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Preparation and characterization of antibacterial ointment from cashew nut shell liquid-an agricultural by-product

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ABSTRACT

Cashew nut shell liquid (CNSL) was obtained from Anacardium occidentale L. plant, is a natural resource of phenolic lipid. It was obtained as natural (n) and technical (t), based on its mode of extraction, with unique structural features, such as phenolic hydroxyl, carboxyl, aromatic ring and long alkyl chain that identify it as good platform for chemical transformation into valuable products. The main constituents of CNSL are anacardic acid, cardanol and cardol. Copper (Cu) has been extensively used in bacteriostatic and bactericidal applications since ages. Copper incorporated CNSL combinations from technical CNSL (Cu-tCNSL) are expected to exhibit good antibacterial activity. The aim of this research is to prepare Cu-tCNSL, by simple, solvent-free, cost-effective and less time-consuming approach following "Green Chemistry" protocol, to study the antibacterial activity of Cu-tCNSL, and to investigate the possibility of formulation of ointments therefrom. The structure and morphology of Cu-tCNSL were investigated by FTIR, NMR and TEM analyses, and homogeneity of ointments was studied by homogenization test. The antibacterial activity was investigated against Staphylococcus aureus and Pseudomonas aeruginosa. Our studies revealed that Cu-tCNSL showed antibacterial activity by Gram-positive Staphylococcus aureus only and can be well formulated into ointments.

1. Introduction

Cashew nut is gained from cashew tree, *Anacardium occidentale* L., native to Brazil. It is also available abundantly in India, Bangladesh, Kenya, Africa and some other parts of the world. Cashew nut shell liquid (CNSL) is acquired as a by-product of cashew industry. It is safe, non-toxic, biodegradable and renewable natural source of phenolic lipid. CNSL constitutes three major components: Anacardic acid (AA), Cardanol (CN) and Cardol (CL) [1] (Figure 1). It is obtained as natural CNSL (nCNSL) and technical CNSL (tCNSL), which depends upon the mode of extraction of CNSL from cashew nut. Solvent extraction of cashew nut shell yields nCNSL, which contains 2-methyl cardol (2.90%), monounsaturated AA (82.90%), diunsaturated AA (8.0%), AA (3.60%), and 2.60% unidentified compounds. tCNSL is acquired by hot oil process or by roasting of cashew nut shells at higher temperatures (80-200ºC). Due to decarboxylation during roasting process, AA is converted to CN, and thus tCNSL is rich in CN [2].

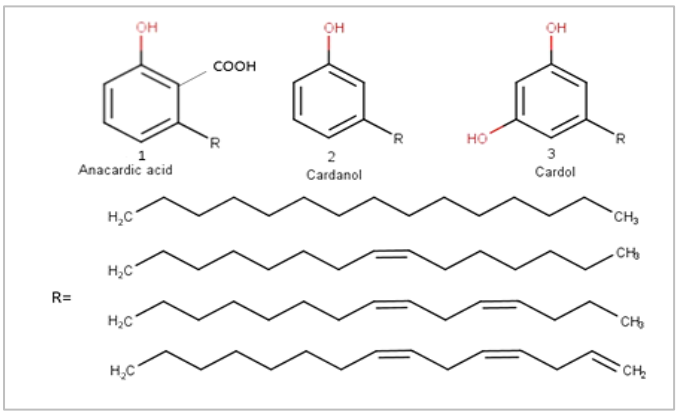

Figure 1: Chemical structures of main constituents of CNSL

CNSL has unique structural features such as the presence of phenolic group, aromatic ring and C15 long alkyl chain, amenable to several chemical transformations, thus producing CNSL based epoxies, polyesters, polyols, polyurethanes, phenalkamines, polyureas, benzoxazines, resoles, novalacs, and others, which find various applications as flame retardants, plasticizers, surfactants, antioxidants, foams, antibiofilm, antibacterial and anticorrosive coatings [3-8]. The most attractive feature of CNSL is that it is obtained as a by-product. Thus, it is available as a cheaper and environmentally benign substitute to petro-based chemicals. The constituents of CNSL, AA, and CN have also been used in pure forms to produce several derivatives, with versatile applications [4, 9-13].

CNSL derivatives have been used in pharmaceuticals, and as food stabilizers. In some studies, CNSL has shown biological activities. Similar to lipids, due to unsaturation containing side chains, CNSL and its derivatives have undergone crosslinking in ambient conditions, producing transparent, and scratch free polymeric films. As it contains phenolic lipids, CNSL/ CN has shown reduction of metal ions, in *situ*, resulting in their corresponding metal nanoparticles [11].

Today, considerable attention is being paid on the utilization of bio-resources, from environmental, economical, and safety viewpoints. The research work described in this study reports the in-*situ* synthesis of CuO in tCNSL matrix, without any surfactant, catalyst, or solvent, by simple approach producing a nanocomposite, from CNSL-a bio-resource, by in-*situ* preparation of copper oxide nanoparticles in tCNSL matrix, using copper acetate as inorganic precursor. Nano-sized CuO have shown antibacterial activity against broad range of bacteria [14, 15][5,6]. Thus, it is expected that the incorporation of CuO in tCNSL would introduce antibacterial behavior in final product, rendering it useful in ointment formulation.

In this study, we have attempted to enrich/modify tCNSL with nano CuO keeping under consideration the following viewpoints: adopting environmentally friendly operations, devoid of toxic chemicals, with low cost and improved performance, less time and energy consuming protocol *via* "Green Chemistry" a tool in Pharmaceutical Chemistry,

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and utilizing a renewable resource and industrial waste, CNSL, for an advanced pharmaceutical application.

The aim of research work is to enrich/modify tCNSL with bio-synthesized CuO [Cu-tCNSL] by "Green Chemistry" protocol, to elucidate the structure of CNSL and Cu-tCNSL by FTIR and ^{1}H NMR, to study their antibacterial behavior against Gram-negative and Gram-positive bacteria and to explore the possibility of formulating CNSL, and Cu-tCNSL into ointments.

2. Results and discussion

The synthesis was carried out through the chemical reaction between -OH group of CNSL and Cu-Ac, by one-pot, one-step in-s*itu* reaction, without any solvent, surfactant, crosslinker, catalyst, or any other external agents. CNSL serves as the matrix, diluent, reaction medium, providing functional sites for reaction to occur. The overall strategy suggests utilization of an agricultural by-product by simple method, in very short reaction time, i.e., 15 minutes. The overall approach follows the principles of "Green Chemistry".

The reaction was also accomplished at higher temperatures (60^{o}C and 80^{o}C). It was found that the samples obtained in this way were very viscous and were cured/dried over time; and thus could not be formulated in ointments or properly dissolved in xylene to study their antibacterial behavior. As a result of drying/curing, the samples had a limited pot life, so they could not be kept for future use. To overcome this problem, we repeated the reaction at 30^{o}C. The reaction took place in similar manner. The samples obtained at this temperature were sufficiently viscous, showed free-flowing tendency, and were ideal for ointment formulation. They did not dry/cure and so could be easily secured for future use. The introduction of CuO in tCNSL was confirmed by FTIR and ^{1}H NMR spectral analyses as discussed in proceeding sections and antibacterial activity was also investigated. The ointments could be formulated well from tCNSL, bees wax [BW], white soft paraffin [WP] and Cu-tCNSL (Figure 2). Homogeneity tests were conducted on the formulated ointments, tCNSLO and Cu-tCNSLO, and no separation of contents was observed (Figure 3). The antibacterial study revealed that Cu-tCNSL showed good antibacterial activity.

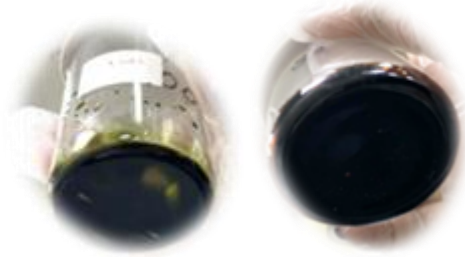


Figure 2a: Cu-tCNSL

Figure 2b: Preparation of BW and WP mixture

(i) (ii) (iii)

Figure 2c: Preparation of Cu-tCNSLO ointments from (i) 5T, (ii) 10T and (iii) 15T

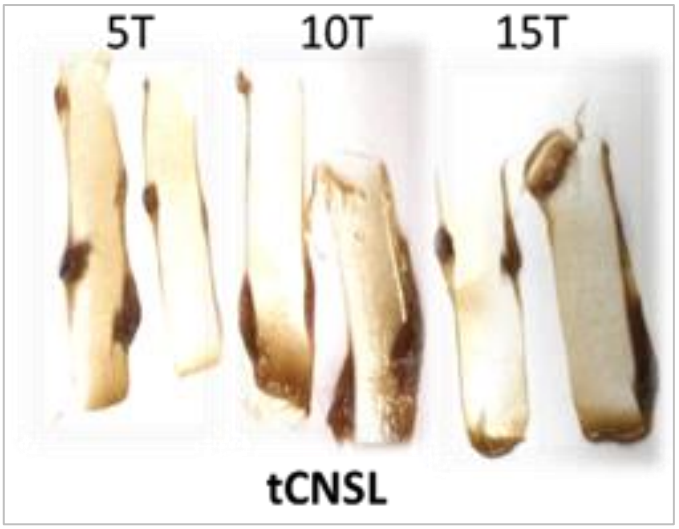


Figure 3: Homogeneity test of the prepared ointments

2.1. *FTIR spectral analysis*

The absorption bands observed in FTIR spectra of tCNSL and Cu-tCNSL are given below:

tCNSL(cm^{-1}): 3400-3300 (–OH, intermolecular hydrogen bonded), 3080 (Ar C=C-H), 3010 (C=C-H), 2926(asymm CH_2/CH_3) and 2854 (symm CH_2/CH_3), 1600-1500 (Ar C=C and C=C), 1486 (C-H bending), 1350 (O-H bending), 1268 and 1155 (phenolic C-O), 1074 (phenolic C-OH), 997(ArC=C), 912 and 876 (=C-H), 780 and 694(-C=C-H , out of plane bending).

Cu-tCNSL(cm^{-1}): Changes were observed in absorption bands of phenolic –OH, C=C str and C–OH, along with the presence of an additional band at 648 cm^{-1} (Cu–O).

As mentioned above, Cu-tCNSL showed the presence of absorption bands in FTIR typical to those present in FTIR spectrum of tCNSL. However, in FTIR spectrum of Cu-tCNSL, the -OH band was suppressed, indicating that during chemical reaction with Cu-Ac, -OH of CNSL was consumed. There is an additional band at 648 cm^{-1} (Cu–O), supporting the presence of CuO, formed due to chemical reaction between phenolic -OH and CuAc.

2.2. *^{1}H NMR spectral analysis*

^{1}H NMR spectra of tCNSL and Cu-tCNSL were given in Figures 4 and 5. ^{1}H NMR spectrum of tCNSL showed the presence of methyl, methylene protons, unsaturation and phenolic hydroxyl. These peaks were also present in ^{1}H NMR spectrum of pure Cardanol [11]. Thus, Figure 4 confirms that tCNSL used in this research has Cardanol. The unmarked peak in the spectrum at 3.9ppm indicated the presence of (non hydrogen bonded) hydroxyls. In ^{1}H NMR spectrum of Cu-tCNSL, peak for -OH disappears confirming that the chemical reaction between Cu-Ac and tCNSL occurs at phenolic -OH, which was also observed in FTIR spectra discussed above.

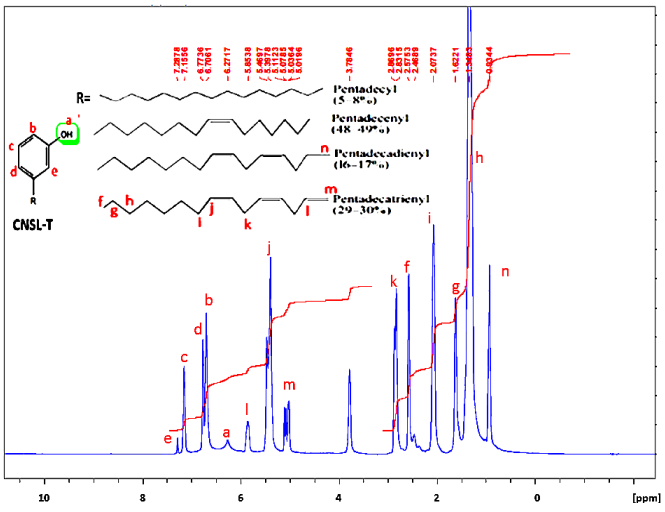


Figure 4: ^{1}H NMR spectrum of tCNSL

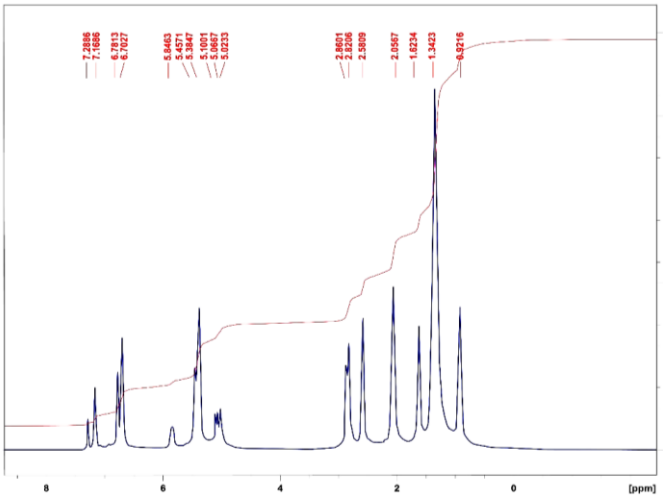


Figure 5: ^{1}H NMR spectrum of Cu-tCNSL

2.3. TEM image analysis

Transmission electron micrograph of Cu-tCNSL (Figure 6) showed the presence of nanosized CuO which occurred as nanosized spheres with well-defined boundaries, embedded in tCNSL matrix.

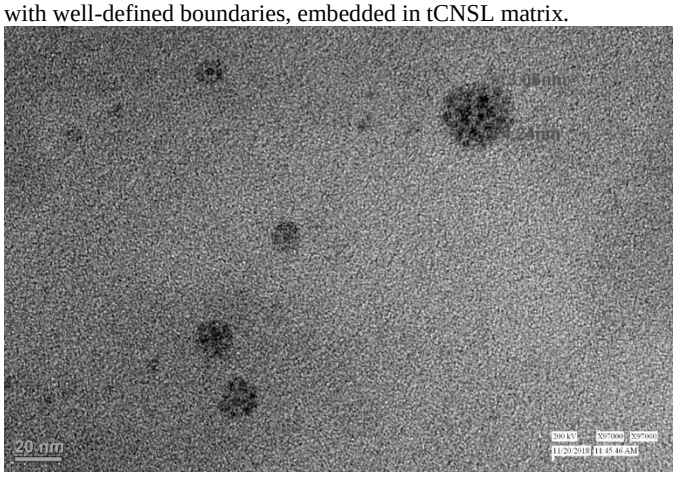
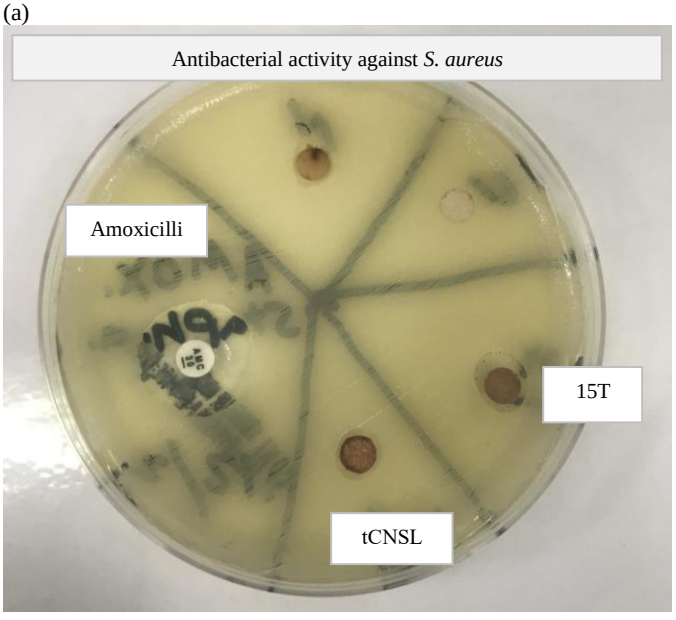


Figure 6: Transmission electron micrograph of Cu-tCNSL

2.4. Antibacterial activity

Antibacterial behavior of test samples was observed as zone of inhibition of bacterial growth. In addition, all samples dissolved well in xylene and antibacterial investigation was carried out for all samples. The concentration of test samples for antibacterial activity was taken as 150µg/mL, in xylene. It was observed that sample 15T showed good antibacterial activity (Figure 7) against *Staphylococcus aureus*, while with the Gram-negative *Pseudomonas aeruginosa*, all the product samples showed no antibacterial activity.

(a)



(b)

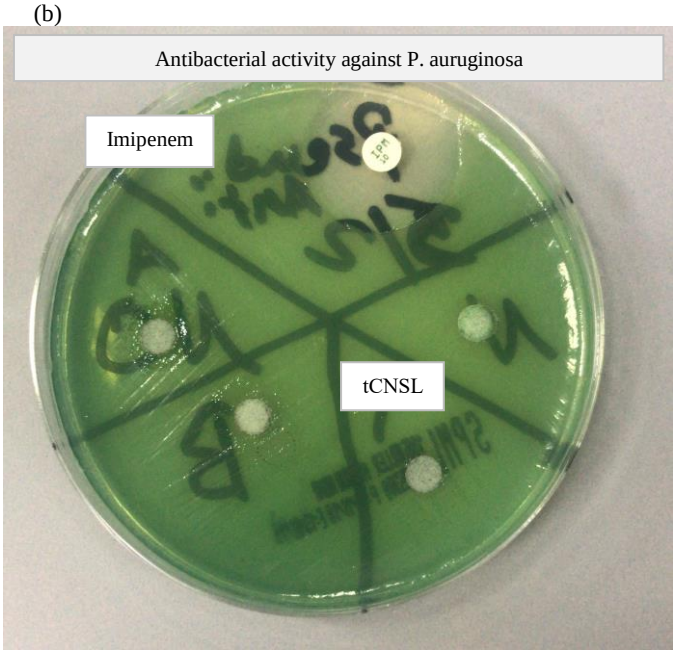


Figure 7: Antibacterial activity of tCNSL and 15T against (a) *S. aureus* (showing small inhibition zone with 15T) and (b) *P. auruginosa* (showing no inhibition zone for all test except the positive control Imipenem) at 150µg/mL, in xylene

Table 1: Antibacterial activity of the samples at different concentrations against S. aureus (A-250 µg/mL, B-325 µg/mL and C-500 µg/mL, in xylene)

Cu-tCNSL			
A	15%	20%	25%
	9 mm	7 mm	8 mm
B	15%	20%	25%
	8 mm	8 mm	7 mm
C	15%	20%	25%
	7 mm	8 mm	No inhibition zone

The samples were further prepared in three concentrations: A-250 µg/mL, B-325 µg/mL and C-500 µg/mL, in xylene. In *Staphylococcus aureus*, Cu-tCNSL (15%) and tCNSL showed antibacterial activity, at 150µg/mL, in xylene (Figure 7) and also at A-250 µg/mL, B-325 µg/mL and C-500 µg/mL, in xylene (Table 1). Good antibacterial activity was expected at higher concentration (above 250 µg/mL of these materials, however, at higher loading of CuAc (20 and 25 %, Table 1) and at higher concentrations (325 µg/mL and C-500 µg/mL, in xylene), the antibacterial activity deteriorated (Table 1). Good results were obtained at A-250 µg/mL concentration and 15% loading of Cu-Ac (Table 1). The research work still needs more investigation, in terms of optimization of reaction conditions and the concentration of Cu-Ac.

3. Materials and Methods

tCNSL was procured from Golden Cashew Product Pvt. Ltd., Pondicherry, India.

Cu $(CH_3COO)_2.H_2O$ (Cu-Ac), molecular mass- 199.7 g/mol, bees wax [BW], white soft paraffin [WP] were used as received.

3.1. Preparation of Cu-tCNSL

tCNSL was placed in a flask, and calculated amount of finely powdered CuAc was added, in small portions, with continuous stirring at 30±5 ºC. After complete addition of CuAc, stirring was continued until the end of the reaction, to obtain Cu-tCNSL (Figure 2). The percent composition of Cu-tCNSL is given in Table 2, coded as 5 T, 10T, 15T, 20T and 25T.

Table 2: Percent composition of Cu-tCNSL

Cu-tCNSL	CuAc
5T	5%
10T	10%
15T	15%
20T	20%
25T	25%

3.2. Preparation of BW and WP mixture

Calculated amount of BW was taken in a clean mortar and placed on a hot water bath to dissolve beeswax. Next, required amount of WP was added to the mortar containing molten beeswax, and both were mixed thoroughly with pestle to obtain a homogenous mixture of BW and WP (Figure 2b).

3.3. Preparation of ointment

tCNSL and Cu-tCNSL were added to the homogenous mixture of BW and WP, in separate mortars, and again with a pestle mixed thoroughly for homogenization. Homogenization test was conducted to confirm homogenous mixing of contents. The prepared ointments were named as tCNSLO, and Cu-tCNSLO respectively (Figure 2c).

3.4. Sample photos

The photographs of samples were taken by Sony XZ phone camera.

3.5. FTIR

The spectra were taken on Tensor 37, Bruker Germany (by Opus 6.5 software).

3.6. ^{1}H NMR

The spectra were recorded on Bruker Germany spectrometer.

3.7. TEM

The image was taken on TECNAI G2 30S-TWIN instrument.

3.8. Homogenization test

This test was conducted to confirm the formation of homogenous mixture of ointment. *3.9. Antibacterial activity*

Two bacterial isolates used in this experiment, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, were sub-cultured on blood agar and incubated for 18 h at 37ºC. From single colonies formed for these bacterial growths, two-three colonies were inoculated into Nutrient broth media and incubated for 18 h at 37ºC to make a suspension for each bacterium. These suspensions were standardized at 600 nm optical density equal to 0.1 to make assure the inoculum prepared have same number of cells to prepare for antibacterial activity test. The disc diffusion method was adopted according to the CLSI for antimicrobial susceptibility (CLSI, 2010) each bacterial suspension applied by a cotton swab was then spread on Muller-Hinton agar media evenly to be prepared for the application of the discs contain antibacterial. The discs prepared by impregnating each disc (6mm diameter, 0.9mm thick) of filter paper into the solution prepared for each sample. We estimated that each disc contains 20µl of the sample solution. Four prepared filter paper discs were impregnated into the different chemical preparation then by using sterile forceps these discs were applied onto the surface of the prepared media. One disc (Amoxicillin for *S. aureus* and Impenem for *P. aeruginosa*) was added in each plate as a standard positive control. Two plates were prepared with the discs for each bacterial species. The plates were incubated for 24 h at 37ºC. All prepared samples were diluted in xylene.

4. Conclusions

Our results revealed that 3-4 nm sized spherical CuO particles were embedded in CNSL matrix by solvent free approach. The possibility to formulate ointments from tCNSL and Cu-tCNSL, in different % loading of copper acetate, was investigated, and homogenous ointments were obtained. This research demonstrates that we can prepare ointment from Cu-tCNSL, which showed antibacterial activity to kill Gram-positive bacteria. In this study we found that we can benefit from an agricultural waste material by its chemical conversion into a valuable pharmaceutical product, thus adding significant value to an otherwise waste material. Because CNSL is a natural product, it is cheap and safe and does not have any side effects.

Our recommendations and future plans are to carry out skin irritation tests and study the antifungal behavior of the samples. The research work has not been previously carried out and extensive studies are required to be done so that we can optimize the reaction conditions and concentration of copper, to find the best composition.

NOTE: Authors claim there is no conflict of interest

Author Contributions: All authors contributed to the study conception, design, material preparation, data collection and analysis. Drs Eram Sharmin, Najla Obaid and Mariam Mojally read and approved the final manuscript.

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

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