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Biological Activities of Some New Environmentally Safe 2-Aminobenzothiazole Complexes of Copper (II) Derived Under Microwave Irradiation

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ABSTRACT
Chemistry of present era aims to build a pollution free environment. For the same, it targets to create some alternatives which are eco-friendly and nature loving. Present research work is a step towards achieving such alternatives. For this, complexes copper (II) palmitate with 2-amino-6-chlorobenzothiazole and 2-amino-6-methylbenzothiazole were synthesized under microwave irradiation. These compounds were also obtained with conventional heating procedures to compare them with those obtained with microwave and characterized by elemental analysis, IR, NMR, ESR spectral studies. Their purity was checked by thin layer chromatography. The fungi toxicities of the ligands and complexes have been investigated using Antifungal Disk Diffusion susceptibility testing of yeasts of Candida species approved guideline (M44-A, NCCLS, USA). The fungi toxicity results indicate that the strain of Candida species are susceptible towards complexes of benzothiazole and suggests that with the increase in concentration of copper palmitate complexes it may increase further.
KEY WORDS: *2-amino-6-chloro benzothiazole, 2-amino-6-methylbenzothiazole, copper (II) palmitate, antifungal activities, Disk Diffusion Method*

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INTRODUCTION

Co-ordination chemistry of transition metal ion has a great interdisciplinary relevance in day to day life. Improved pharmaceuticals are no doubt gift of above mentioned field and indirectly demand to control the harmful effects of bacteria, fungi and viruses. It definitely creates zeal to synthesize such improved versions. These significant properties seem to be a result of chelation behavior between ligands and transition metal ion.

So, attempts are made to prepare binuclear macro cyclic complexes of azole ring derivatives with transition metal ions (special emphasis on copper), which possess beneficial properties like antibacterial, antimalarial, antifungal, antibacterial, anticancerous, antiallergic, anticonvulsant, antibiotic etc [1-10].

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 [11-12]. Hence, they share an important account in industries, modern engineering and pharmacy. Aforesaid fact motivates us to design novel complexes of biological interest related to our daily life.

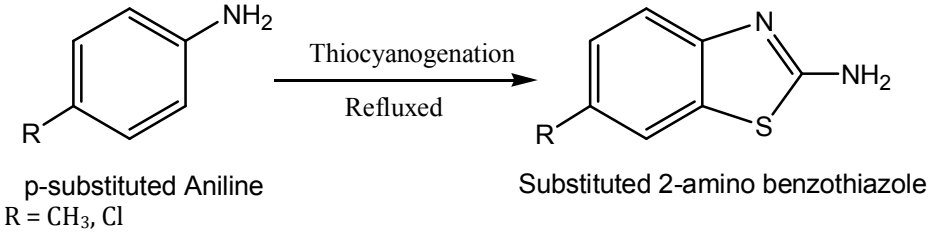
METHOD AND MATERIALS

Preparation of Substituted Benzothiazole

In the Thiocyanogenation method 12.3 g p-chloro aniline/13.8 p-methyl aniline (0.1 mole) was treated with a mixture of 7.6 g 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

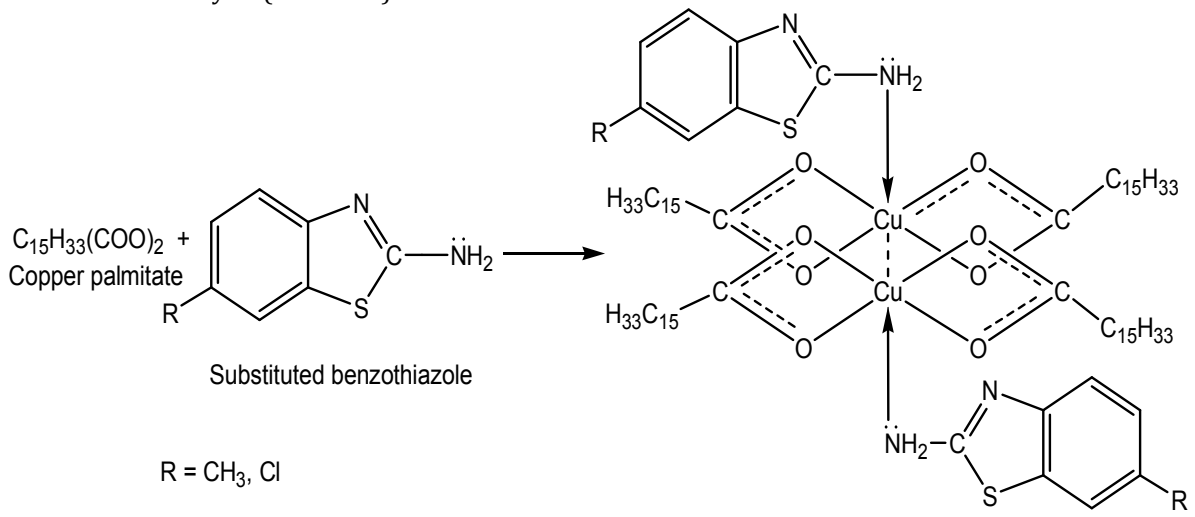
saturated solution of sodium carbonate (Na_2CO_3) is added to neutralize it, till the solid was formed. The precipitate was filtered washed with cold water, dried and recrystallised with ethanol (Scheme 1).



Scheme 1. Benzothiazole of substituted aniline

Preparation of complexes

The complexes of copper palmitate and benzothiazole were prepared by adding 0.6295 g (0.001 mole) copper palmitate with 0.002 mole 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 (Scheme 2).



Scheme 2. Complex of copper palmitate and substituted benzothiazole

Susceptibility testing

DD method was performed on Mueller – Hinton agar supplemented with 2% glucose and 0.5 □g of methylene blue per ml due to the ability of that medium to produce enhanced definition of growth margins. To prepare the medium, stock solutions of MB (0.1g/20 ml) was made in distilled water. 100 □l of this solution per liter of agar suspension were added then 20 g of glucose per liter of agar suspension were added.

The GMB stock solution was filter sterilized and cooled in a 45 to 50°C water bath, Mueller-Hinton agar were prepared by pouring stock solution into Petri dishes on a level horizontal surface to give a uniform depth of approximately 4 mm and allowing it to absorb for 4 to 6 hour. If excess surface moisture was present in the incubator (10 – 30 minutes) with the lids agar until the excess moisture has evaporated. The surface should be moist, but with no droplet on the agar surface or the Petri dish cover. Petri dishes were stored in refrigerator (4°C). The agar medium had pH between 7.2 and 7.4 at room temperature. These Petri dishes were kept for 24 hours in other incubator at 37°C for sterility test.

To control the precision (repeatability) and accuracy (trueness) of the results obtained with disk diffusion test procedure, several quality control strains were obtained from reliable source.

(1) Candida albicans ATCC 90028
(2) Candida krusei ATCC 6258

The quality control strains were tested by the standard disc diffusion test using the same materials and methods that are used to test clinical isolates. Quality control strains were stored in a way that minimizes the possibility of mutation in the organism.

Inocolum preparation

Inoculums were prepared by picking five colonies of approximately 1 mm in diameter from a 24 hour old in 5 ml of sterile 0.145 mol/liter saline (8.5 g/liter NaCl; 0.85% saline).

Inoculum suspensions

The inoculum suspensions were prepared as described for the CLSI M44-A, USA method [13-14]. The turbidity was measured with a spectrophotometer at 625 nm and was adjusted to match a 0.5 McFarland density standard, resulting in a concentration of 1×10^6 to 5×10^6 cells/ml. These inoculums were used directly for inoculation of agar plate's growth. To standardize the inoculums density for a susceptibility test, a $BaSO_4$, suspension with turbidity equivalent to 0.5 McFarland standards or its optical equivalent to a 0.5 McFarland standard or its optical equivalent was used as turbidity standard for inoculums.

Preparation of Mcfarland nephlometry Standard:

- Required reagents

I. $BaCl_2$ $2H_2O$: 0.048M/Lit or 1.175% [W/v]
II. H_2SO_4: 0.18 M/Lit or 1%
III. Distilled water 200 ml

Procedure

100 ml distilled water filled in two 250 ml capacity sterile flasks. In one flask 1.175% $BaCl_2$ $2H_2O$ solution is prepared. From the other flask 1 ml of distilled water is discarded and 1 ml pure H_2SO_4, is added to make a 1% [v/V] solution.

From the H_2SO_4 solution, 0.5 ml is discarded and to the remaining 99.5 ml, $BaCl_2$. $2H_2O$ solution is added drop by drop at constant stirring. The solution thus prepared is now consisting of 0.5 standards of McFarland standards which is equivalent to 1.5×10^8 cell/ml. The solution is distributed into 2 – 3 large [18×150 mm] test tubes in approximately 4 – 6 ml amount. The transmittance of the solution at 625 nm was between 0.08 – 0.1 and is standard.

Inoculation of Test Plates

A sterile cotton swab was dipped into the suspension. Dipping a sterile cotton swab into the dried surface of inoculums and evenly streaking the swab in three directions (60 each) over the entire surface of the plate inoculated the agar plates. The plates were allowed to dry for at least 15 minutes before the disks were applied to the surface.

Preparation of Dispensing Disks

For disk diffusion tests we require two types of antimicrobial disks.

1. Antimicrobial disks
   They were used as standard for quality control. Here we use amphotericine and ketoconozole antifungal agents for this purpose.
2. Test disks (for complexes and ligands)
   To prepare the disk of testing samples we required Watmann filter paper No. 2 and vials. First of all 6 mm diameter disks of Watmann filter paper were punched. 100 disks were kept in each vial. These vials were carefully sealed with cotton plug and sterilized by heating in oven.

All the requirements used in dispensing were sterile. Sterile vials containing 100 disks, which absorbed all the solution. This dispensing was done in sterile hood/chamber that was already cleaned with methanol and exposed to U.V. light and blower; solutions were dispensed in the vials near the lighted sprit lamp kept in the hood. The testing compounds of concentration 0.5 mg/disk are listed and abbreviated as follows:

1. Complex of copper palmitate with 2-amino-6-methylbenzothiazole. CP [BTA]CH_3 - C1
2. Complex of copper pamitate with 2-amino-6-chlorobenzothiazole. CP [BTA]Cl - C2
3. Ligand; 2-amino-6-methylbenzothiazole-(BTA)CH_3 - C3
4. Ligand; 2 –amino chlorobenzothiazole--(BTA)Cl - C4
5. Standard – Amphotoricine
6. Standard – Ketoconozole

RESULT AND DISCUSSION

Micro analytical data of the compounds were recorded at Regional Sophisticated Instrumentation Center, Central Drug Research Institute, Lucknow (RSIC, CDRI). The melting points were determined in an electrically heated apparatus. The IR spectra were recorded in KBr pellets (400-4000 cm^{-1}) on Perkin Elmer

spectrophotometer. The NMR spectra were recorded in $CDCl_3$ at CDRI, Lucknow with TMS as the internal reference. ESR spectrophotometer equipped with 100 KHz field modulations. Tetracyanoethylene (TCNE) was used as the standard. Solvents were purified according to standard procedures.

BIOCIDAL STUDY

In the present study, we investigated the applicability disc diffusion (DD) method for determining the susceptibility of Candida species against newly synthesized complexes, which were prepared by mixing copper palmitate with substituted 2-aminobenzothiazoles. For this purpose some standard antifungal compounds were used as references.

These antifungal research powders were stored at -20°C until they were used. We use two standard antifungal agents for this test viz. Amphotericine and Ketoconozole.

Table 4: Antifungal effect of copper(II) palmitate with substituted ligands and complexes against *Candida albicans* and *Candida krusei* (expressed as inhibition zone in mm).

Disks (6 mm) 0.5 mg	*Candida albicans* (in mm)	*Candida krusei* (in mm)
$CP(BTA)CH_3$	13	17
CP(BTA)Cl	18	14
$(BTA)CH_3$	12	6
(BTA)Cl	11	10

Table 5: Antifungal effect of copper(II) palmitate with substituted ligands and complexes against *Candida albicans* and *Candida krusei* (expressed as inhibition zone in mm).

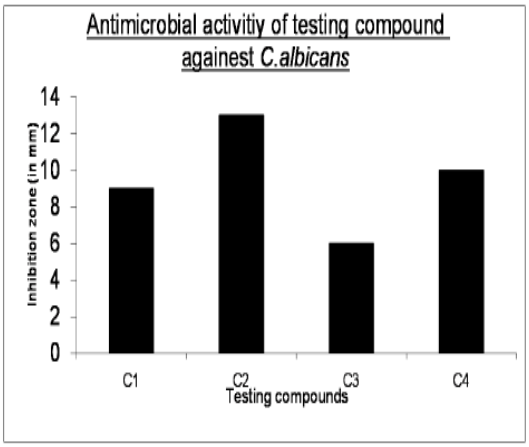
Disks (6 mm) 0.1 mg	*Candida albicans* (in mm)	*Candida krusei* (in mm)
$CP(BTA)CH_3$	7	7
CP(BTA)Cl	-	6
$(BTA)CH_3$	-	5
(BTA)Cl	3	2

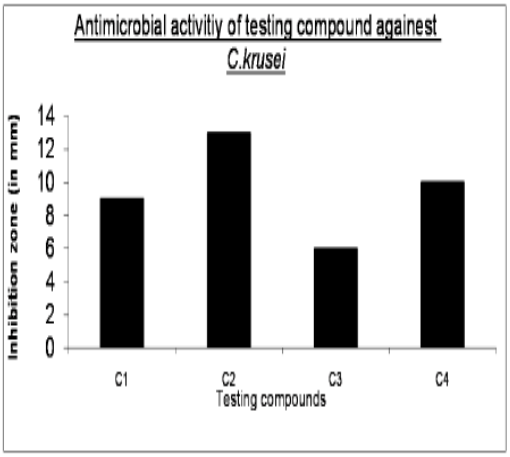
Table-6: Disk-diffusion test results for Standard Disks

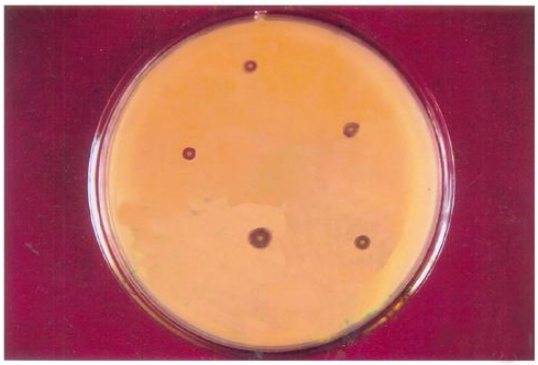
Antimicrobial disks	*Candida albicans* (in mm)	*Candida krusei* (in mm)
Amphotericine	19	21
Ketocanazole	22	21

On the basis of our results, it is suggested that the Disc Diffusion test is a useful method for testing the activity of synthesized compounds against *Candida* species. It is very attractive due to its simplicity, reproductivity, and lack of requirements for specialized equipment. The ability of Disc Diffusion test to determine the susceptibility of an individual within 24 hrs seems to be a noticeable advantage.

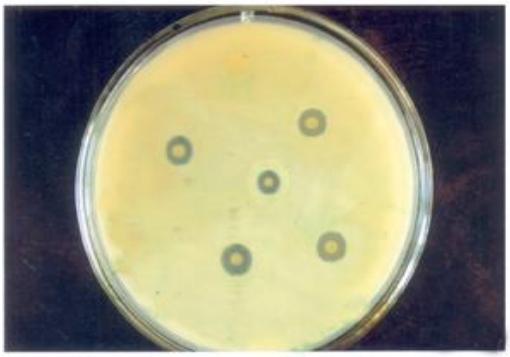
On the other hand, it is clearly observed that activity of complexes is enhanced as compared to free ligands mainly due to synergistic mechanism.



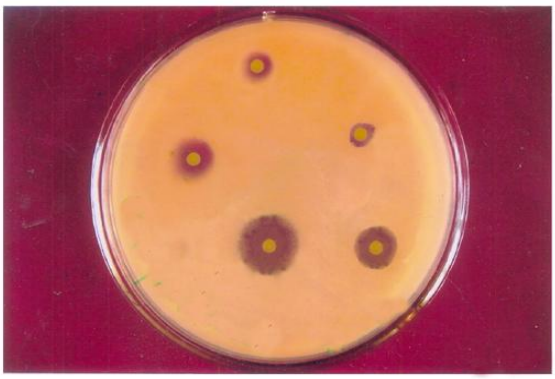




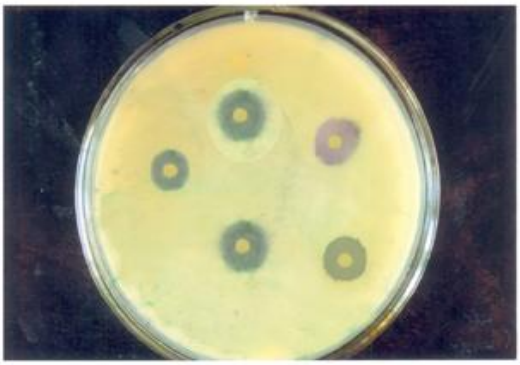
Test Disk : At Lower concentration for Copper Palmitate and its complexes against Krusei



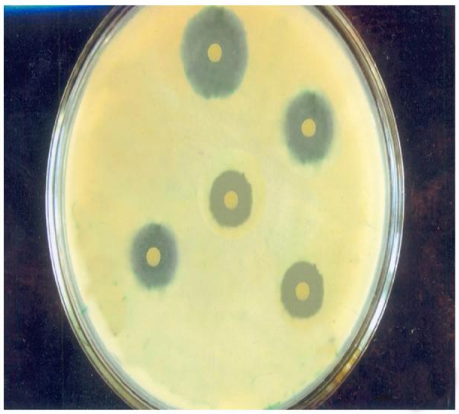
Test Disk : At Lower concentration for Copper Palmitate and its complexes against Albicans



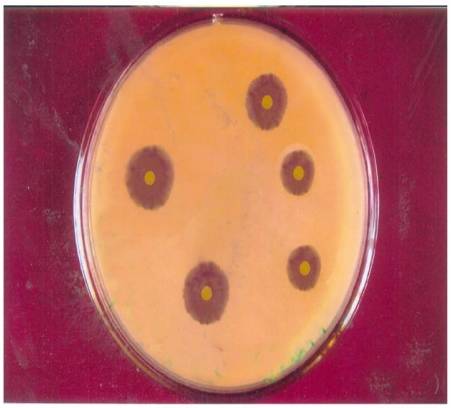
Test Disk : At Higher concentration for Copper Palmitate and its complexes against Krusei



Test Disk : At Higher concentration for Copper Palmitate and its complexes against Albicans



Test Disk : Standard Disk against Albicans



Test Disk : Standard Disk against Krusei

CONCLUSION

The antifungal activities of free ligands and their corresponding complexes have been evaluated by the disk diffusion test. The results are expressed in millimeter. The two antimicrobial disks with amphotericine and ketoconozole were taken as standards and the sample disks were compared with it.

A scrutiny of table 4 reveals that all complexes show higher activities then pure ligands suggesting that complexes are more powerful agents. Benzothiazoles and other N and S containing compounds are able to enhance the performance of copper soaps. It was observed that enhanced activity of complexes was also due to synergistic mechanism, i.e. free ligand and pure soap show less activity but on complexation they show enhanced activity. These results support out our studies where pure soaps and ligands show less inhibition whereas on complexation the inhibition enhanced.

Here it must be noted that studies against *Candida albicans* revealed that among the substituent's present on benzothiazole ring, the chloro group show higher activity as compared to the methyl group, but in case

of *Candida krusei* the results were just opposite. This may be attributed to the fact that the atom introduced into the complex through the ligand also plays an important role in enhancing the effectiveness of fungicidal molecule.

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