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Synthesis and Characterization of Bactericidal Agents Derived from Neem (*Azadirecta Indica*) Oil

Priyanka Kumawat[1]*, Rashmi Sharma[1] and Nitin Sharma [2]

[1]*Department of Chemistry, SPC Govt. College Ajmer*

[2]*Department of Biochemistry, Dr. S.N. Medical College Jodhpur*

***Corresponding Author:** Priyanka Kumawat, Department of Chemistry, SPC Govt. College Ajmer.

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Abstract

In this study, we evaluated the antimicrobial potency of copper (II) soap and copper soap complex synthesized/formulated in our laboratory. The solid copper (II) soap derived from Neem (*Azadirecta Indica*) oil and its complex with ligand containing nitrogen and oxygen atoms like urea has 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 bacterial studies of soap and complex have been investigated. The derived compound was 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.

Key words: *Copper (II) soap; Complex; IR Spectroscopy; Biological studies*

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Introduction

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, tri-clorocarbanilide and chloroxylenol, most of which are thought to be car-cinogenic, mutagenic and or generate allergic reactions. Triclosan (2, 4, 4'trichloro-2'hydroxydiphenyl ester) has been used in soaps, shampoo and fabrics, as an antimicrobial agent [1].

Copper (II) soap derived from non-edible Neem (*Azadirecta Indica*) oil play a vital role in various fields due to their surface active properties. This oil was particularly chosen as it is easily available commercially and biodegradable in nature. Copper soap have a tendency of complexation with 'nitrogen' and 'oxygen' containing ligands. Using urea as a ligand, complexation of synthesized copper soap has been done to obtain its complex.

Since copper metal is toxic in nature, literature survey reveals that the synthesized copper soap and its urea complex may play a significant role in biological activities. The interest in co-ordination chemistry is increasing continuously with the preparation of organic ligands containing a variety of donor groups and it is multiplied many folds when the ligand have biological importance [2–4].

Copper metal as well as nitrogen and oxygen containing compounds play a vital role in fungicidal activities. Various non-edible oils are widely used, easily available, and are eco -friendly. Also, vegetable oil based lubricants are slowly replacing the mineral oils due to their extraordinary bio-degradability and many other specific properties [5-7]. These facts led us to synthesize copper soaps of non-edible oils and its complex with ligand containing nitrogen and oxygen elements, investigate the role of copper and other elements in biological activities. It is anticipated that it will generate new hopes in agrochemical, industrial and pharmacological field [8-11].

Micro-Organisms taken for study are as follow

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

Staphylococcus aureus (S. aureus)

Staphylococcus aureus is an anaerobic, Gram-positive *cocci*, which appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with *hemolysis*, when grown on blood agar plates. The golden appearance is the etymological root of the bacteria's name; aureus means "golden" in Latin. *S. aureus* may occur as a commensal on human skin; it also occurs in the nose frequently (in about a third of the population) and throat less commonly [12-13].

Coagulase-negative staphylococci (CoNS)

The definition of the heterogeneous group of *coagulase-negative staphylococci* (CoNS) is still based on diagnostic procedures that fulfill the clinical need to differentiate between *Staphylococcus aureus* and those staphylococci classified historically as being less or nonpathogenic. Due to patient- and procedure-related changes, CoNS now represent one of the major nosocomial pathogens, with *S. epidermidis* and *S. haemolyticus* being the most significant species.

They account substantially for foreign body-related infections and infections in preterm newborns. While *S. saprophyticu* has been associated with acute urethritis, *S. lugdunensis* has a unique status, in some aspects resembling *S. aureus* in causing infectious endocarditis. In addition to CoNS found as food-associated saprophytes, many other CoNS species colonize the skin and mucous membranes of humans and animals and are less frequently involved in clinically manifested infections.

This blurred Coagulase-Negative Staphylococci gradation in terms of pathogenicity is reflected by species- and strain-specific virulence factors and the development of different host-defending strategies. Clearly, CoNS possess fewer virulence properties than *S. aureus*, with a respectively different disease spectrum. In this regard, host susceptibility is much more important. Therapeutically, CoNS are challenging due to the large proportion of methicillin-resistant strains and increasing numbers of isolates with less susceptibility to glycopeptides [14-15].

Acinetobacter baumanii

Acinetobacter species are saprophytic and ubiquitous and can be found in natural (e.g. soil, water, food) and hospital environment. *Acinetobacter* is considered as a part of commensal flora of man (e.g. axillae, groin, digit webs) where they occasionally present as opportunistic pathogens. Cutaneous colonization can be seen in approximately 25% of population. 7% of adults and children show transient pharyngeal colonization.

It is often difficult to distinguish between the colonization and the infection with this organism and hence attribute the exact morbidity and mortality associated with infections due to this organism. The other genomospecies are unnamed. DNA groups 1, 2, 3 and 13 are sachharolytic strains and are collectively referred to as Acinetobacter calcoaceticus-A. baumannii complex [16-17].

Pseudomonas aeruginosa (P. aeruginosa)

Pseudomonas aeruginosa is an opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection. It is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility. The word Pseudomonas means false unit, from the Greek pseudo- (Greek: ψευδο, false) and Monas (Latin: Monas, from Greek: μονος, a single unit). The stem word Mon was used early in the history of microbiology to refer to germs, e.g., Kingdom Monera.

The species name aeruginosa is a Latin word meaning *copper rust*. This also describes the blue-green bacterial pigment seen in laboratory cultures of the species. This blue-green pigment is a combination of two metabolites of P. aeruginosa, pyocyanin (blue) and pyoverdine (yellow), which impart the blue-green characteristic color of cultures. Another assertion is that the word may be derived from the Greek prefix ae- meaning "old or aged, and the suffix ruginosa means wrinkled or bumpy [18-19].

Micrococcus

Micrococcus luteus is a gram positive, spherical, saprotrophic bacterium in obligate aerobe M. luteus is found in soil, dust, water and air and as part of the normal flora of the mammalian skin. The bacterium also colonies the human mouth, mucosae, oropharynx and upper respiratory tract. *M. luteus* is cogulase negative bacitracin susceptible and forms bright yellow colonies on nutrient agar. To confirm it is not Staphylococcus aureus a bacitracin susceptibility test performed [20-22].

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 complex under study was prepared in two steps. In the first step, copper soap was prepared and in the second step complexation of copper soap was done with ligand like urea [23]. 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 [24].

Copper soap was prepared by refluxing the non-edible oil i.e. Neem (*Azadirecta Indica*) oil, in its pure form, of an 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 [25-27].

Name of oil	% Fatty acid				
	16:00	18:00	18:01	18:02	Other acid (C_{20}– C_{24})
Neem oil	14.9	14.4	61.9	7.5	1.3

Table1: *Fatty acid composition of oil used for copper soap/complex synthesis.*

The ligand urea was taken directly. The purified copper soap derived from non-edible oil was refluxed with ligand (urea) 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 [28-29].

The complex obtained was dark green in colour. The complex was soluble in benzene and other organic solvents but insoluble in water. The complex was stable at room temperature, its physical parameters like saponification value (S.V.), saponification equivalent (S.E.) and molecular weights are recorded in Table 2 On the basis of their elemental analysis, 1:1 (metal: ligand) type of stoichiometry has been suggested [30-32].

Name of soap/complex	Colour	M.P.	Metal Content %		S.V.	S.E.	Average M.W.
			Observe	Calculate			
Copper Neem Soap	Dark Green	50°c	10.16	10.07	198	283.33	630.16
Copper Neem Soap Urea Complex	Dark Green	72°c	9.31	9.19	-	-	690.76

Table2: *Analytical and physical data of copper soap and its complex derived from Neem oil.*

In order to study the structure of soap and complex, the infrared (IR) absorption spectra of compounds were obtained on a ABB Horizon MB 3000 series instrument spectrophotometer (4000–600 cm^{-1}) from SPC Govt. College, Ajmer.

Antimicrobial Evaluation

The biological activities of copper soap and its corresponding complex with ligand urea 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.

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). Petriplates 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 and the standard deviation has been measured by the conventional measure of repeatability and the average was taken as final reading [33-37].

Results and Discussion

The copper soap and complex are abbreviated as follows-

1. Copper - Neem Soap (CN)
2. Copper – Neem Soap Urea Complex (CNU)

IR spectral analysis

The absorption bands observed at 2925 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 1665 cm^{-1} and another weaker band at 1373 cm^{-1} were due to carboxylate ion COO–, C–O anti symmetric and symmetric stretching respectively. Also >C=O stretching bands were observed at 1744 cm^{-1} small peak corresponding to $-CH_2$ twisting and wagging has been observed at 1311 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 480 cm^{-1} [23-25].

Absorption bands	CN (cm^{-1})	CNU (cm^{-1})
Corresponding to soap moiety		
Olefinic =C–H stretching	3010	3009
CH_3 and CH_2, C–H Anti sym. stretching (vas)	2916	2925
CH_3 and CH_2, C–H sym. stretching (vs)	2854	2854
> C = O Stretching	1744	1744
C = C stretching [Cis Unconjugated]	1665	-
COO -, C–O Anti-sym. Stretching	1582	1589
CH_2, C–H Bending (δ) (Scissoring)	1465	1458
C–H, Deformation, =C–H Rocking	1443	-
COO–, C–O Sym. Stretching	1373	1373
CH_2, C–H Bending (δ) (Twisting and Wagging)	1311	1311
CH_3, C–H Rocking	1157	-
CH_2, C–H Rocking	725	765
=C-H, Out of Plane Bending of C-H	679	705
Cu–O Stretching	480	560
Corresponding to ligand moiety		
NH_2, N–H Stretching	-	3500
Sym. NH_2, N–H Stretching	-	3420
N-H Bending	-	1651
C–N Stretching	-	1149

Table3: *IR spectral data for copper (II) Neem Soap and its complex.*

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 [38-40]. The C–N stretching band of primary amide was observed at nearly 1149 cm^{-1} the absorption band 1744 cm^{-1} was found to be representative of amide > C = O group. A broad band near 3500-3420 cm^{-1} was observed corresponding to N–H stretching of amides. Also C–H stretching band due to deformation out of plane (in Benzene) was also observed at 705 cm^{-1} (Table 3) [41-44]. Thus on the basis of above observations it can be safely assumed that complexation of copper soap has taken place with urea.

Concertration (PPM)	CN *S.aureus* (cm)	CNU *S. aureus* (cm)	CN Cons (cm)	CNU Cons (cm)	CN *A. baumanii* (cm)	CNU A. *baumanii* (cm)	CN *P. aeruginosa* (cm)	CNU *P. aeruginosa* (cm)	CN Micrococcus (cm)	CNU Micrococcus (cm)
3 * 104	1.7	1.6	1.7	1.7	1.6	1.5	1.6	1.5	1.5	1.4
$1.5 * 10^4$	1.5	1.4	1.5	1.5	1.4	1.3	1.4	1.2	1.3	1.2
$.75 * 10^4$	1.3	1.2	1.2	1.3	1.2	1.1	1.2	1	1.2	1
$.3750 * 10^4$	1	1	1	1.1	1	1	1	0.8	1	0.8
Solvent	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3

Table 4: *Bactericidal data for copper (II) soap and its complex derived from Neem oil.*

Biological Activities

Neem urea soap complex show higher antibacterial activity than pure soap suggesting that complex 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 complex as compared to the soap can possibly be explained on the basis of presence of donor atoms N and O as well as the structural compatibility with molecular nature of the toxic moiety [34,45]. The activity of copper Neem soap and copper Neem soap urea complex derived from Neem oil were found in the follow order soap and complex respectively:

For Soap

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

For Complex

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

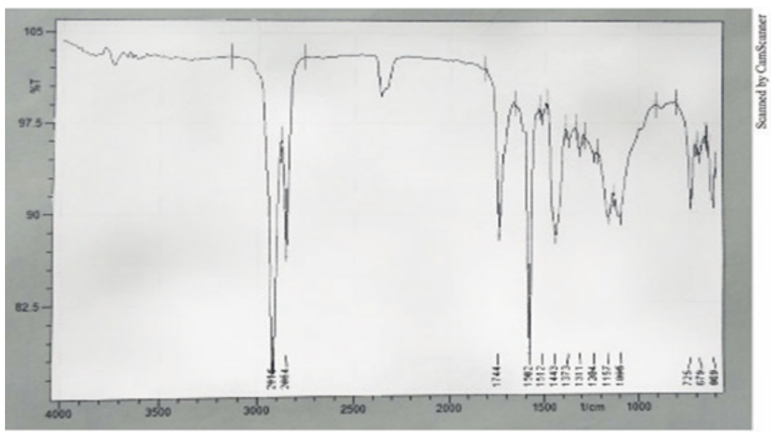

Figure 1: *IR Spectra of Copper Neem Soap.*

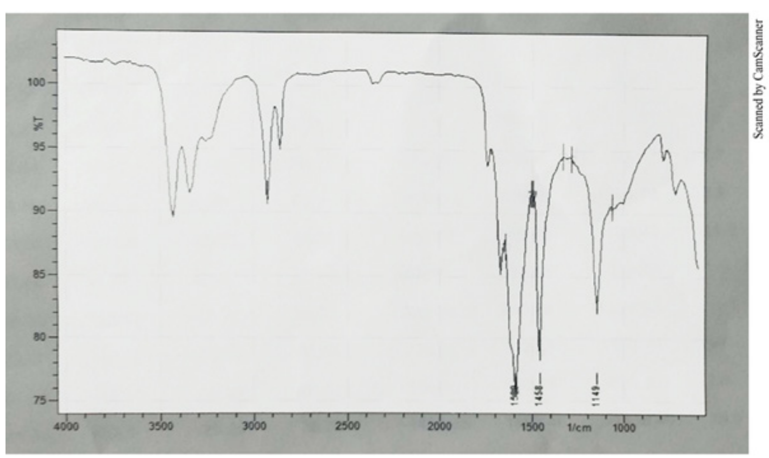

Figure 2: *IR Spectra of Copper Neem Soap Urea Complex.*

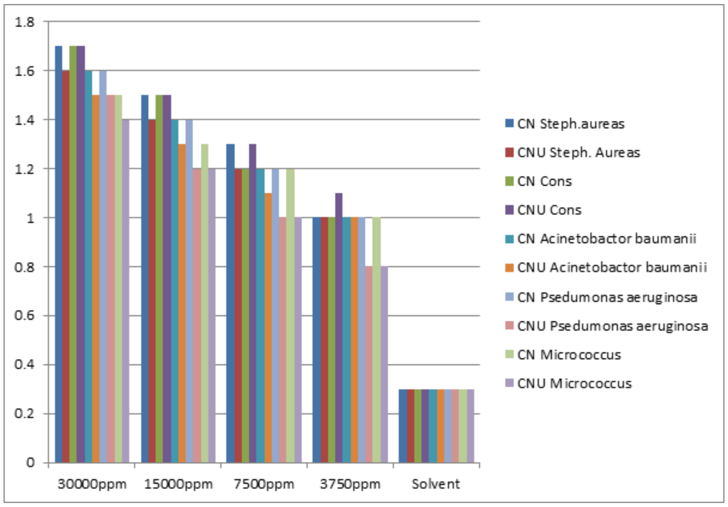

Figure 3: *The Plot data for copper (II) soap and its complex derived from Neem oil.*

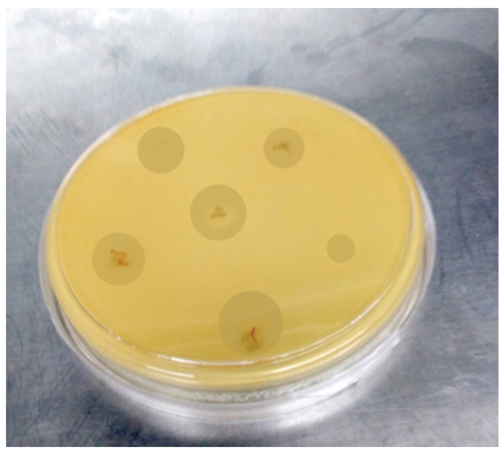

Figure 4: *Zone of Copper Neem Soap for Staphylococcus aureus.*

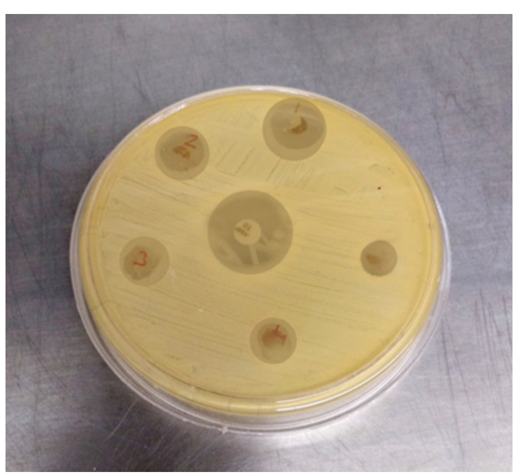

Figure 5: *Zone of Copper Neem Soap Urea Complex for Coagulase-negative staphylococci (CoNs).*

Conclusion

Antiseptic soap, sometimes called antibacterial soap or anti-fungal soap, is a regular soap in liquid or solid form. Though these compounds are considered to have low toxicity, their 2-hydroxy isomers have been shown to undergo thermal and photo-chemical ring closure to form polychlorinated dibenzo-p-dioxins which are highly toxic. In addition, it is though that bacteria could develop resistance to triclosa and this could lead to development of resistance and change in microbial community structure. These shortcomings of antiseptic soap (treated with synthetic antiseptics) generate the need to develop more environment friendly toilet soaps [46-48].

Neem oil was particularly chosen as it is easily available commercially and biodegradable in nature. Copper soap have a tendency of complexation with 'nitrogen' and 'oxygen' containing ligands. The interest in co-ordination chemistry is increasing continuously with the preparation of organic ligands containing a variety of donor groups and it is multiplied many folds when the ligand have biological importance. These facts led us to synthesize copper soaps of non-edible oils and its complex with ligand containing nitrogen and oxygen elements, investigate the role of copper and other elements in biological activities [49-50]. The compounds studied are comparatively better antibacterial agents than Imipenem [IPM[10]] which showed lower antibacterial activity.

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