 5. Antibacterial Sensitivity of Synthesized complex against the bacteria *Lactobacillus Acidophilus*

Table 1. Antibacterial Sensitivity of Synthesized Complexes against Bacteria under Study

S.No.	Micro-organism	Zone of Inhibition (mm)	Activity Index	Activity
1.	*Bacillus Subtilis*	18	2	20
2.	*Escherichia Coli*	15	1.6	16
3.	*Pseudomonas Aeruginosa*	28	3.1	31
4.	*Lactobacillus Aciedophillus*	24	2.6	26

Diameter of the zone of inhibition for the complex under study is given.
Diameter of the well was found to be 8 mm.
DMSO (negative control) showed 9 mm zone against bacteria under observation.
Activity index = zone of inhibition of sample[S]/zone of inhibition of reference [R] and
The activity can be found out by:

$$\text{Activity} = \text{activity index} * 10$$

Note the following observations-

1. If the activity is less than 13, it means that the extract is inactive,
2. If the activity is between 13-18, it means that the extract is bioactive,
3. If the activity is greater than 18, it means that the extract is highly active.

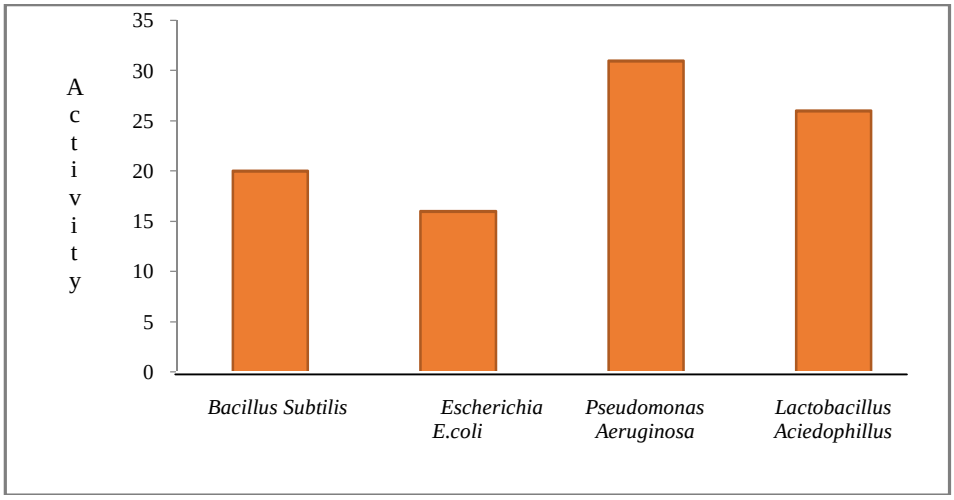


Figure 6. Plot depicting the Activity of Synthesized Complexes against microorganisms.

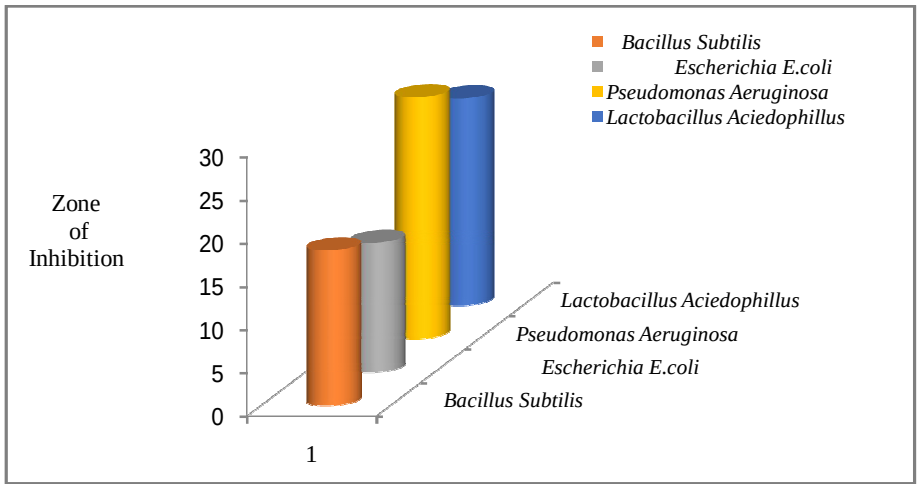


Figure 7. Plot depicting the Zone of Inhibition of Synthesized Complexes against microorganisms.

RESULTS AND DISCUSSION

Binuclear aromatic on complexation with transition metals shows significant biological importance due to their unusual configurations. This makes them exceptionally suitable for antibacterial and antifungal activity. The enhanced biological activity of the macrocyclic complexes can be explained on the basis of Overtone's concept and Tweed's Chelation theory. Lipids, polysaccharides are some important constituents of cell walls and cell membranes, which contains many amino phosphates, carbonyls and cysteinyl ligands to maintain the integrity of the membrane by acting as a diffusion barrier and also provide suitable sites for binding. On the other hand, it has been suggested that Chelation reduces the polarity of the metal ion to a greater extent because of partial sharing of the positive charge of the metal ion with donor groups within the chelate ring. Further, this coordination process increases the delocalization of pi-electrons over the whole chelate ring and enhances the lipophilicity of the complexes which leads to the breakdown of the permeability barrier of the cell. Subsequently, the interaction between the metal ion and the lipid is favored resulting in penetration through the lipid layer of cell membranes and blocking of the metal binding sites in the enzymes of microorganisms thus destroying them more aggressively.

Some studies support that the percentage ratio of the metal ions and the geometry of the complexes play a significant role in the biological behaviour of the metal chelate. If the geometry and charge button around the molecule are incompatible with the geometry and charge distribution around the pores of the bacterial cell wall, penetration through the wall by the toxic agent cannot take place and his will prevent the toxic reaction within the pores. These factors evincible the overall mechanism of permeation through the pod layer of the organisms thus killing them more effectively and efficiently. Few researchers also view that metal complexes perturb the respiration process of the cell by blocking the synthesis of the proteins, which restricts further growth of the organism. This indicates the Chelation tends to make more powerful and potent antimicrobial agents then the parent moieties. Therefore, it is justified but the process of Chelation dominantly affects the biological behavior of the complexes.

In addition to these, some important factors that contribute to activity are the nature of the metal ion, the nature of the ligand, coordinating sites and geometry of the complex, concentration, hydrophilicity, lipophilicity, stability, conductivity, solubility, different dipole moment and the presence of co-ligands. Certainly, steric and pharmacokinetic factors also play an important role in deciding the potency of an antimicrobial agent. Not only this, the presence of lipophilic and polar substituent is expected to power the antibacterial activity.

Heterocyclic ligands with multi functionality have a greater chance of interaction either with nucleoside bases or with biologically essential metal ions present in the biosystem and can be promising candidates as bactericides since they always look to enact especially with some enzymatic functional groups to achieve a higher co- ordination number. Thus, the antibacterial property of metal complexes cannot be ascribed to Chelation alone but it is an attribute of all the above contributions.

APPLICATION

The metal complexes bearing N-C=S linkage possess a range of biological applications namely tranquilisers, sedatives, neuroleptic, anti-histamines, anti-helminthes, anti-inflammatory, antipsychotic, anti-viral, anesthetics, anti-malarial, antibacterial, antifungal, diuretic, anticancerous, anticonvulsant, antibiotic etc. Some nitrogen and sulphur ligands are used in therapy, photo-sensitisers, indicators-dyes and heat stabilizers. The benzothiazole ring is present in various marine and terrestrial natural compounds which have biological significance. Heterocycles containing thiazole moiety are present in many natural products such as epothilone A, bleomycin, lyngbyabellin A and dolostatin. Being a heterocyclic compound, benzothiazole finds use in research as a starting material for the synthesis of usually larger bioactive structures.

CONCLUSION

The antibacterial activity of the synthesized complexes has been evaluated by the Kirby-Bauer and Stokes' method. The results are expressed in millimeter. The antimicrobial disks with streptomycin (for anti-bacterial) were taken as standards and the sample disks were compared with it. A scrutiny of table 1 reveals the zone of inhibition and activity index of the experiment under study. The studies reveals that complexation enhances the zone of inhibition and also complexes show higher activities then pure ligands suggesting that complexes are more powerful agents then individual moieties. It is seen that N, S, O etc containing aromatics are able to enhance the performance of copper soaps. Hence, we can conclude that Benzothiazoles and other N and S containing compounds are able to enhance the performance of copper soaps. Maximum activity was shown against *Pseudomonas aeruginosa* and minimum for *E.coli*.

Future Scope: Surface active agents are an immense part of biological systems and are found to be promising for pharmaceutical industries & medical systems. Such aromatics shows immense applications as lubricants, wood preservatives, detergents, emulsifiers, foaming, wetting, paints, varnishes, anticancer, anti tubercular, antibiotic, antimicrobial and antifungal, anticonvulsant, analgesic and anti-inflammatory anti-protozoal, anti-helmintics, anti-HIV, antihepatic, antiulcer activities, antibacterial, antimalarial, anti-allergic, antibiotic agents. The continued interest to proliferate structural novelties of such complexes is due to their wide application in medicinal, biochemical, bioinorganic, environment, industrial and photochemistry. The vital information plays an important role in various industrial process as well as biological applications.

Conflict of Interest: The authors declare no conflict of interest.

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