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A FACILE SYNTHESIS AND BIOLOGICAL EVALUATION OF SOME MACROCYCLIC COPPER COMPLEXES

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Keywords:

Biocidal activity, Antibacterial, Antifungal, Copper Palmitate, Copper Caprylate, 2-amino 6-bromo benzothiazole

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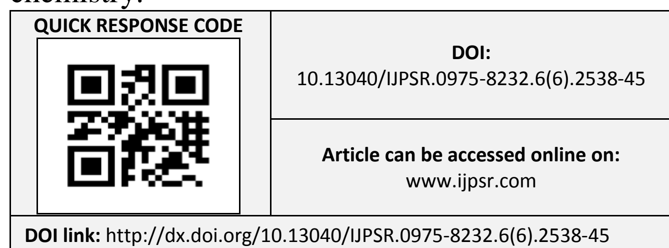
ABSTRACT: Biologically potent compounds are one of the most important classes of materials for the upcoming generations. Increasing number of microbial infectious diseases and resistant pathogens create a demand and urgency to develop novel, potent, safe and improved variety of antimicrobial agents. This initiates a task for current chemistry to synthesize compounds that show promising activity as therapeutic agents with lower toxicity. Therefore, a substantial research is needed for their discovery and improvement. Transition metal complexes share an important place in this regards. Further, it is evidenced that complexation of above metal ions with nitrogen and sulphur donor ligands increases the efficiency of biocidal activity. For the same, metallic soaps of copper (derived from common fatty acids) were complexed with substituted 2-amino benzothiazole derivatives. Complexes were characterized by elemental analysis IR, NMR and ESR spectral data. Their purity was checked by Thin Layer Chromatography. Their antimicrobial efficacy / biological activity was examined against selective bacteria such as Pseudomonas aeruginosa (Gram negative), Lactobacillus aciedophillus (Gram positive), antifungal activity against Fusarium semitectum and Trichophyton ruburum by Kirby-Bauer and Stokes' method. The transition metal complexes showed good antibacterial and antifungal activity than the free ligands because chelation increases the anti-microbial potency.

INTRODUCTION: The chemistry of macrocyclic nitrogen and sulphur donor ligands and their complexes with transition metal ions has been an interesting and fascinating area of research activity all over the World since last few decades. The continued interest to proliferate structural novelties of such complexes is due to their wide application in medicinal, biochemical, bioinorganic, environment, industrial and photochemistry. Afore said complexes have received much attention in recent years on account of their rational design and synthesis in coordination chemistry because of their potential wide applications as functional materials, enzymatic reaction mechanism and in bioinorganic chemistry.



In biological system, the metal ions are co-ordinated to ligands rather than existing as free ions. Interactions such as intercalation, hydrogen bonds, electrostatic, vander Waals force and so on widely existing in them makes considerable effect on biological processes. Depending on their concentration they either contribute towards the health of organism or cause toxicity.

No doubt, complexes of copper ion shares important application in above said areas of research. The chelation of metal ion with nitrogen and sulphur donor moieties is worth mentioning [1-4].

Such copper complexes are applied in various activities such as anticancer, anti tubercular, antibiotic, antimicrobial and antifungal, anticonvulsant, analgesic and anti-inflammatory anti-protozoal, anti-helmintics, anti-HIV, anti-hepatic, antiulcer activities, antibacterial, anti-malarial, anti-allergic, antibiotic etc agents [5-11].

Their increasing physiological importance and their active role in co-ordination chemistry creates zeal to synthesize, characterize and improve this class of compounds. Extensive structural and physico-Studies[12-15] of such compounds are crucial for increasing the understanding of the structural insight, type of bonding and electronic interactions between proximate metal centers and involved ligands. For the same, we proceed with the procedure of synthesis and summary of antibacterial and antifungal sensitivity testing by Kirby-Bauer and Stokes' method [16-21]. The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism.

MATERIALS AND METHODS:

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 this method (0.1mol) p- bromo aniline was treated with a mixture of 7.6 gm ammonium thiocyanate and 80 ml glacial acetic acid with (0.1mole) of cupric chloride 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 thiocyanogenation of aryl amine takes place in the presence of thiocynogen gas, which is generated insitu by the reaction of cupric chloride and ammonium thiocynate. After cooling the reaction mixture, add 100 ml concentrated HCl, and heat again for half an hour, then cool it and then saturated solution of sodium carbonate (Na_2CO_3) is added to neutralize it, till the solid was formed. The solid separated out was filtered and washed with cold water, dried and recrystallised with ethanol.

Synthesis of Copper Surfactants:

Copper palmitate / Copper Caprylate was prepared by mixing one gm of Palmitic acid / Caprylic acid 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 Palmitic acid / Caprylic 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 g 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.

1. **Preparation of Complexes**

The complexes of copper palmitate / Copper caprylate and benzothiazole were prepared by adding (0.001mole) copper palmitate / copper caprylate 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-d6 using Bruker DPX-300 spectrophotometers using TMS as internal reference.

2. **Antimicrobial Susceptibility Testing** (Kirby-Bauer and Stokes' methods.)

- Preparation of Mueller-Hinton agar medium (antibacterial screening) and Sabouraud Dextrose Agar medium (antifungal screening).
- Preparation of positive control Streptomycin (5mg (w/v) for antibacterial and Itraconozole for antifungal, also negative control DMSO.
- Antibacterial and Antifungal sensitivity testing by Kirby-Bauer and Stokes' methods

Hinton susceptibility test agar:

Mueller-Hinton agar medium (antibacterial screening) and Sabouraud Dextrose Agar (antifungal screening) is the only susceptibility test medium that has been validated by NCCLS. Mueller-Hinton agar should always be used for disk diffusion susceptibility testing.

McFarland turbidity standard:
A McFarland 0.5 standard should be prepared and quality controlled prior to beginning susceptibility testing. If tightly sealed to prevent evaporation and stored in the dark, the standard can be stored for up to 6 months. The McFarland standard is used to adjust the turbidity of the inocculum for the susceptibility test.

Preparation of inoculums:
Each culture to be tested should be streaked onto a non inhibitory agar medium to obtain isolated colonies. After incubation at 35°C overnight, select 4 or 5 well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline or non selective broth (Mueller-Hinton broth, Peptone water) and vortex thoroughly. The bacterial suspension should then be compared to the 0.5 McFarland standards. The turbidity standard should be agitated on a vortex mixer immediately prior to use. If the bacterial suspension does not appear to be the same density as the McFarland 0.5, the turbidity can be reduced by adding sterile saline or broth or increased by adding more bacterial growth.

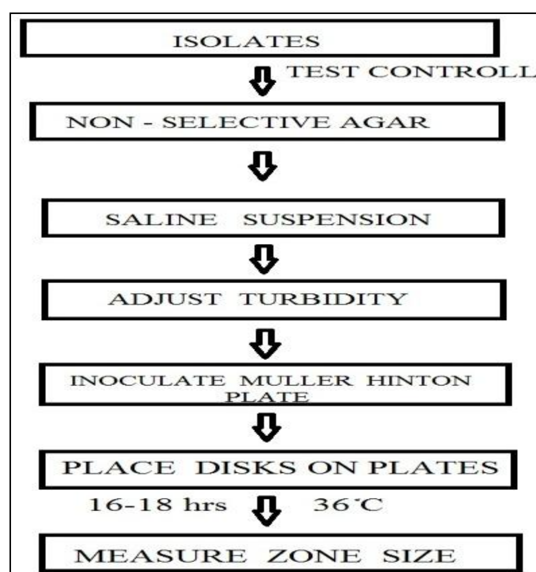


FIG. 1: FLOW CHART PRESENTING THE STEPS INVOLVED IN KIRBY-STOKS' METHOD

Inoculation procedure:
Within 15 minutes after adjusting the turbidity of the inoculums suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculums. Finally, swab all around the edges of agar surface. The Mueller-Hinton plate should be swabbed over the entire surface of the medium three times, rotating the plate 60 degrees after each application.

Loading the plate with Positive, negative control and sample:

a) The working supply of antibiotic (streptomycin, positive control) should be stored in the refrigerator (4°C). Upon removal of the antibiotic from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1hour to allow the temperature to equilibrate.

b) 50µl of the antibiotic suspension was dispensed in the well labelled with C (control) to the plates as soon as possible, but no longer than 15 minutes after inoculation. Diffusion of the drug in the well begins immediately.

c) 50µl of the sample (S) and 50µl of the reference (R, negative control) was dispensed in the well labelled with C (control) to the plates as soon as possible, but no longer than 15 minutes after inoculation.

Recording and interpreting results:
After the Loading of C, S, R on the plate, invert the plate and incubate at 35°C for 16 to 18 hours. After incubation, measure the diameter of the zones of complete inhibition (including the diameter of the well) and record it in millimetres. The measurements can be made with a ruler on the under surface of the plate without opening the lid. The zones of growth inhibition should be compared and recorded as susceptible, intermediate, or resistant to each drug tested.

Colonies growing within the clear zone of inhibition may represent resistant variants or mixed inoculums. The distance from the colony(ies) closest to the well to the centre of the well should be measured and then doubled to obtain a diameter. The diameter of the outer clear zone should be recorded as well and an interpretation recorded for

each diameter. The colony(ies) inside the zone should be picked, re-isolated, re-identified, and retested in the well diffusion test to confirm the previous results. The presence of colonies within a zone of inhibition may predict eventual resistance to that agent.

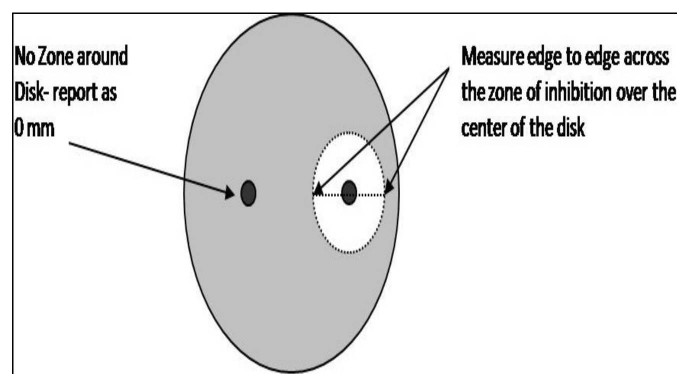


FIG. 2: MEASURING ZONE OF INHIBITION

Note:

a) DMSO (negative control) show slight activity against test organism.

b) Streptomycin (5mg (w/v) per well) serves as a positive control as anti bacterial screening and Itraconozole as positive control as antifungal screening (5mg (w/v) per well).

c) Sample Compound A (Complex of copper palmitate with 2-amino 6- bromo benzothiazole) and B (complex of copper caprylate with 2-amino 6-bromo benzothiazole) with concentration 5mg/ well (w/v).

RESULTS AND DISCUSSION:

Biological activity is simply an expression describing the beneficial or adverse affect of drug on living matter. Innovations of newer, cheaper and more potent analogs of molecules with already well recognized biological activities from a key part of research in the pharmaceutical field. Bringing about modifications by manipulating the parent structures serves to enhance the activity of the potent analogs and eliminates adverse effects or toxicity associated with the parent drug is the orientated goal of present chemistry. The study described here is a step to achieve such a goal.

Note:

Sample A = Complex of Copper Palmitate with 2-amino 6-bromo Benzothiazole.

Sample B = Complex of Copper Caprylate with 2-amino 6-bromo Benzothiazole.

TABLE 1: ANTIBACTERIAL SENSITIVITY OF SYNTHESISED COMPLEXES AGAINST SOME BACTERIA

S. No.	Microorganism	Sample	Zone of Inhibition (mm)		Activity Index
	Positive Control (Streptomycin) [5mg(w/v)]			Sample	
1	*Pseudomonas*	Sample A	45	28.5	3.17
	Aeruginosa	Sample A	45	30	3.33
2	*Lactobacillus*	Sample A	38	23.5	2.61
	Aciedophillus	Sample B	38	24.5	2.72

S. No.	Microorganism	Sample	Zone of Inhibition (mm)		Activity Index
		Positive Control (Itraconozole) 5mg(w/v)		Sample	
1	*Fusarium*	Sample A	Nil	32	3.55
	semitectum	Sample B	Nil	19	2.11
2	*Trichophyton*	Sample A	25	Nil	Nil
	Ruburum	Sample B	25	15.5	1.72

Diameter of the zone of inhibition is given.
Diameter of the well was 8mm.
DMSO (negative control) showed 9mm zone against test organisms.
Activity index = zone of inhibition of sample[S] /zone of inhibition of reference [R] and the activity can be found out by:

Activity = activity index * 10

If the activity is

- < 13 = it means that the extract is inactive,
- 13 – 18 = it means that the extract is bioactive,
- 18 = it means that the extract is highly active.

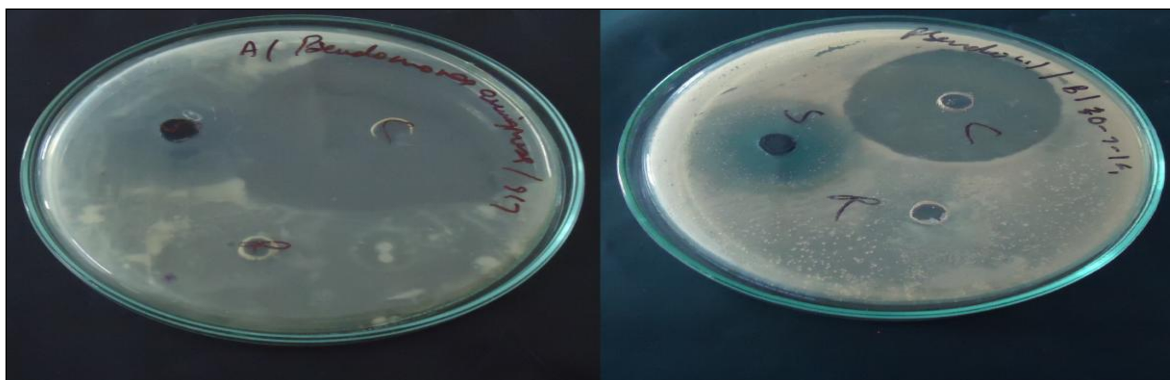


FIG. 3: TEST DISKS PRESENTING SENSITIVITY OF COMPLEXES AGAINST *PSEUDOMONAS AERUGINOSA*

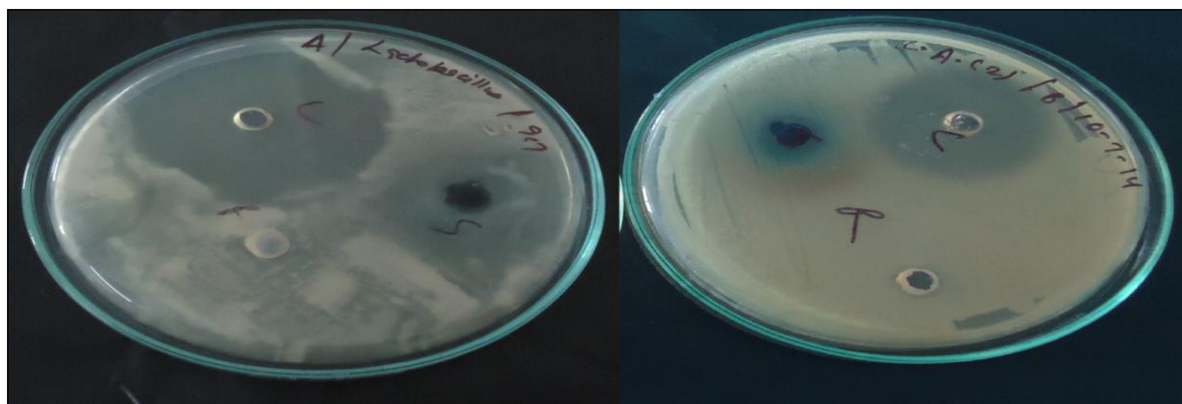


FIG. 4: TEST DISKS PRESENTING SENSITIVITY OF COMPLEXES AGAINST *LACTOBACILLUS ACIEDOPHILLUS*

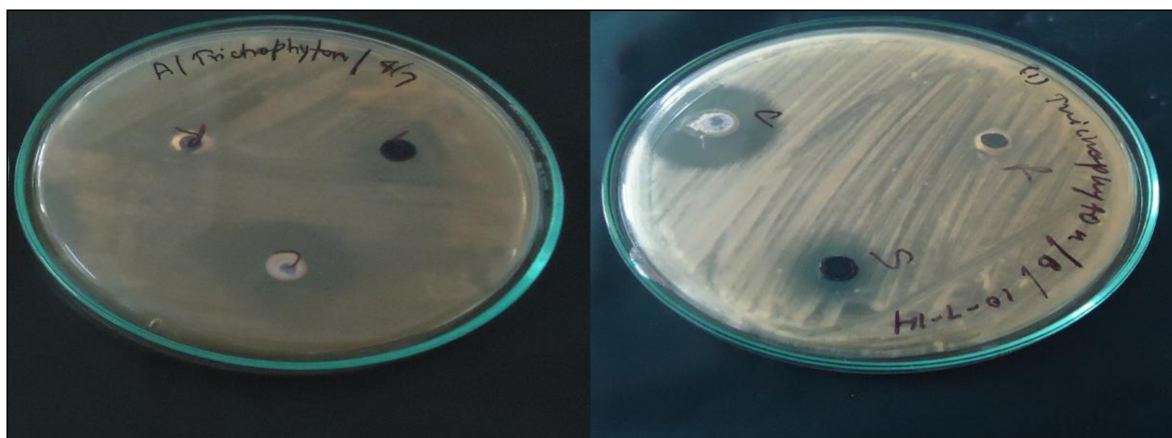


FIG. 5: TEST DISKS PRESENTING SENSITIVITY OF COMPLEXES AGAINST *TRICHOPHYTON RUBURUM*

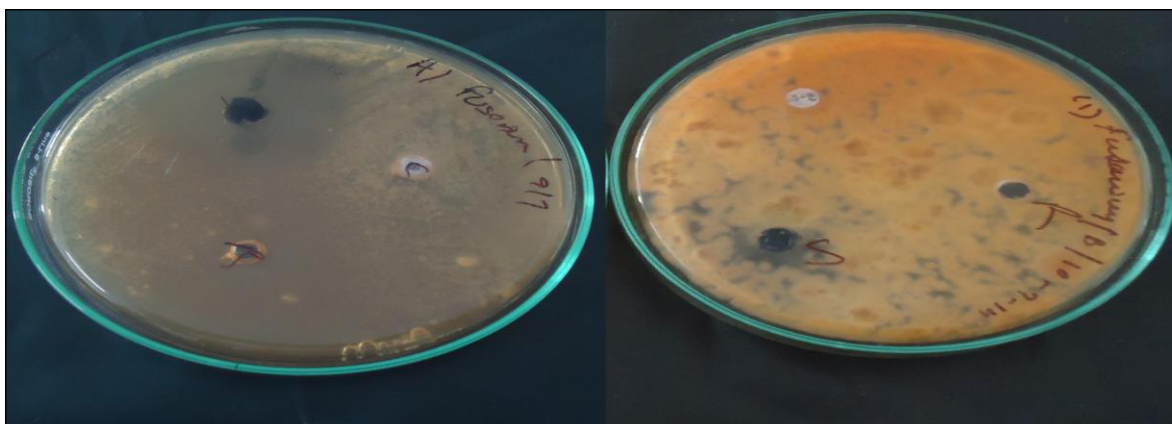


FIG 6: TEST DISKS PRESENTING SENSITIVITY OF COMPLEXES AGAINT *FUSARIUM SEMITECTUM*

The enhanced biological activity of the macrocyclic complexes can be explained on the basis of Overtone's concept and Tweed's Chelation theory. According to this theory, it has been suggested that coordination 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 also increases the delocalization of π-electrons over the whole chelate ring and enhances the lipophilicity of the complexes which subsequently enhances the 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.

These complexes also perturb the respiration process of the cell and thus block the synthesis of the proteins that restricts further growth of the organism. In addition to this, many other factors, such as solubility, dipole moment, conductivity, stability and geometry of the complexes which are influenced by the metal ion may be the possible reasons for the antimicrobial activities of these metal complexes.

Most commonly such agents inhibit or kill the microbes by following basic mechanism:

- Transition metal complexes bind the microbes or their metabolites through N O S donor.
- Damage the cell wall or inhibit of cell wall synthesis alters alternation of permeability of cytoplasmic membrane.
- Alternation of permeability of cytoplasmic membrane.
- Alternation physical state of protein and nucleic acids.
- Inhibition of enzyme action.
- Substrate competition with essential metabolites.

Following graphs are the comparative interpretations for the antimicrobial study for the synthesized complexes:

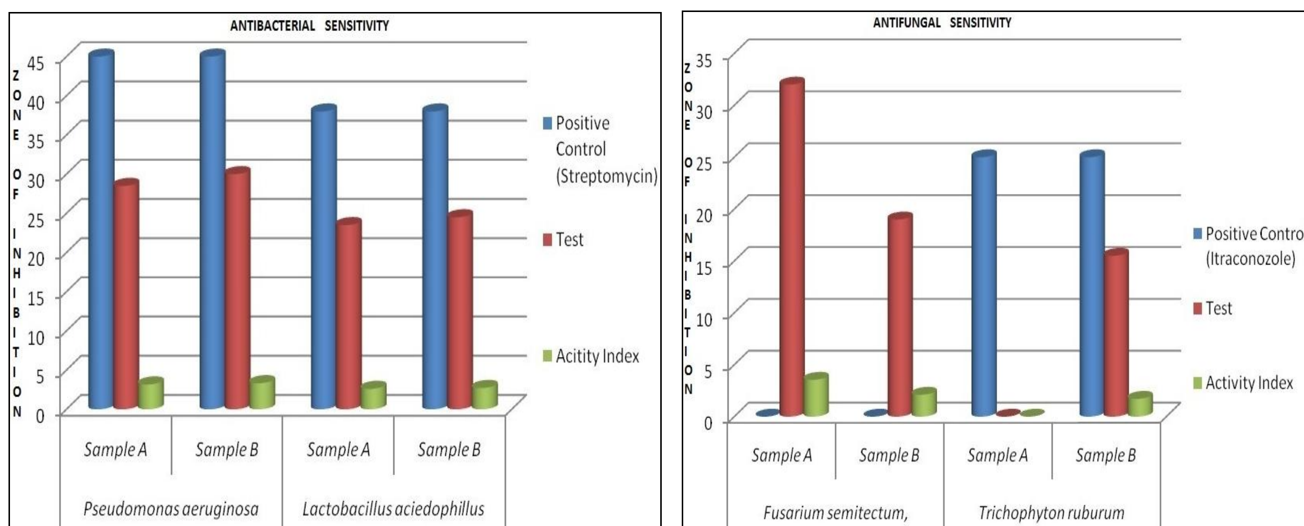


FIG. 7: PLOTS PRESENTING THE COMPARATIVE STUDY OF COMPLEX SENSITIVITY AGAINST DIFFERENT MICROBES UNDER STUDY

The antimicrobial screening of the Complex A (namely Copper palmitate with 2-amino 6-bromo Benzothiazole) and Complex B (namely Copper Caprylate with 2-amino 6-bromo Benzothiazole) were performed against two pathogenic bacteria *Pseudomonas aerugenosa* (gram negative), *lactobacillus acidophilus* (gram positive) and two fungi namely *Trichophyton ruburum* and *Fusarium semitectum*. Since DMSO was used as a solvent, it was also screened against all organisms. The diameter of zone of inhibition induced by DMSO was 9mm. The results of the investigated samples were summarized in **Table 1** and **Table 2**.

The antibacterial activity results revealed that both the complex showed less activity as compared to standard streptomycin. The inhibition of the synthesized metal complexes against all tested bacteria is in the order- complex B > complex A

On the other hand, the antifungal activity results revealed that the complex showed more activity as compared to standard Itraconozole in case of *Fusarium semitectum* but less for *Trichophyton ruburum.* Specially, Complex of copper palmitate with substituted benzothiazole (Sample A) was totally inactive for Trichophyton. The inhibition of the synthesized metal complexes against all tested fungi is in the order-

complex A > complex B (for the case of *Fusarium semitectum*)

complex B > complex A (for the case of *Trichophyton ruburum*)

CONCLUSION: The antibacterial and the antifungal activity of the synthesised complexes have been evaluated by the Kirby-Bauer and Stokes' method. The results are expressed in millimeter. The two antimicrobial disks with streptomycin (for anti-bacterial) and (Itraconozole for anti-fungal) were taken as standards and the sample disks were compared with it. A scrutiny of **Table 1** and **2** reveals that both pure soaps and ligands show less inhibition whereas on complexation the inhibition enhanced and also complexes show higher activities then pure ligands suggesting that complexes are more powerful agents. Hence, we can conclude that Benzothiazoles and other N and S containing compounds are able to enhance the performance of copper soaps.

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