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ANTICANCER AND ANTIMICROBIAL ACTIVITIES OF SINGLE CRYSTALS OF L ALANINE ADDED NICKEL (II) AND COPPER (II) SULPHATES

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ABSTRACT

L alanine added nickel sulphate hexa hydrate (LANSH) and L alanine added copper sulphate penta hydrate (LACS) crystals were grown by slow evaporation method in aqueous solution at ambient temperature. The grown crystals were characterized structurally and biologically. Structural details were analysed through single crystal X ray diffraction (SXRD) and FTIR studies. The SXRD study revealed that the crystal LANSH belongs to monoclinic system with space group $P_{2(1)/c}$. The crystal LACS belongs to triclinic system with space group P_1. The grown crystals were tested for their anticarcinogenic effects on cervical cancer cells and antimicrobial behaviour against select bacteria. The results of the tests indicated that these crystals are biologically active. Moreover it is confirmed that the crystal LACS is more efficient than LANSH in both anticancer and antibacterial activities.

KEYWORDS: Inorganic crystals, L alanine, crystal structure, anticancer, antimicrobial.

1. INTRODUCTION

In addition to wide range of applications in various fields, metal based complexes have significant anticancer, antibacterial and antiviral effects.[1] Currently there is a tendency to use amino acid residues during the pro drug design process. The literature reports that bioactive compounds show enhanced activity when linked to amino acids.[2] In view of the importance of amino acids in metabolic processes they have been an important target in the design of anti metabolites.[3] Cancer is undoubtedly one of the main health concerns and one

of the primary targets regarding medicinal science. Cancer is the abnormal growth of cells in our bodies that can lead to death. These cells are born due to imbalance in the body and by correcting this imbalance cancer may be treated.[4] Continuous demand for new anticancer drugs has stimulated chemotherapeutic research, based on the use of metals since potential drugs developed in this way may be less toxic and more prone to exhibit anti-proliferative activity against tumors.[5] Even though platinum based complexes had been in primary focus of research on chemotherapy agents, interests in this field have shifted to non platinum based agents, in order to find different metal complexes with less side effects and similar or better cytotoxicity. Thus, a wide variety of metal complexes are being intensively studied to replace platinum.[6]

Infectious diseases such as bacterial and virus are the leading cause of death world-wide. Antibiotic resistance has become a global concern. The clinical efficacy of many existing antibiotics is being threatened by emergence of multi drug resistant pathogens. There is continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanism of action for new and re-emerging infectious diseases.[7]

Aiming at discovering new useful materials with efficient anticarcinogenic and antimicrobial behaviour, in the present study, single crystals of L alanine added nickel sulphate hexa hydrate (LANSH) and L alanine added copper sulphate penta hydrate (LACS) have been grown by slow evaporation method and investigated the effect of L alanine on the properties of nickel sulphate hexa hydrate (NSH) and copper sulphate penta hydrate (CS).

2. MATERIALS AND METHODS

Solution growth- slow evaporation method was adopted for the preparation of crystals. As per the estimated solubility data, saturated solutions of NSH (62.5 g/100 ml), CS (37.8 g/100 ml) and L alanine (16.72 g/100 ml) were prepared separately using doubly distilled water at ambient temperature with continuous stirring using magnetic stirrer (REMI 1MLH) and filtered twice by Whatman No.1 filter paper. The saturated solutions of NSH and L alanine were added in the stoichiometric ratio 3:1(pH 3) and the saturated solutions of CS and L alanine were added in the stoichiometric ratio 3:1(pH 1). Both the above mixtures were stirred well separately for 5 hours to get good homogeneity. The beakers containing the solutions were closed with a pin holed aluminium foil and kept at room temperature in a dust and vibration free environment for slow evaporation. A highly transparent, good quality LANSH and LACS single crystals of dimensions 14x13x10 mm^3 and 42x33x20 mm^3 were

harvested on 61st and 73rd day respectively and are shown in figures 1a and 1b. Recrystallization was carried out repeatedly to enhance the purity of the crystal.

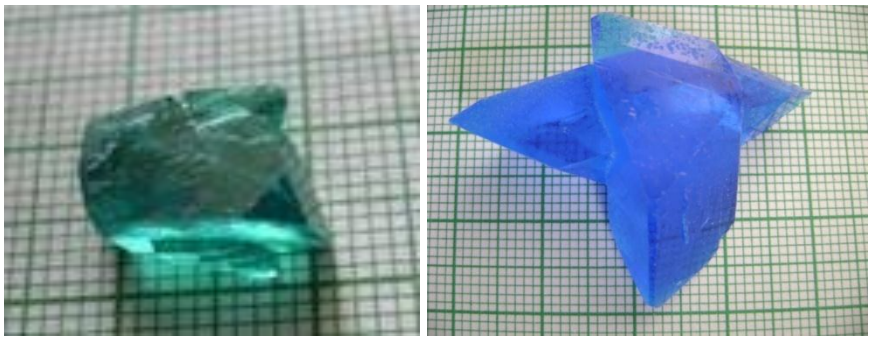


Fig. 1a. LANSH. **Fig. 1b. LACS.**

Fig. 1 Photographs of grown crystals.

3. RESULT AND DISCUSSION

Results of various studies carried out with the grown crystals are discussed hereunder.

3.1 Single crystal X-ray diffraction (SXRD) analysis

As grown crystals of LANSH and LACS have been subjected to SXRD employing a Bruker AXS diffractometer using MoKα radiation (λ = 0.71073Å). The lattice parameter values of the grown crystals are listed in Table 1 enabling a comparison.

Table 1. Lattice parameters of LANSH and LACS crystals with their parent compounds.

Crystal	a (A^0)	b (A^0)	c (A^0)	α (^{0})	β (^{0})	γ (^{0})	Cell volume (A^{03})	Crystal system
LANSH	6.24	12.47	9.18	90	106.93	90	683.35	Monoclinic
NSH	9.88	7.21	24.07	90	98.37	90	1696.6	Monoclinic
LACS	5.96	6.11	10.71	77.46	82.37	72.70	362.30	Triclinic
CS	5.99	6.14	10.74	77.33	82.27	72.57	359.60	Triclinic

The SXRD results reveals that the crystal LANSH belongs to monoclinic system with space group $P_{2(1)/c}$. It is seen that there is observable change in the lattice parameters and decrease in cell volume of the grown LANSH crystals compared to that of NSH. This might be due to the presence of L alanine in the lattice of LANSH crystals.[8] The crystal LACS belongs to triclinic system with space group P_1. The cell parameters of LACS had undergone tiny changes and resulted in increase in volume which attributes the presence of L alanine in the crystal lattice of CS.[9]

3.2. FTIR analysis

The FTIR spectra of the grown crystals of LANSH and LACS had been recorded in the KBr phase in the frequency region 4000-400 cm^{-1} using Perkin Elmer spectrophotometer and are shown in figure 2.

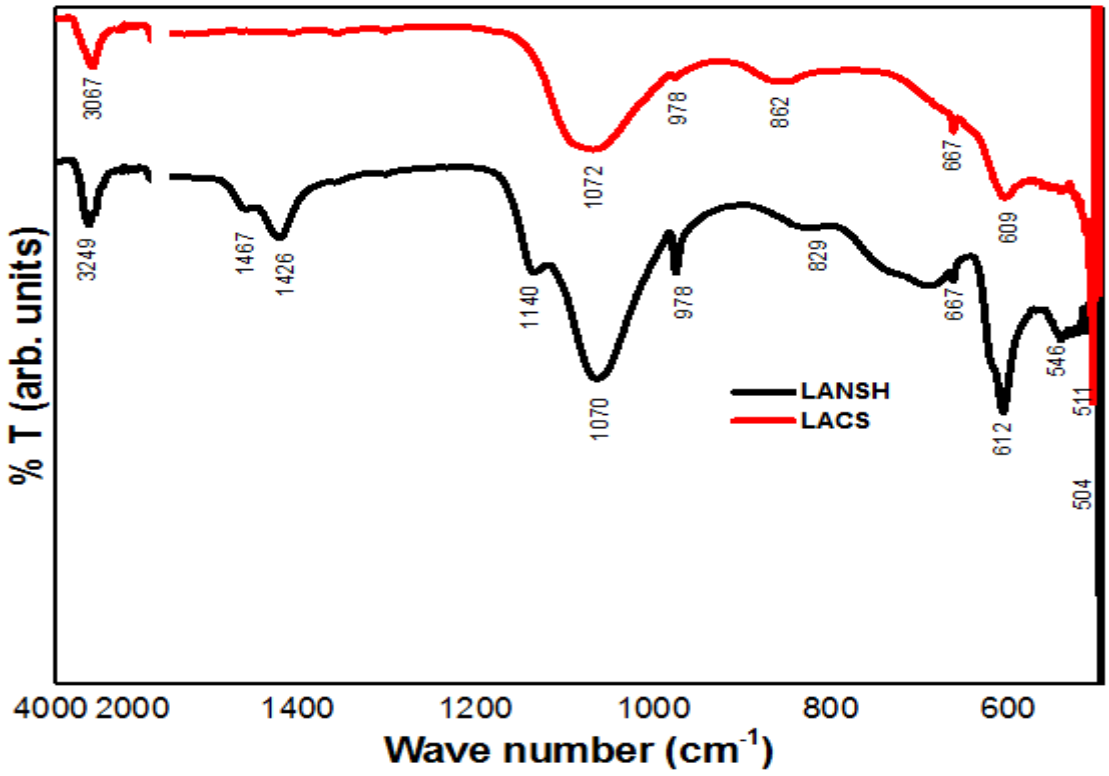


Fig. 2. FTIR spectra of grown crystals.

The broad envelope around 3200-2500 cm^{-1} indicates the presence of water and it belongs to free water symmetry stretch.[8] The asymmetric stretch of sulphate (v_3) appears at around 1200-950 cm^{-1}. The bending modes of sulphate (v_4) are positioned at 667cm^{-1}.[10] The peaks observed at 1467, 1426 cm^{-1} represent CH_3. The peaks at 1140,862 and 829 cm^{-1} represent C-H and O-H groups.[11,12] The peaks at 1467, 1426, 546, 504 and 511 cm^{-1} indicate the presence of carboxylic group. The presence of Ni is confirmed through the peaks at 1070 and 978 cm^{-1} in LANSH and Cu through 862 and 511 cm^{-1} in LACS crystals.[13-19] The spectra observed for the grown crystals are similar to that reported in the literature for $MgSO_4.7H_2O$ and $ZnSO_4.7H_2O$.[18, 19]

3.3. Anticancer studies

Cancer is among the most dreaded of human diseases. It is recognized as a major threat to health. Cervical cancer is the leading cause of death in women world- wide and one fifth of

all new cases are diagnosed in India.[20] Cervical cancer commonly occurs due to chronic infection with human Papilloma virus.[21] Metal complexes have unique properties of enhancing their role as antitumor agents. An important property is the ability of metals to form positively charged ions in an aqueous solution that can bind to negatively charged biological molecules. The high electron affinity of metal ions can significantly polarize the groups that are coordinated to them, leading to the generation of hydrolysis reactions.[22] In vitro anticancer studies of several nickel(II) complexes on MCF7 human breast cancer cells reveal that nickel(II) complexes are actively inhibiting cell proliferation.[23,24] Hence LANSH, a water soluble nickel compound was investigated for its anticancer activity against human cervical cancer cells.

Organic chelators of copper serve the role as inhibitors of angiogenesis. Upon administration with a specific copper chelator, copper complexes would be formed at a relatively high level in tumors.[25-27] There are some copper-chelating compounds with anticancer activity available in the market like D penicillamine, Tetrathiomolybdate, Captopril, Trientine, Clioquinol. But all these compounds have their own side effects.[24]

Hence based on literature, an effort is made to add L alanine, a biological molecule with NSH and CS to make them as anticarcinogenic agents.

METHODOLOGY

MTT assay method was utilized to estimate the anticancer activity of the grown crystals.

Cell Line

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science, Pune, India and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum. The cells were maintained at 37^0C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week.

Cell Treatment Procedure

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (ETDA) to make single cell suspensions and viable cells were counted using a hemo cytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. One hundred micro litres per well of cell suspension were seeded into 96-well plates at plating density of

10,000 cells/well and incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dispersed in phosphate buffered saline and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations.

Following sample addition, the plates were incubated for an additional 48 h at 37^0C, 5% CO_2, 95% air and 100% relative humidity. The control and triplicate was maintained for all concentrations.[28, 29]

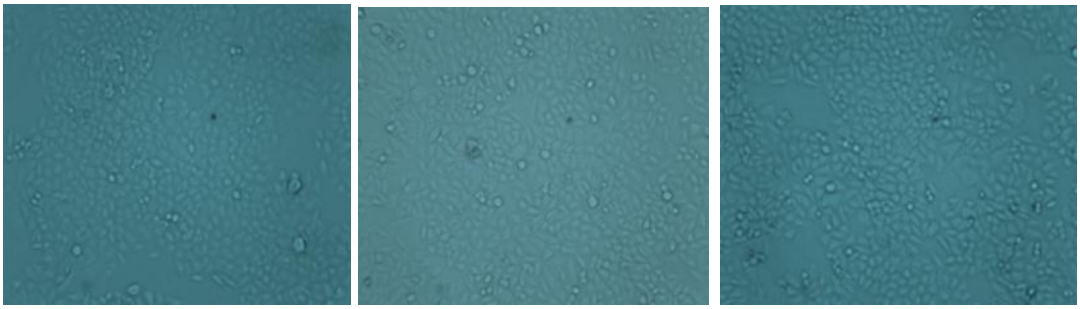
MTT Assay

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader.

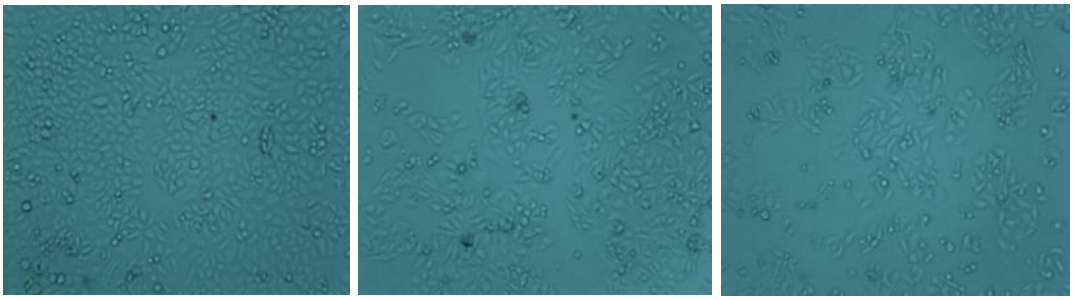
The percentage cell growth was then calculated with respect to control as follows:
% Cell Growth = [A] Test / [A]control x 100.

The % of cell inhibition was determined using the following formula.
% of Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

The results obtained for five different sample concentrations of LANSH and LACS crystals are shown in figures 3a and 3b in which control shows the picture of untreated cancerous cervical cell line (HeLa). The spherical shaped images represent dead cells and the remaining images are of cervical cell line (HeLa) affected by cancer.

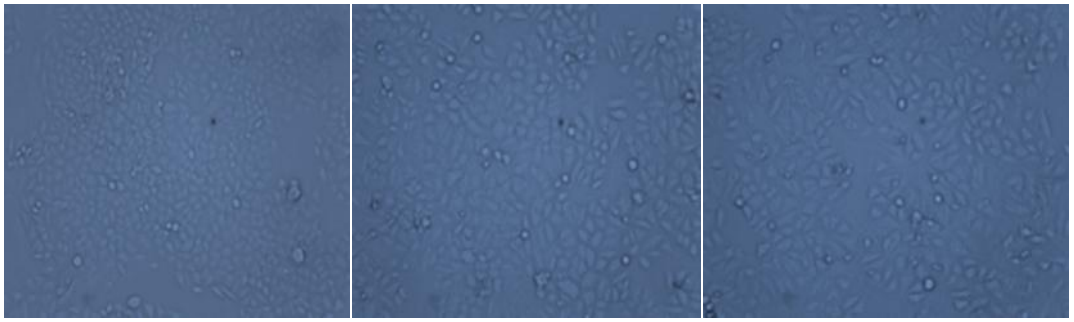


Control **12.5 (µg/ml)** **25 (µg/ml)**

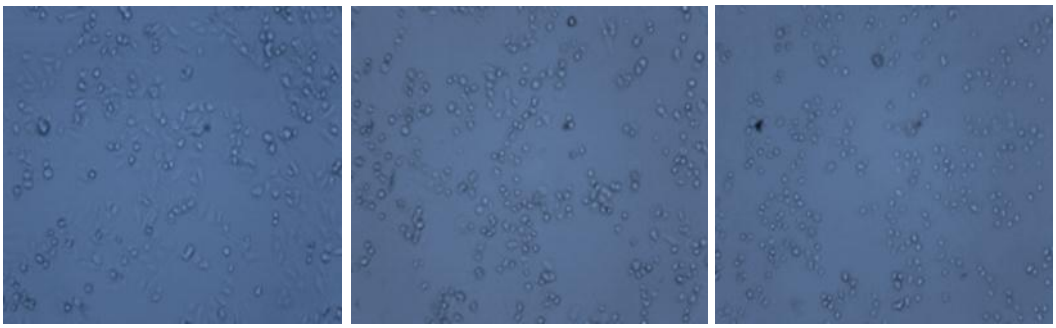


50 (µg/ml) **100 (µg/ml)** **200 (µg/ml)**

Fig. 3a. LANSH.



Control **12.5 (µg/ml)** **25 (µg/ml)**



50 (µg/ml) **100 (µg/ml)** **200 (µg/ml)**

Fig. 3b. LACS.

Fig. 3. Photographs showing cancer cell inhibition for different concentrations.

The data obtained for percentage of cancer cell inhibition and average cell absorption of LANSH and LACS along with control are consolidated in table 2.

Table 2. Percentage of cancer cell inhibition and average cell absorption.

Sample Concentration (µg/ml)	Cell Inhibition (%)		Average Cell Absorption (µg)	
	LANSH	LACS	LANSH	LACS
12.5	0.638468	18.99441	0.416	0.338
25	1.516361	49.64086	0.398	0.210
50	10.69433	65.92179	0.319	0.142
100	47.00718	92.73743	0.218	0.030
200	57.62171	100	0.177	0
Control			0.4177	0.4177

Percentage of cancer cell inhibition for different sample concentration is depicted as a bar diagram in figure 4.

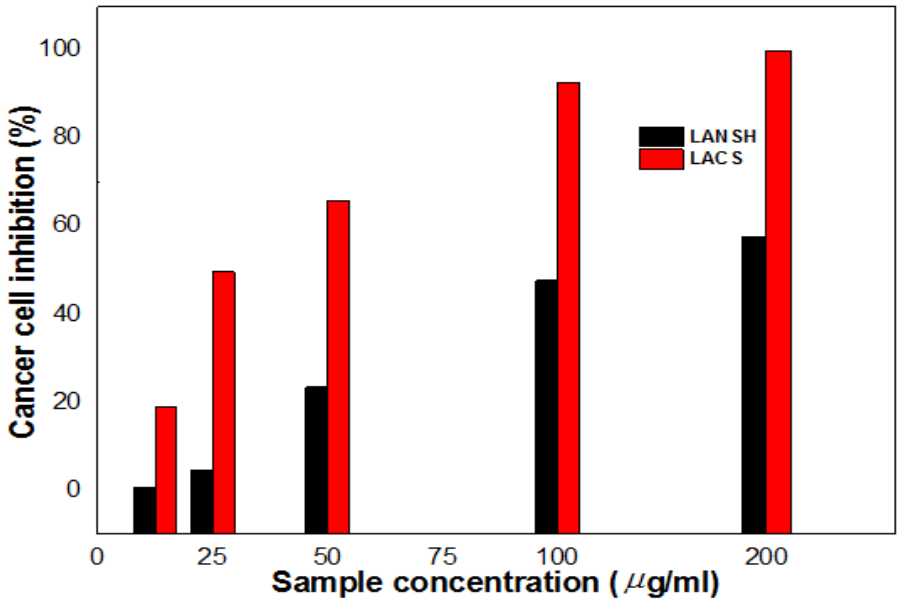


Fig. 4. Plot of cancer cell inhibition of LANSH and LACS for different concentrations.

It is clear from the above results that LANSH crystal exhibit good anticarcinogenesis activity at higher concentration (57.6% for 200 µg /ml) and its IC_{50} value is 0.506 µM. LACS crystal is active for all the concentrations and it is 100% efficient at the concentration of 200µg/ml with the IC_{50} value of 0.112µM. This result is in line with the existing literature.[23, 24]

3.4. Antimicrobial analysis

Even though metal compounds when taken in isolation were inactive or weakly active towards bacterial strains, the grown crystals showed antimicrobial activity. The biological

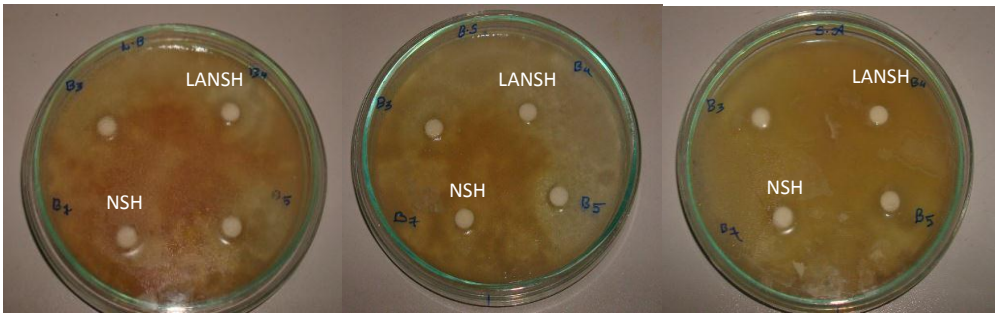
screening is effective and the results show a significant antibacterial activity for the grown LANSH and LACS crystals over both types of bacterial strains.

METHODOLOGY

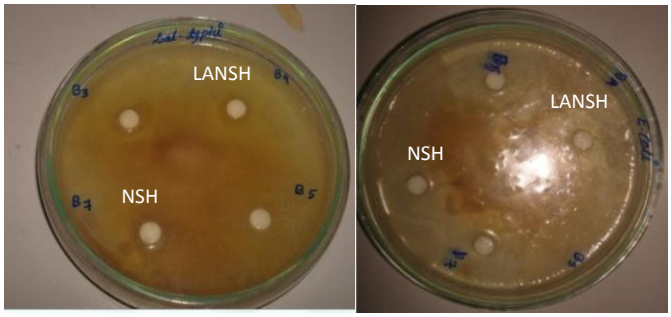
Disc Diffusion Method: Agar disc diffusion is a widely accepted in vitro investigation method to test microorganisms.[30] Bacteria were maintained at 4^0C on broth media before use. The required number of petri plates was prepared and autoclaved at 121°C for 15 minutes and they were allowed to cool under laminar air flow. About 20 ml of media was aseptically transferred into each sterile petri dish and allowed to solidify. 0.2 ml inoculum suspension was spread uniformly over the agar medium using sterile L shaped glass rod to get uniform distribution of bacteria. Sterile discs were loaded with sample and the plates were incubated at 5°C for 1 hour to permit good diffusion and then transferred to an incubator at 37°C for 24 hours. The zone of inhibition was compared with standard disc containing Streptomycin as control. The antimicrobial activity was recorded by measuring the width of the clear inhibition zone around the disc using zone reader.[31]

The grown crystals were screened for gram positive *(Lactobacillus, Bacillus subtilis and Staphylococcus aureus)* and gram negative *(Salmonella typhi and Escherichia coli)* bacteria. The inhibition zone diameters in the assays for LANSH, NSH, CS and LACS crystals are given in figures 5a and 5b and in Table 3 enabling a comparison.

It is clear that the antibacterial activity of the NSH crystal was enhanced for *L.bacillus, B.subtilus and S. typhi,* remains same for *S.aureus* and decreases for *E.coli* by the addition of L alanine. Among the five species, *L.bacillus* is more sensitive to the grown LANSH crystal. The sensitivity follows the order as *Lactobacillus>Bacillus subtitlis>Staphylococcus aureus >Salmonella typhi>Escherichia coli.*

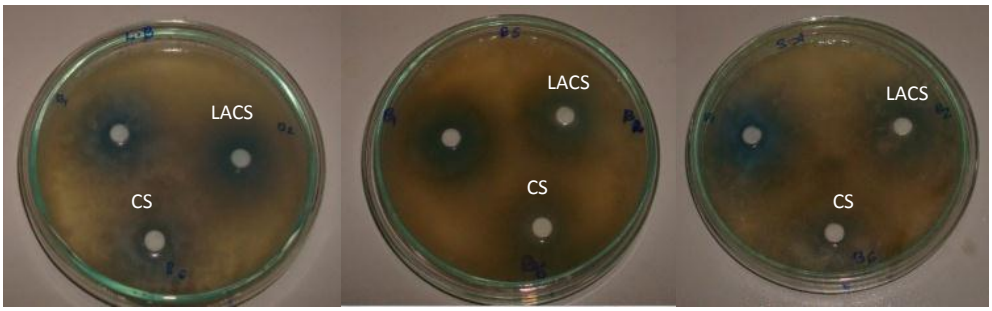


L.bacillus **B.subtilus** **S.aureus**

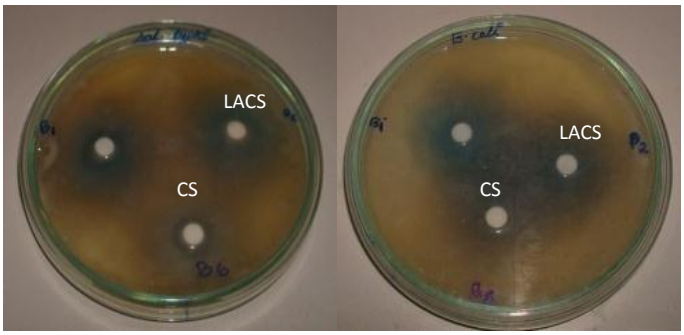


S.typhi **E.coli**

Fig. 5a. Photographs of Zone of Inhibition (mm) of LANSH and NSH against select bacteria.



L.bacillus **B.subtilus** **S.aureus**



S.typhi **E.coli**

Fig. 5b. Photographs of Zone of Inhibition (mm) of LACS and CS against select bacteria.

Table 3. Zone of Inhibition against select bacteria (mm).

Sample	*L.bacillus*	*B.subtilus*	*S.aureus*	*S.typhi*	*E.coli*
LANSH	14 ± 0.2	13 ± 0.3	13 ± 0.1	11 ± 0.2	10 ± 0.6
NSH	12 ± 0.6	9 ± 0.3	13 ± 0.8	10 ± 0.1	12 ± 0.2
LACS	19 ± 0.8	16 ± 0.3	17 ± 0.4	19 ± 0.5	21 ± 0.9
CS	18 ± 0.8	15 ± 0.2	14 ± 0.9	16 ± 0.3	16 ± 0.1

From table 3, it is also clear that inhibitory activity of CS is enhanced by the addition of L alanine for all the five bacteria. The order of sensitivity against LACS crystal is *Escherichia*

coli> *Lactobacillus*> *Salmonella typhi*> *Staphylococcus aureus*> *Bacillus subtitlis*. It is noticed that the diameter of zone of inhibition of LACS is more compared to LANSH since, Cu_2^+ exhibited higher zone of inhibition than other crystals. The reason may be due to greater chance of delocalization of Cu_2^+ in the presence of secondary nitrogen of L alanine.[13, 32-34]

Hence L alanine addition is more effective in CS than in NSH crystal as for as antimicrobial activity is concerned and both LANSH and LACS crystals may be considered for pharmacological applications.

4. CONCLUSION

The LANSH crystal belongs to monoclinic structure with space group $P_{21/c}$ and LACS belongs to triclinic structure with space group P_1. The FTIR study confirms the presence of various functional groups. Both LANSH and LACS crystals prove their anticancer and antibacterial behaviour. LANSH is moderately active whereas LACS is highly efficient and can be used as a potential candidate for cancer treatment and bacterial infections. Incorporation of L alanine is responsible for increased anticancer and antibacterial activity. Hence the grown crystals may be considered for pharmacological applications in designing better and more active drugs.

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