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Synthesis and Analysis of Copper Neem (*Azadirechta Indica*) Soap-Nitro and Ethoxy Benzothiazole Complexes for Anti-Bacterial Activity Related with Skin Diseases

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Abstract

The solid copper(II) soap derived from Neem *(Azadirechta Indica)* oil and its complex with ligand containing nitrogen, sulphur and oxygen atoms like 2-amino -6-nitro benzothiazole and 2-amino- 6-ethoxy benzothiazole have been synthesized and characterised by elemental analysis, IR spectroscopy and biological studies. From the analytical data, the stoichiometry of the complex has been observed to be 1:1 (metal:ligand). The derived compounds were found active against *Staphylococcus aureus, Coagulase-negative staphylococci (CoNs), Acinetobacter baumanii, Pseudomonas aeruginosa* and *micrococcus* bacteria. These findings have high medical, industrial and economic significance as copper (II) soap and copper (II) soap complex could be harnessed in the formulation of medicated soaps.

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Introduction

The current global attention towards the production of biodiesel especially from non-edible sources has made research into production of non-edible vegetable oil a necessity. Biodiesel is a clean, biodegradable, non-toxic, renewable and environmentally friendly energy source made from fat and oil via transesterification processes. Neem *(Azadirechta Indica)* oil is generally light to dark brown, bitter and has a rather strong odour that is said to combine the odours of peanut and garlic. It comprises mainly triglycerides and large amounts of triterpenoid compounds, which are responsible for the bitter taste. It also contains azadirachtin, meliantriol, salannin, nimbin and nimbidin. [1-2]

Neem *(Azadirechta Indica)* oil is widely used as insecticides, lubricant, drugs for variety of diseases such as diabetes and tuberculosis. This oil could also prolong leather goods when it is applied on them. There are several methods to obtain neem *(Azadirechta Indica)* oil from the seeds like mechanical pressing, supercritical fluid extraction, and solvent extraction. Antiseptic soap, sometimes called anti-bacterial soap or anti-fungal soap, is a regular soap in liquid or solid form. According to Osbore and Grobe antibacterial soaps can remove 65 to 85% bacteria from human skin. Contemporary commercial antimicrobial soaps contain synthetic chemicals such as triclosan, triclorocarbanilide and chloroxylenol, most of which are thought to be car-cinogenic, mutagenic and or generate allergic reactions. [3-4]

Thiazole a heterocyclic nucleus played influential role in the advancement of different medicinally important moiety. They are mainly intrinsically toxic to microorganism especially to fungi and bacteria. Substituted benzothiazoles and phenylthiourea constitute an important class of compounds. The basic structure of benzothiazole consist of benzene ring fused with 4, 5 position of thiazole. Copper has efficiency to bind with fatty acids and oils to form copper soaps. Copper (II) soaps in polar and non polar solvents are used in emulsification, wetting, lubricating and foaming due to their surface active properties. Combination of copper soaps with heterocyclic ligands play wonderful role in various fields. They show fungicidal, pesticidal, insecticidal and nematocidal activities. The above mentioned applications give us the reason to synthesize the complexes of copper soap with N-donor ligands. With the help of elemental analysis, melting points IR spectroscopy; characterization of these complexes were done. [5-7]

Micro-Organisms Taken for Study are as Follow

Micro-oraganisms employed to study and explain the bactericidal processes of complex synthesized in our laboratory were as follows:

Staphylococcus Aureus

S. aureus are cocci that form irregular grape-like clusters. They are non-motile, non-sporing and catalase positive. They grow rapidly and abundantly under aerobic conditions. On blood agar, they appear as glistening, smooth, entire, raised, translucent colonies that often have a golden pigment. [8]

Coagulase Negative Staphylococci (CoNS)

Coagulase negative staphylococci (CoNS) are normal commensals of the skin, anterior nares, and ear canals of humans. They have long been considered as non-pathogenic, and were rarely reported to cause severe infections. However, as a result of the combination of increased use of intravascular devices and an increase in the number of hospitalized immunocompromised patients, CoNS have emerged as a major cause of nosocomial bloodstream infections. [9]

Acinetobacter Baumanii

Acinetobacter species are Gram negative nonfermentative bacteria commonly present in soil and water as free living saprophytes. They are isolated as commensals from skin and throat. There have been frequent changes in their taxonomy so that their pathogenic role is understood only recently. *Acinetobacter* has emerged as an important nosocomial pathogen involved in outbreaks of hospital infections. [10]

Pseudomonas Aeruginosa

Pseudomonas aeruginosa, a worldwide prevalent pathogen mainly associated to respiratory infections, is ubiquitous in nature, with the water as its preferred habitat. Hydropathic facilities, commonly named *thermae*, are used to treat respiratory health conditions by deep inhalation of unprocessed natural mineral water. [11]

Micrococcus Luteus

They are mostly arranged in tetrads. They are positive for catalase and oxidase. They grow in circular, entire, convex and creamy yellow pigmented colonies having diameters of approximately 4mm after 2-3 days at 37°C. Several uncommon strains produce raised colonies with translucent, depressed centres. Colony pigmentation varies considerably but are usually different shades of yellow or cream-white. [12]

Experimental

All the chemicals used were of LR/AR grade. Solvent was purified according to standard procedures before use. Elemental analysis was done for soap and complex for their metal content following standard procedures. The complexes under study were prepared in two steps. In the first step, copper soap was prepared and in the second step complexation of copper soap was done with ligands like nitro benzothaizole and ethoxy benzothaizole. [13]

Benzene was dehydrated by storage over sodium wire for 2–3 days and by refluxing for about twenty hours, it was then distilled and redistillation was carried out azeotropically with ethanol. [14]

Copper soap was prepared by refluxing the non edible oils i.e. Neem oil, in its pure form, of a available in the Indian market, with alcohol and 2N KOH solution for 3 hours (Direct metathesis). The neutralization of excess KOH present was done by 1N HCl. Saturated solution of copper sulphate was then added to it for conversion of neutralized soap into copper soap. Copper soap so obtained was then washed with hot water and dried. The soap was recrystallized using hot benzene. The fatty acid composition of the non edible oil was confirmed through gas liquid chromatography [GLC] of its methyl esters and is given in Table 1. [15]

The ligands 2-amino -6-nitro benzothaizole and 2-amino -6-ethoxy benzothaizole were synthesized using thiocyanation method. The purified copper soap derived from non edible oil was refluxed with ligands (2-amino-6 -nitro benzothaizole and 2-amino-6-ethoxy benzotha-izole) in 1:1 ratio using benzene as a solvent for one hour. It was then filtered hot, dried, recrystallized and purified in hot benzene. Thin layer chromatography [TLC] using silica gel was used to check the purity of the complex. [16]

In general, all the soild complexes obtained were green in colour. The complexes were soluble in benzene and other organic solvents but insoluble in water. The complexes were stable at room temperature, their physical parameters like saponification value (S.V.), saponification equivalent (S.E.) and molecular weights are recorded in Table 2.

On the basis of the elemental analysis, 1:1 (metal:ligand) type of stoichiometry has been suggested.

Material & Methods

In order to study the structure of soap and complexes, the infra–red (IR) absorption spectra of compounds were obtained on a ABB Horizon MB 3000 series instrument spectrophotometer (4000 – 600 cm^{-1})

Table 1. Fatty acid composition of oil used for copper soap / complexes synthesis

Name of oil	% fatty acid				
	16:0	18:0	18:1	18:2	Other acid (C_{20} – C_{24})
Neem oil	14.9	14.4	61.9	7.5	1.3

Table 2. Analytical and physical data of copper soap and their complexes derived from Neem oil

Name of soap /complexes	Colour	M.P.	Metal Content %		S.V.	S.E.	Average M.W.
			Observe	Calculate			
CN	Dark Green	50^0c	10.16	10.07	198	283.33	628.16
CNB	Green	62^0c	8.46	8.26	-	-	768.28
CEB	Green	60^0c	8.46	8.27	-	-	767.34

from SPC Govt. College, Ajmer.

The biological activities of copper soap and its corresponding complex with ligand nitro and ethoxy benzothaizole have been screened against *Staphylococcus aureus, Coagulase-negative staphylococci (CoNs), Acinetobacter baumanii, Pseudomonas aeruginosa,* and *micrococcus* bacteria at 3 * 10^4 ppm, 1.5* 10^4 ppm, .75* 10^4 ppm, and .375 * 10^4 ppm using disc of these solutions by Mueller Hinton Agar plates.

The following Bacteria: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *CoNS, Micrococcus* and *Acinetobactor*. All bacterial strains were maintained on nutrient agar medium at ±37°C. These cultures are obtained from the Department of Microbiology, Dr. S.N. Medical College Jodhpur.

Antimicrobial Evaluation

The antimicrobial activity of newly synthesized compounds was evaluated using agar disc diffusion assay. Briefly, a 24 and 48 hours old culture of selected bacteria was mixed with sterile physiological saline (0.9%) and the turbidity was adjusted to the standard inoculum of Mac Farland scale 0.5 (106 colony forming units (CFU) per ml). Petri plates containing 20 ml of Mueller Hinton Agar was used for antibacterial activity. The inoculum was spread on the surface of the solidified media and Whatman No. 1 filter paper discs (5 mm in diameter) impregnated with the test compound (20 µl/ disc) were placed on the plates. Ampicillin (10 mg/disc) was used as positive control for bacteria. A paper disc impregnated with petroleum ether was used as a negative control. Plates inoculated with the bacteria were incubated for 24 hour at 37° C. The inhibition zone diameters were measured in centimeters. All the tests were performed in triplicate, The E test (AB Biodisk) which is a quantitative method for antimicrobial susceptibility testing was applied the dilution of antibiotic and diffusion of antibiotic into the medium and the average was taken as final reading. A method is considered repeatable if independent repeats of the same experiment in the same laboratory produce nearly the same results. The conventional measure of repeatability is the standard deviation or some multiple of the standard deviation, standard error, variance etc. [17-18] The ANOVA (software) also provided a variance component analysis to assess the variance within experiments and the variance between experiments. The square root of the sum of those two variances was sr, which was interpreted as the typical difference, sign neglected, between the log density for a single (randomly chosen) experiment. [19]

Results and Discussion

The copper soap and complexes are abbreviated as follows

a. Copper - Neem Soap (CN)
b. Copper – Neem Soap 2-amino -6-nitro Benzothaizole Complex (CNB)
c. Copper – Neem Soap 2-amino -6-ethoxy Benzothaizole Complex (CEB)

IR Spectral Analysis

The absorption bands observed at 2924 cm^{-1} and 2854 cm^{-1} corresponds to asymmetric and symmetric stretching of methylene ($-CH_2$) group. The presence of absorption bands at 1458 cm^{-1} is representative of symmetric bending of nearly 3010 cm^{-1} corresponds to olefinic =C–H stretch. The strong absorption band at 1589 cm^{-1} were due to carboxylate ion COO–,C–O anti symmetric respectively. Also >C=O stretching bands were observed at 1744 cm^{-1}. Also peaks corresponding to $–CH_3$ and $–CH_2$ rocking have been seen at 1157 cm^{-1} and 725 cm^{-1} respectively. Copper–oxygen (Cu–O) stretching bands have been distinguished at 424 cm^{-1} . [20]

The above-mentioned absorption bands (Table 3) were found to be common with the absorption bands observed for pure copper soap of non edible oils. Apart from these absorption bands the following bands were also observed corresponding to the ligand moiety. [21]

The C–N stretching band of primary amide was observed at nearly 2361 cm^{-1} the absorption band 1744 cm^{-1} was found to be representative of amide >C=O group. A broad band near 3564 cm^{-1} was observed corresponding to N–H stretching of amides. Also unconjugated C-N stretching band was observed at 1095 cm^{-1}. C–H stretching band due to deformation out of plane (in benzene) was also observed at 649 cm^{-1}. (Table 3) [22], (Figure 1).

Thus on the basis of above observations it can

Table 3. IR spectral data for copper (II) Neem Soap and their Complexes

Absorption bands	CN (cm^{-1})	CNB (cm^{-1})	CEB (cm-1)
Corresponding to soap moiety			
Olefinic =C–H stretching	3010	3010	3010
CH_3 and CH_2, C–H Anti sym. stretching (v_{as})	2916	2924	2924
CH_3 and CH_2, C–H sym. stretching (v_s)	2854	2854	2854
C=C Stretching	2200	2152	2160
>C=O Stretching	1744	1744	1744
C=C Stretching (Cis Unconjugated)	1665	1651	1655
COO^-, C–O Anti-sym. stretching	1582	1589	1582
CH_2, C–H Bending (δ) (scissoring)	1465	1458	1458
C–H, deformation, =C–H Rocking	1443	1427	1420
CH_3, C–H Rocking	1157	1157	1157
CH_2, C–H Rocking	725	717	825
=C-H, Out of Plane Bending of C-H	679	648	725
Cu–O stretching	480	560	560
Corresponding to ligand moiety			
Asymm. NH_2, N–H stretching	-	3672	3742
NH_2, N–H stretching	-	3564	3564
C–N stretching	-	2361	2361
Unconjugated C–N stretching	-	1095	1095
C–H, deformation due to benzene (out of plane)	-	648	649

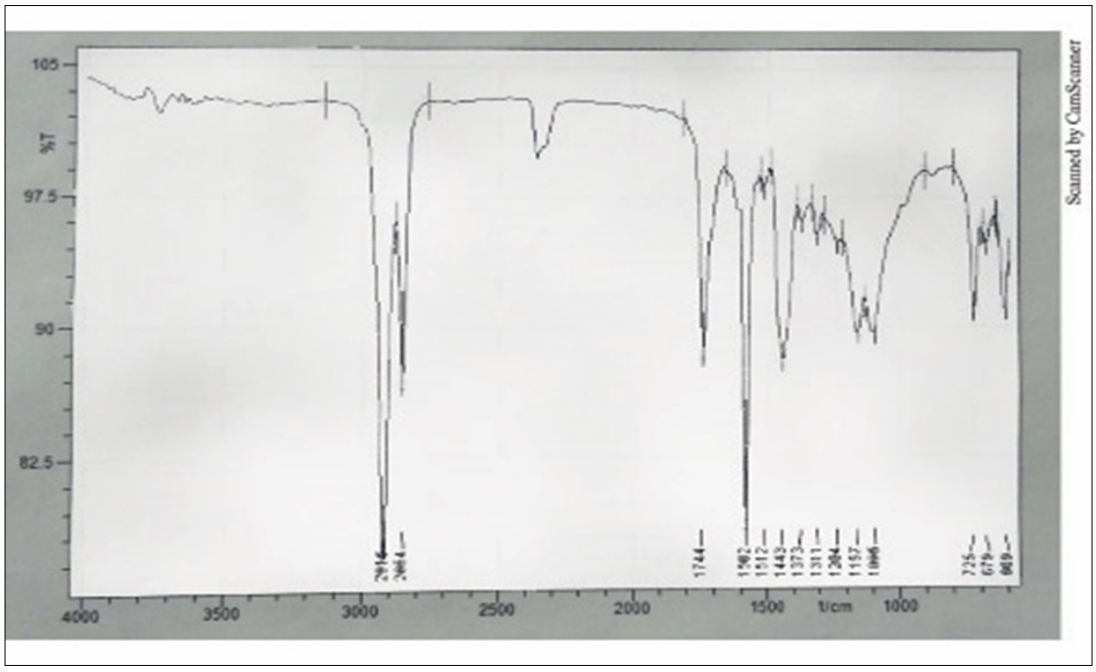

Figure 1. IR Spectra of Copper Neem Soap

Table 4. Bactericidal data for copper (II) soap and their complexes derived from Neem oil

Concertration (PPM)	3 * 104	1.5 * 104	.75 * 104	.375* 104	SOLVENT
CN S.A. S. aureus (cm)	1.7	1.5	1.3	1	0.3
CEB S.A. S. aureus (cm)	1.8	1.7	1.5	1.3	0.3
CNB S. aureus (cm)	1.7	1.5	1.3	1.1	0.3
CN Cons (cm)	1.7	1.5	1.2	1	0.3
CEB Cons (cm)	1.7	1.5	1.3	1.1	0.3
CNB Cons (cm)	1.8	1.6	1.4	1.2	0.3
CN A. baumanii (cm)	1.6	1.4	1.2	1	0.3
CEB A. baumanii (cm)	1.6	1.4	1.2	1	0.3
CNB A. baumanii (cm)	1.6	1.4	1.2	1	0.3
CN P. aeruginosa (cm)	1.6	1.4	1.2	1	0.3
CEB P. aeruginosa (cm)	1.5	1.3	1.2	1	0.3
CNB P. aeruginosa (cm)	1.6	1.4	1.2	1	0.3
CN Micrococcus (cm)	1.5	1.3	1.2	1	0.3
CEB Micrococcus (cm)	1.5	1.3	1.1	0.9	0.3
CNB Micrococcus (cm)	1.5	1.3	1.1	0.9	0.3

be safely assumed that complexation of copper soap has taken place with 2-amino -6-nitro benzothaizole and 2-amino -6-ethoxy benzothaizole.

Biological Activities

Neem soap ethoxy complex and Neem soap nitro complex show higher antibacterial activity than pure soap suggesting their complexes is more powerful antibacterial agent and other N & S etc. containing compounds are able to enhance the performance of copper soap. The enhanced activity of newly synthesized complexes as compared to the soap can possibly be explained on the basis of presence of donor atoms N and S as well as the structural compatibility with molecular nature of the toxic moiety. [23-26]

The activity of copper soap and complexes derived from Neem oil were found in the follow order soap and complexes respectively:-

For CN Soap

Staphylococcus aureus = Coagulase-negative staphylococci (CoNs) > Acinetobactor baumanii = Pseudomonas aeruginosa > Micrococcus

For CEB Complex

Staphylococcus aureus > Coagulase-negative staphylococci (CoNs) > Acinetobactor baumanii > Pseudomonas aeruginosa = Micrococcus

For CNB Complex

Coagulase-negative staphylococci (CoNs) > Staphylococcus aureus > Acinetobactor baumanii = Pseudomonas aeruginosa > Micrococcus (Tables 4-6), (Figure 2-6).

Conclusions

Copper neem soap and its complex has been synthesized and evaluated for their anti-microbial activity against Gram-positive and Gram-negative bacteria. Many of pharmaceuticals and related chemicals, however, are toxic to humans and hard to degrade within the environment. Biomedical and biodegradable products will be in the near future largest application of antimicrobial studies, which must be the area of interest for the scientists to explore the new drug formulation.

Table 5. Descriptive statistics bactericidal data for copper (II) soap and their complexes derived from Neem oil

Bacteria	Soap / Complex	Group	Count	Sum	Average	Variance	Std. Deviation	Std. Error	Coff. Variance
S. aureus	CN	$3* 10^4$	3	5	1.67	0.003	0.058	0.033	0.035
		$1.5*10^4$	3	4.4	1.47	0.003	0.058	0.033	0.040
		$.75*10^4$	3	3.8	1.27	0.003	0.058	0.033	0.046
		$.375*10^4$	3	3.1	1.03	0.003	0.058	0.033	0.056
	CEB	$3* 10^4$	3	5.5	1.77	0.003	0.058	0.033	0.033
		$1.5*10^4$	3	5	1.67	0.003	0.058	0.033	0.035
		$.75*10^4$	3	4.4	1.47	0.003	0.058	0.033	0.040
		$.375*10^4$	3	3.8	1.27	0.003	0.058	0.033	0.046
	CNB	$3* 10^4$	3	5	1.67	0.003	0.058	0.033	0.035
		$1.5*10^4$	3	4.4	1.47	0.003	0.058	0.033	0.039
		$.75*10^4$	3	3.8	1.27	0.003	0.058	0.033	0.046
		$.375*10^4$	3	3.2	1.07	0.003	0.058	0.033	0.054
Cons	CN	$3* 10^4$	3	5	1.67	0.003	0.058	0.033	0.035
		$1.5*10^4$	3	4.4	1.47	0.003	0.058	0.033	0.039
		$.75*10^4$	3	3.7	1.23	0.003	0.058	0.033	0.047
		$.375*10^4$	3	3.1	1.03	0.003	0.058	0.033	0.056
	CEB	$3* 10^4$	3	5	1.67	0.003	0.058	0.033	0.035
		$1.5*10^4$	3	4.4	1.47	0.003	0.058	0.033	0.039
		$.75*10^4$	3	3.8	1.27	0.003	0.058	0.033	0.046
		$.375*10^4$	3	3.2	1.07	0.003	0.058	0.033	0.054
	CNB	$3* 10^4$	3	5.3	1.77	0.003	0.058	0.033	0.033
		$1.5*10^4$	3	4.7	1.57	0.003	0.058	0.033	0.037
		$.75*10^4$	3	4.1	1.37	0.003	0.058	0.033	0.042
		$.375*10^4$	3	3.5	1.17	0.003	0.058	0.033	0.049

A. baumanii	CN	$3*10^4$	3	4.7	1.57	0.003	0.058	0.033	0.037
		$1.5*10^4$	3	4.1	1.37	0.003	0.058	0.033	0.042
		$.75*10^4$	3	3.5	1.17	0.003	0.058	0.033	0.049
		$.375*10^4$	3	2.9	0.97	0.003	0.058	0.033	0.060
	CEB	$3*10^4$	3	4.7	1.57	0.003	0.058	0.033	0.037
		$1.5*10^4$	3	4.1	1.37	0.003	0.058	0.033	0.042
		$.75*10^4$	3	3.5	1.17	0.003	0.058	0.033	0.049
		$.375*10^4$	3	2.9	0.97	0.003	0.058	0.033	0.060
	CNB	$3*10^4$	3	4.7	1.57	0.003	0.058	0.033	0.037
		$1.5*10^4$	3	4.1	1.37	0.003	0.058	0.033	0.042
		$.75*10^4$	3	3.5	1.17	0.003	0.058	0.033	0.049
		$.375*10^4$	3	2.9	0.97	0.003	0.058	0.033	0.060
P. aeruginosa	CN	$3*10^4$	3	4.7	1.57	0.003	0.058	0.033	0.037
		$1.5*10^4$	3	4.1	1.37	0.003	0.058	0.033	0.042
		$.75*10^4$	3	3.5	1.17	0.003	0.058	0.033	0.049
		$.375*10^4$	3	2.9	0.97	0.003	0.058	0.033	0.060
	CEB	$3*10^4$	3	4.4	1.47	0.003	0.058	0.033	0.039
		$1.5*10^4$	3	3.8	1.27	0.003	0.058	0.033	0.046
		$.75*10^4$	3	3.5	1.17	0.003	0.058	0.033	0.049
		$.375*10^4$	3	2.9	0.97	0.003	0.058	0.033	0.060
	CNB	$3*10^4$	3	4.7	1.57	0.003	0.058	0.033	0.037
		$1.5*10^4$	3	4.1	1.37	0.003	0.058	0.033	0.042
		$.75*10^4$	3	3.5	1.17	0.003	0.058	0.033	0.049
		$.375*10^4$	3	2.9	0.97	0.003	0.058	0.033	0.060
Micrococcus	CN	$3*10^4$	3	4.4	1.47	0.003	0.058	0.033	0.039
		$1.5*10^4$	3	3.8	1.27	0.003	0.058	0.033	0.046
		$.75*10^4$	3	3.5	1.17	0.003	0.058	0.033	0.049
		$.375*10^4$	3	2.9	0.97	0.003	0.058	0.033	0.060
	CEB	$3*10^4$	3	4.4	1.47	0.003	0.058	0.033	0.039
		$1.5*10^4$	3	3.8	1.27	0.003	0.058	0.033	0.046
		$.75*10^4$	3	3.2	1.07	0.003	0.058	0.033	0.054
		$.375*10^4$	3	2.6	0.87	0.003	0.058	0.033	0.067
	CNB	$3*10^4$	3	4.4	1.47	0.003	0.058	0.033	0.039
		$1.5*10^4$	3	3.8	1.27	0.003	0.058	0.033	0.046
		$.75*10^4$	3	3.2	1.07	0.003	0.058	0.033	0.054
		$.375*10^4$	3	2.6	0.87	0.003	0.058	0.033	0.067

Table 6. Anova table bactericidal data for copper (II) soap and their complexes derived from Neem oil

Bacteria	Soap / Complex	SS	df	MS	F	P-value	F crit
S. aureus	CN	0.66	3	0.22	66.25	5.43E-06	4.066
	CEB	0.44	3	0.15	44.25	2.51E-05	4.066
	CNB	0.6	3	0.2	60	7.93E-06	4.066
Cons	CN	0.68	3	0.23	68.33	4.82E-06	4.066
	CEB	0.6	3	0.2	60	7.93E-06	4.066
	CNB	0.6	3	0.2	60	7.93E-06	4.066
A. baumanii	CN	0.6	3	0.2	60	7.93E-06	4.066
	CEB	0.6	3	0.2	60	7.93E-06	4.066
	CNB	0.6	3	0.2	60	7.93E-06	4.066
P. aeruginosa	CN	0.6	3	0.2	60	7.93E-06	4.066
	CEB	0.39	3	0.13	39	4.02E-05	4.066
	CNB	0.6	3	0.2	60	7.93E-06	4.066
Micrococcus	CN	0.39	3	0.13	39	4.02E-05	4.066
	CEB	0.6	3	0.2	60	7.93E-06	4.066
	CNB	0.6	3	0.2	60	7.93E-06	4.066

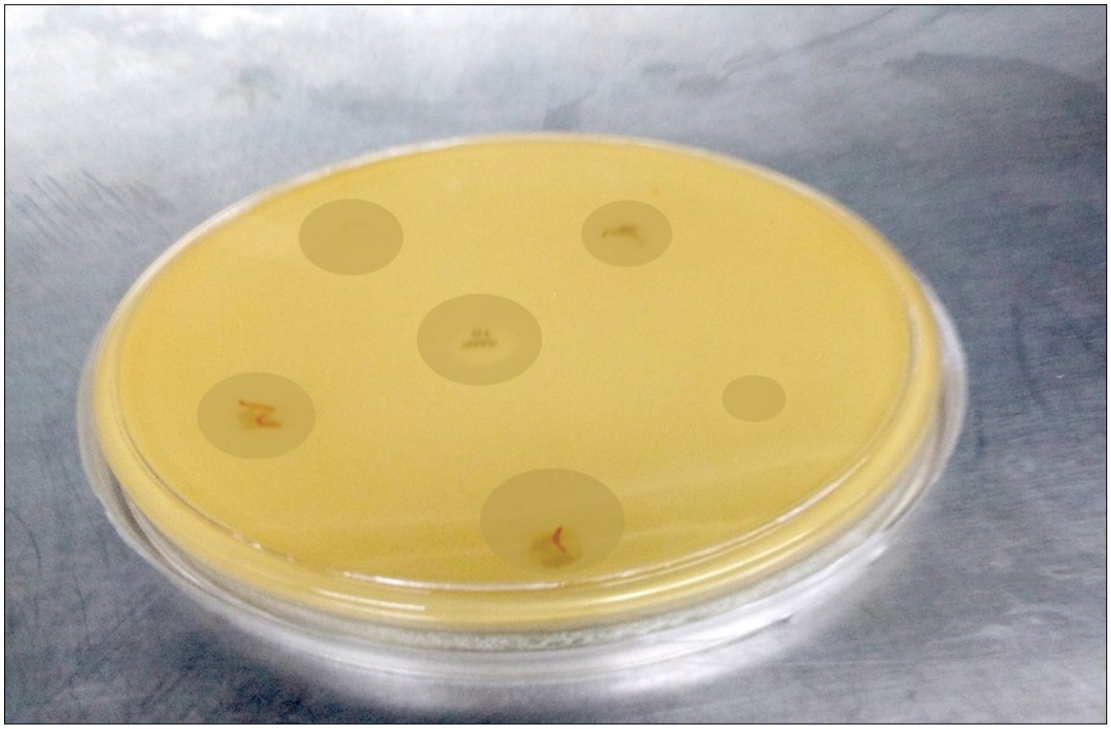

Figure 2. Zone of Copper Neem Soap For Staphylococcus aureus

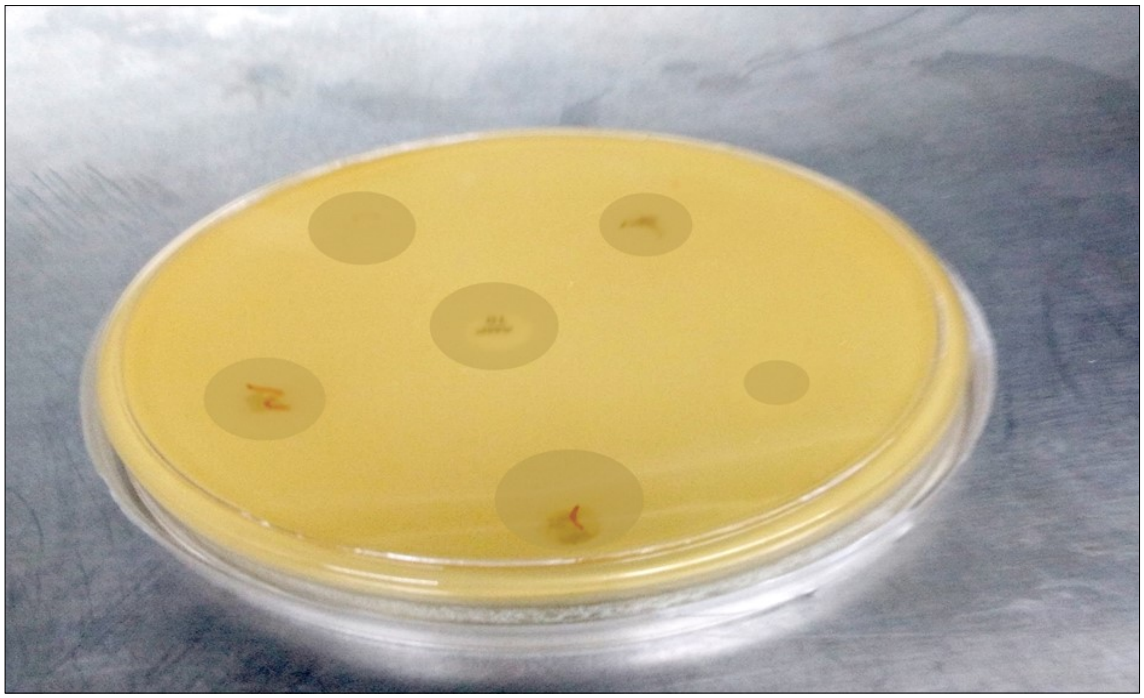

Figure 3. Zone of Copper Neem Soap 2-amino -6-ethoxy Complex for *Staphylococcus aureus*

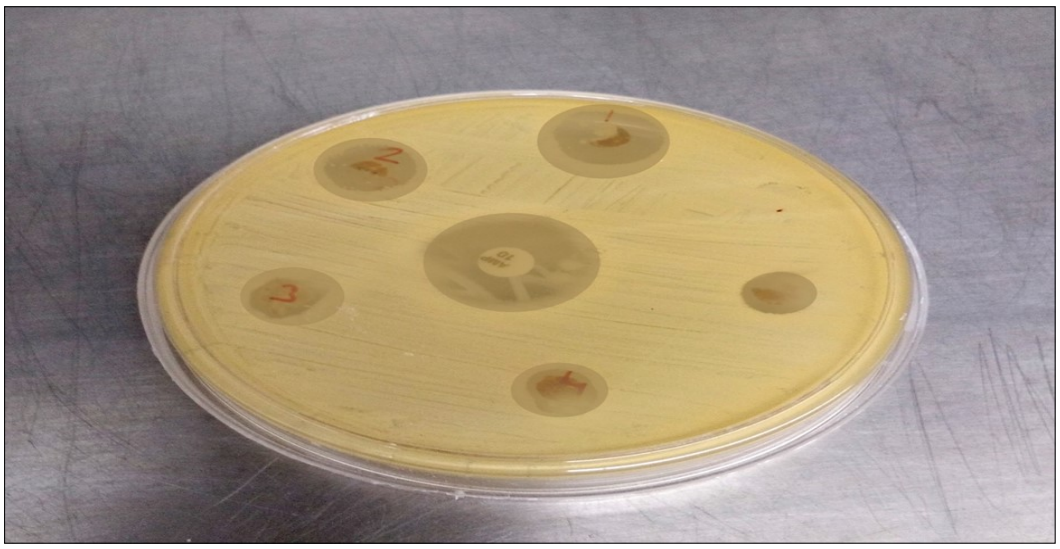

Figure 4. Zone of Copper Neem Soap 2-amino -6-nitro Complex for Coagulase-negative staphylococci (CoNs)

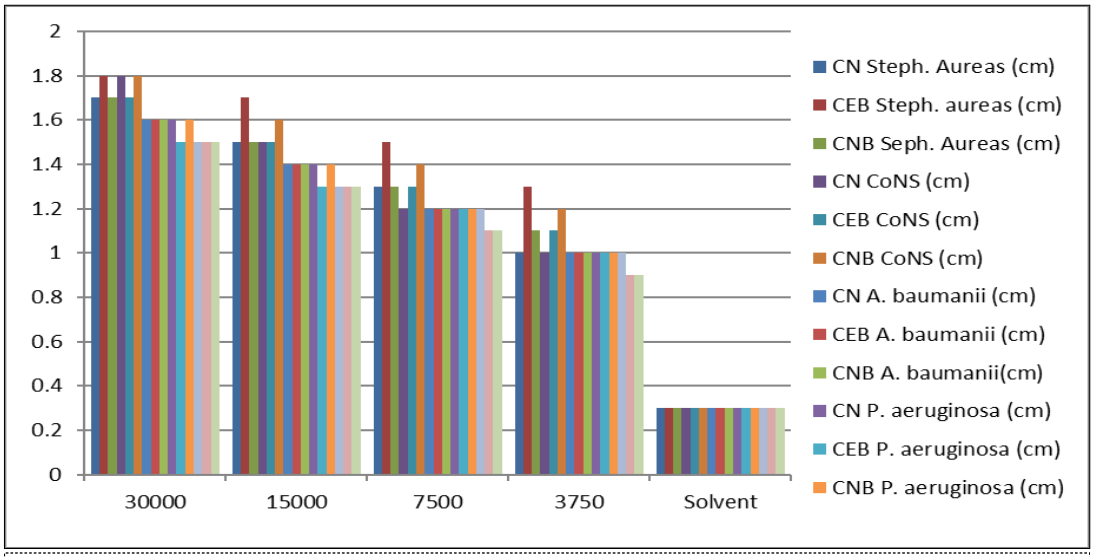

Figure 5. The Plot data for copper (II) soap and their complexes derived from Neem oil

Figure 6. skin disease of Staphylococcus aureus

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