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# Synthesis and Anti-Proliferative Effect of Linalool propionate Loaded Carbon Nanoparticles

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## Abstract

The essential oils (EOs) extracted from plants hold many medicinal properties. But the delivery of the EOs and maintenance of its stability is a challenge. In this study, a linalool associated compound isolated from *Coriander sativum* was conjugated with carbon nanoparticle (CNP). The conjugate was characterized by FTIR, TEM, and PS analysis. The conjugate showed similar antioxidant and anti-proliferative activity when compared to the pure extract. In the case of antibacterial property, conjugate showed good inhibition against both gram-positive and gram-negative bacteria whereas the extract showed inhibition only against gram-negative bacteria. In short, the activity of the compound was not altered by its conjugation with CNP. This conjugated compound was completely

water soluble which shall make it a likely formulation for hydrophilic based drug delivery applications.

**Keywords:**

Carbon nanoparticle, linalool, *Coriander sativum*, MTT assay, DPPH assay, antibacteri-  
al.

# INTRODUCTION

The drug delivery system is a vehicle that delivers a desired pharmaceutical ingredient to the desired target that is classified based on the type of drug and carrier material, route of administration and delivery, and release mechanism. It is composed of a drug, a carrier, and a linker if required. The carriers that are commonly used to develop a drug delivery system includes polymers, nano-materials, lipid-based materials, metal oxides, micro-particles, vesicles and so on [1]. Among them, the use of nano-material is gaining interest due to their ease of preparation, stability and broader surface area. Based on shape, size, morphology, the type of precursor used, chemical and physical characteristics, the nanoparticles are classified as metal nanoparticles, carbon nanoparticles, lipid nanoparticles, nano-spheres, dendrimers, hydrogels, nanotubes, polymer nanoparticles, nano-shells, quantum dots, nano-micelles, and so on [2]. The nano-material can be synthesized either using a top-down or bottom-up approach [3]. In this study, we used the bottom up microwave technique using carbohydrate as a precursor to synthesize carbon nanoparticle (CNP).

Many synthetic drugs are available in the market for various medical ailments but natural resistance may be developed against these drugs in a course of time. Hence, there is a need for a new drug or moiety which may show better activity than the current compound. The phyto-researchers have spent a tremendous amount of work to isolate many new compounds from plant resources. The bioactive compounds isolated from various plants shall showcase different medicinal properties. But all these compounds cannot reach the desired target and cannot give the desired results when administered as an essential oil (EO) all the time. It is because these EOs are very unstable which

may degrade at normal storage temperature and are volatile in nature. The plants synthesize them in response to certain growth and environmental conditions and are usually stored in vesicles. EOs are lipophilic and can be easily metabolized by dermal absorption. It will then reach the systemic circulation to impart its medicinal properties to the target organs. Hence, EOs are easily prepared and used for skin application [4].

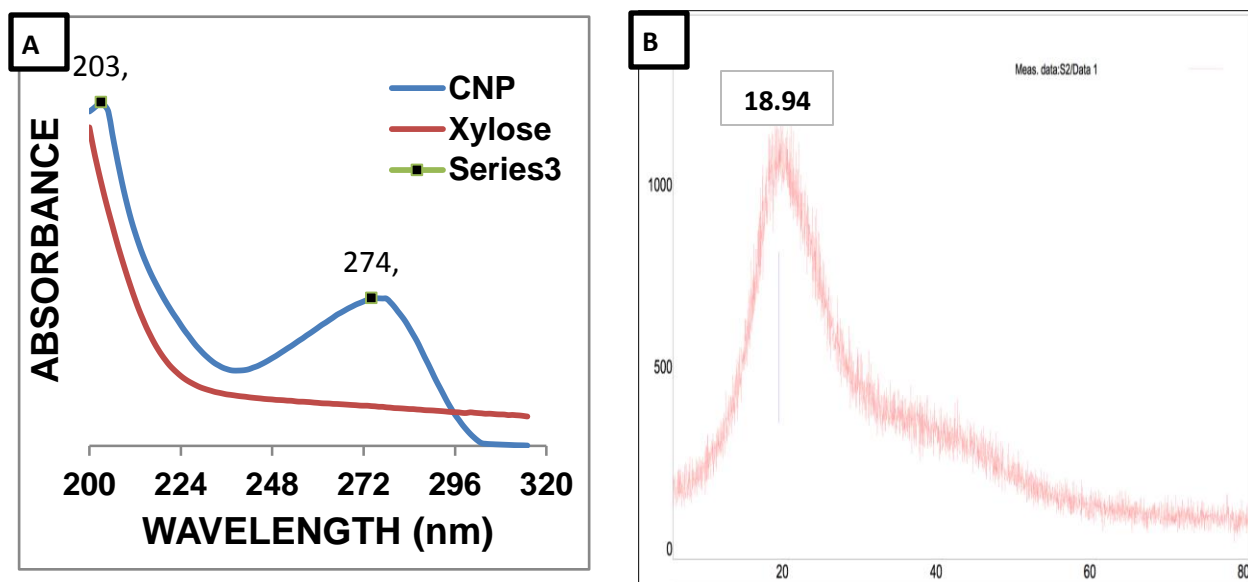
The delivery of an EO by routes other than skin is a real challenge since the carrier may interfere or alter its activity. Mostly, the EOs were encapsulated in a carrier to capture the bioactive compound using techniques like emulsification, precipitation, coacervation, complexation and use of supercritical fluid [4]. Only a few studies reported the loading of bioactive compounds from an EO on a nanomaterial, but the stability towards its activity is still very limited [5]. The choice of plant for the extraction of essential oil for this was made on the abundant availability of the source. *Coriander sativum* is one such source which is easily procured from the local market. Also, the essential oil from *C. Sativum* already showed to hold much medicinal property [6, 7]. But most of these type of essential oil was encapsulated using lipid-based nanomaterials or nanofibers and were tested for its cytotoxicity [8, 9].

In this study, one of the linalool associated bioactive compounds were isolated from *C. sativum* in a pure crystalline form and we have attempted to load the compound on a carbon nanoparticle using the procedure discussed earlier with few modifications [10]. The activity of the conjugate was evaluated against microbes and HeLa cell lines, and the antioxidant capability was also tested.

## Results and discussion

An amber/wine colored powder was obtained when the heating of xylose-acid mixture was stopped. The recovery of CNPs from the monomer after precipitation with ethanol was found to 70-72%. Phosphoric acid is a Lewis acid and it was observed to be efficient due to its non-oxidative and non-volatile property. The effective concentration of phosphoric acid was optimized to be 0.16% after several trials of experiments. The effective temperature for the synthesis was observed to be  $150\pm 5^\circ\text{C}$  for 2 min 30 sec with pulsed heating. Excessive use of Lewis acid or extensive heating resulted in gelling of the CNPs.

The CNPs synthesized were analyzed using UV-Vis spectroscopy (Fig. 1A). The UV-Vis spectrum consisted of a peak at 203 nm and a shoulder marked between 240 nm and 300 nm. The presence of a shoulder peak at 274 nm is characteristics of the electron transition from non-bonding orbital to the  $\pi^*$  orbital ( $n\rightarrow\pi^*$ ) due to the formation of carbonyl group during nanoparticle synthesis. The XRD pattern (Fig. 1B) revealed a peak at  $2\theta = 18.94$  characteristic to that of CNPs and was similar to those reported by earlier studies [11, 12]. Also, there were no additional peaks which correspond to the fact that the synthesized CNPs were devoid of any impurities from the materials used for synthesis and recovery. We can also visualize peak broadening and lowering of the height which implies nanocrystalline nature of the CNPs further confirmed by the nano size (9.65 nm) in the analysis table (S1).



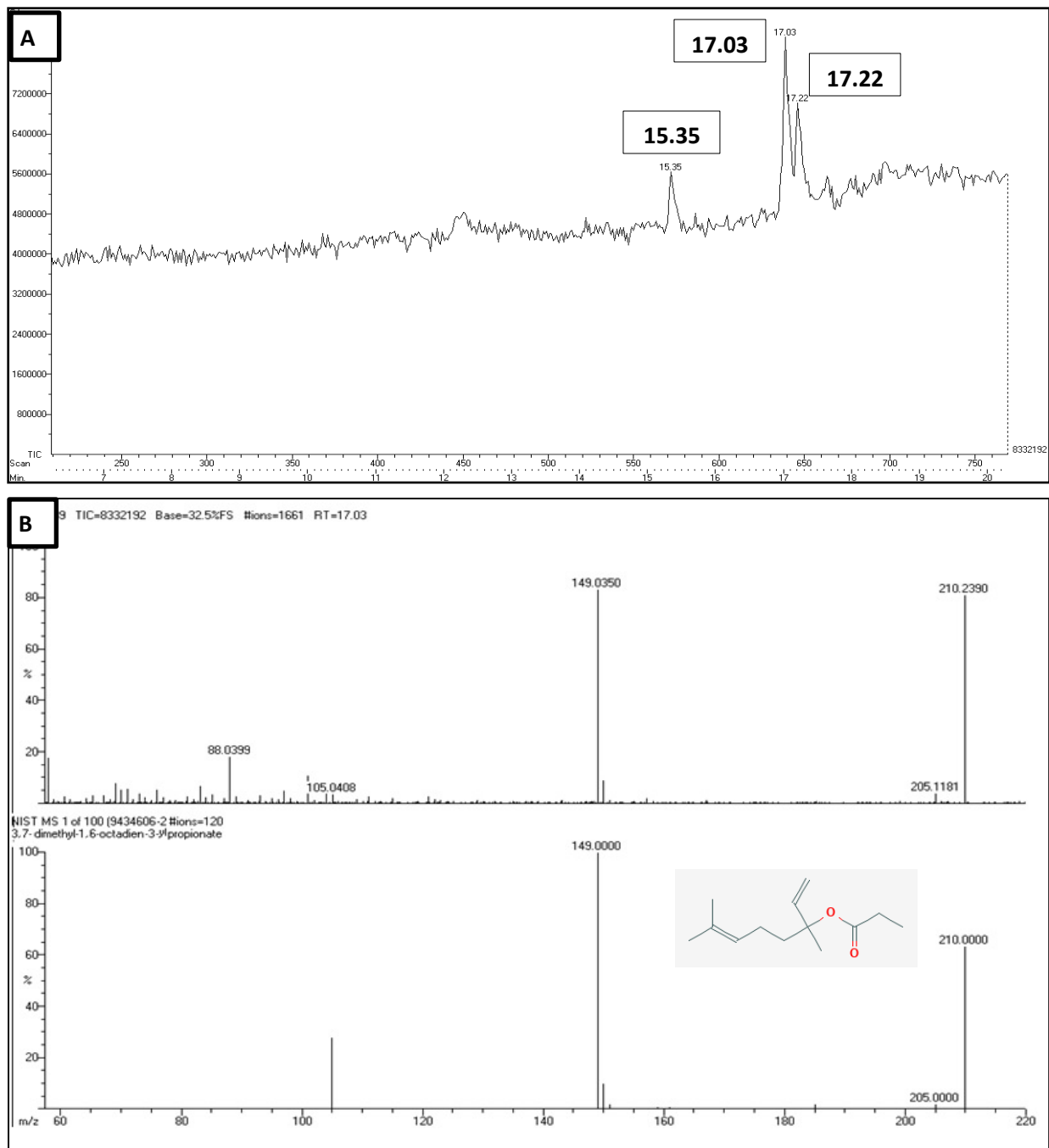
**Figure 1. (A) UV-Vis spectrum of Xylose and microwave synthesized CNP (B) XRD peak at  $2\theta = 18.94$  characteristics of carbon nanoparticle.**

In FTIR analysis (S1), the spike at  $1635.34\text{ cm}^{-1}$  is characteristic of carbonyl stretching and the carboxylate groups were identified by various minor spike between  $1200$  and  $1350\text{ cm}^{-1}$ . The formation of carboxylate group may attribute to the decomposition of hydroxyl bond attached to the carbon atom (C-OH) by the microwave and presence of carbonyl group may be attributed to the ether linkages between any 2 monomer molecules. The stretch at  $3403.74\text{ cm}^{-1}$  may be due to the distribution of the hydroxyl group on the surface of CNP. Similar results were observed in the investigations discussed earlier that supports the formation of CNPs in the synthesized samples [12, 13]. The compound of our interest was linalool and its associated compounds. The essential oil obtained after solvent extraction were analyzed for the presence of linalool or its associated compounds using LC-MS. Hydroxy linalool ( $\text{C}_{10}\text{H}_{18}\text{O}_2$ ), Linalyl propionate

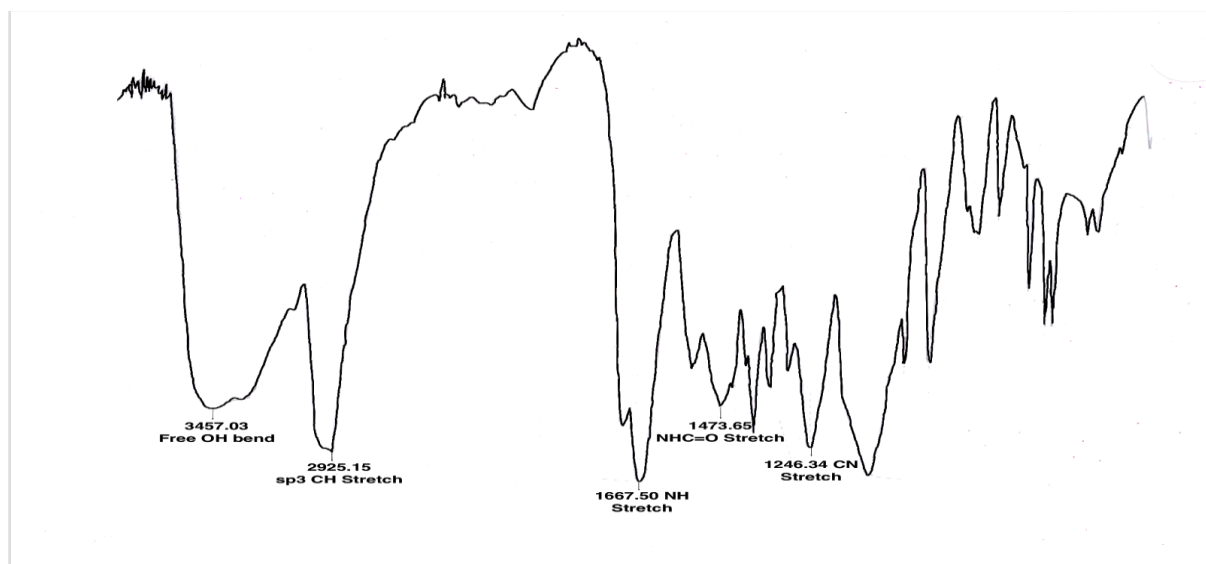
( $C_{13}H_{22}O_2$ ) and Linalool acetate ( $C_{12}H_{20}O_2$ ) were separated at the retention times of 15.35, 17.03 and 17.22 min, respectively (Fig. 2A) and was confirmed by comparing the MS spectrum with the standards. After complete evaporation of the eluate fractions overnight, fine white crystals were obtained. The linalyl propionate (LP) eluate crystal was selected for further studies (Fig. 2B). The amino functionalization of the CNPs followed by its conjugation with linalyl propionate and lyophilization resulted in an ivory colored powder. From the standard graph [14], the LP loading was calculated to be  $28.47 \pm 2.33$  %.

The loading of the LP on CNP and formation of a bond between the compounds was confirmed by FTIR analysis (Fig 3). The amino functionalization of CNPs with urea resulted in a new peak at  $1737\text{ cm}^{-1}$  characteristics of C=O group and C-N-C stretch at  $1697\text{ cm}^{-1}$  from urea. After conjugation, a further new peak appeared between  $2925\text{ cm}^{-1}$  which is characteristics of  $sp^3$  CH group from linalyl propionate and there is a spike at  $1473\text{ cm}^{-1}$  indicating amide bond formation between the carbonyl and amine group.



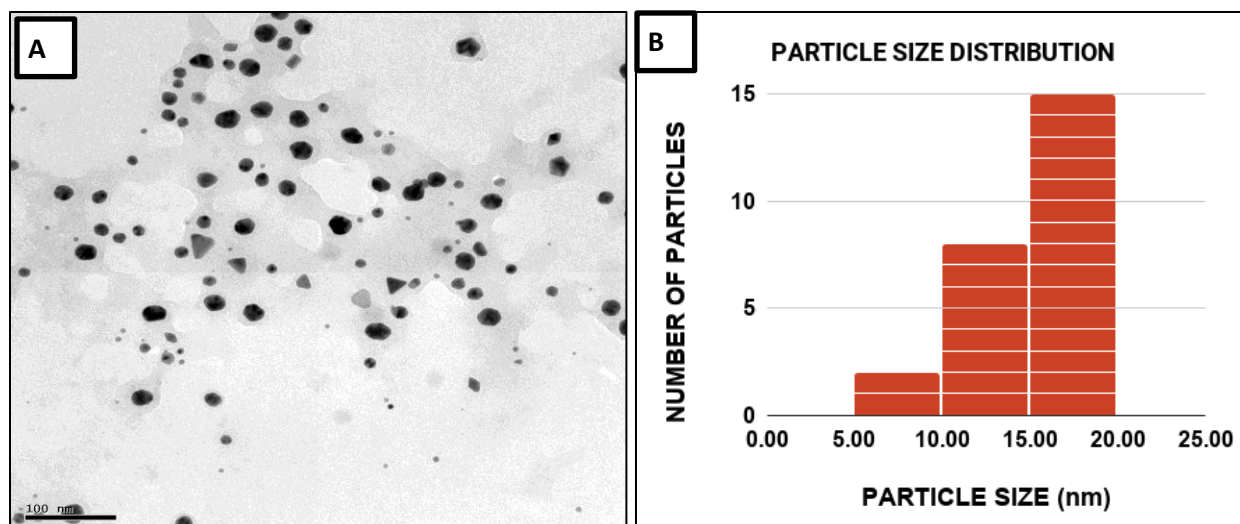


**Figure 2. (A) LC profiles of Hydroxy linalool, Linalyl propionate, Linalyl acetate with RT=15.35, 17.03 and 17.22 min, respectively. (B) MS spectrum of linalyl propionate from the extract (top) and its standard (bottom)**



**Figure 3. FTIR spectrum for LP loaded CNP. It shows spikes which indicate amide bond formation (1473), CH group from LP (2925) and Free hydroxyl from CNP(3457).**

The TEM image of the final sample and the particle size distribution are represented in Fig 4. The particle size distribution analysis was done using ImageJ software. The synthesized LP-CNPs were well dispersed and showed almost spherical shape (Fig 4A). The size of the particles ranged between 5-20 nm with an average particle size of  $14.59 \pm 2.61$  nm (Fig 4B).



**Figure 4. (A) TEM image of spherical LP loaded CNPs which are well dispersed. (B) Particle size distribution of the TEM sample showing the size ranging**

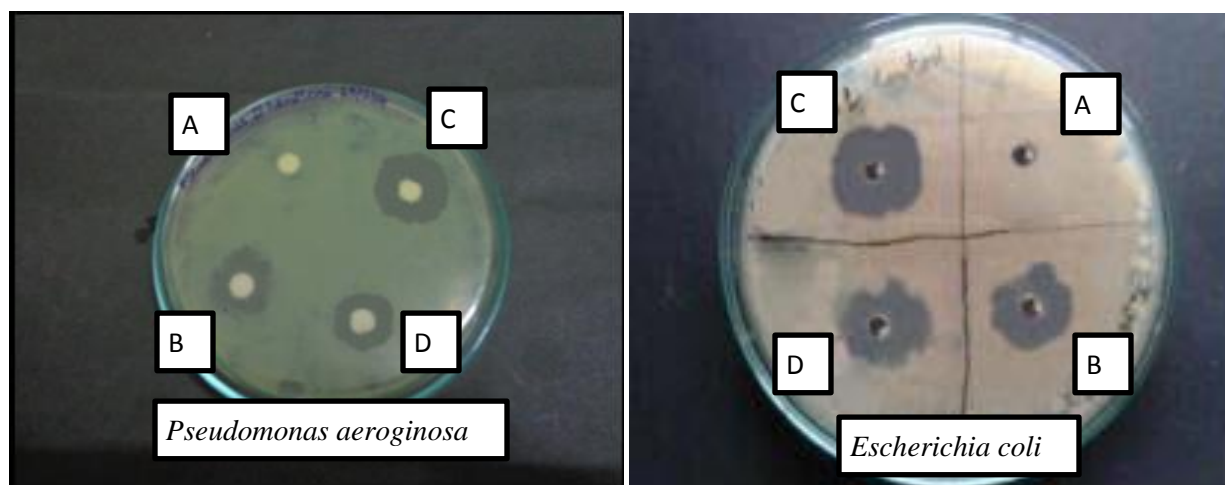
The zone of inhibition (ZOI) caused by the antibacterial activity of the linalyl propionate and the conjugate is shown in table 1. It was observed that the CNPs did not show any antibacterial activity against both gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) and gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) (Fig 5).

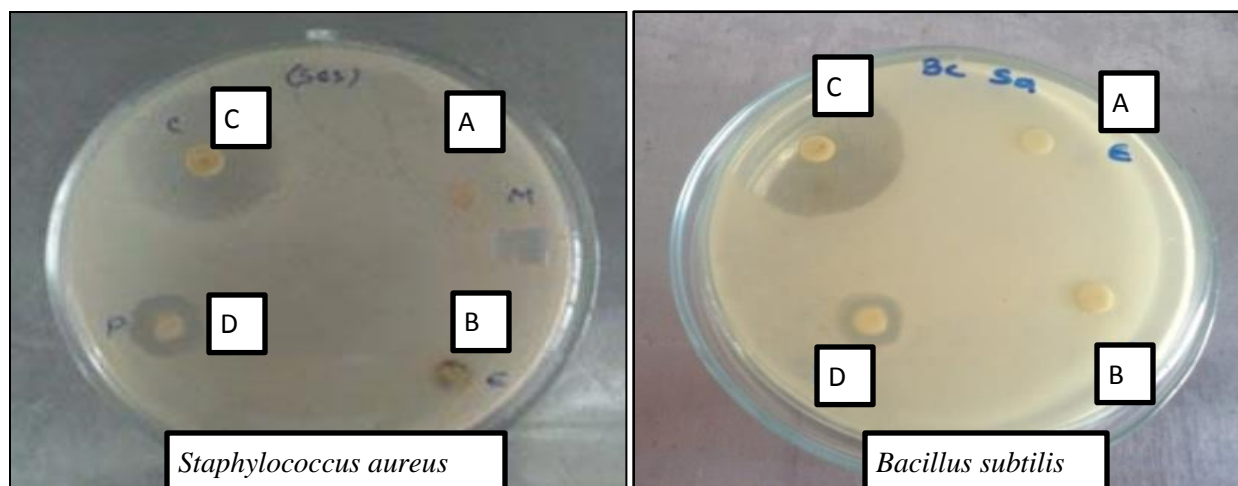
**Table 1. Diameter of the Zone of Inhibition of 2 gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) and 2 gram negative bacteria (*Staphylococcus aureus*, *Bacillus subtilis*). Trials are done in triplicates.**

Name of the organism	Diameter of the zone of inhibition (mm)				
	25 µg/mL		50 µg/mL		Positive control
	LP	LP-CNP	LP	LP-CNP	

<i>Pseudomonas aeruginosa</i>	11±1.25	10±0.62	20±0.76	19±0.33	29±0.87
<i>Escherichia coli</i>	7±0.55	6.8±0.76	13±0.45	15±0.32	25±0.6
<i>Staphylococcus aureus</i>	-	3±0.33	-	7±0.25	32±0.32
<i>Bacillus subtilis</i>	-	2±0.78	-	5±0.46	33±0.11

From the table, it can be concluded that the LP extract showed activity only against gram-negative bacteria and no ZOI was observed in gram-positive bacteria. To the contrary, the conjugates showed a small ZOI in gram-positive bacteria. We all know that a gram positive bacterium has peptidoglycan layer which may be interrupting the penetration of the linalyl propionate. From FTIR analysis, it is clear that a free hydroxyl groups shall form a bond with the amine group of the urea. Similarly, the conjugate with free hydroxyl group left ( $\approx 60\%$ ) might have formed a bond with the amino group of the protein present in the peptidoglycan layer and might have crossed the membrane. This may be the reason that the conjugate is active against gram-positive bacteria.

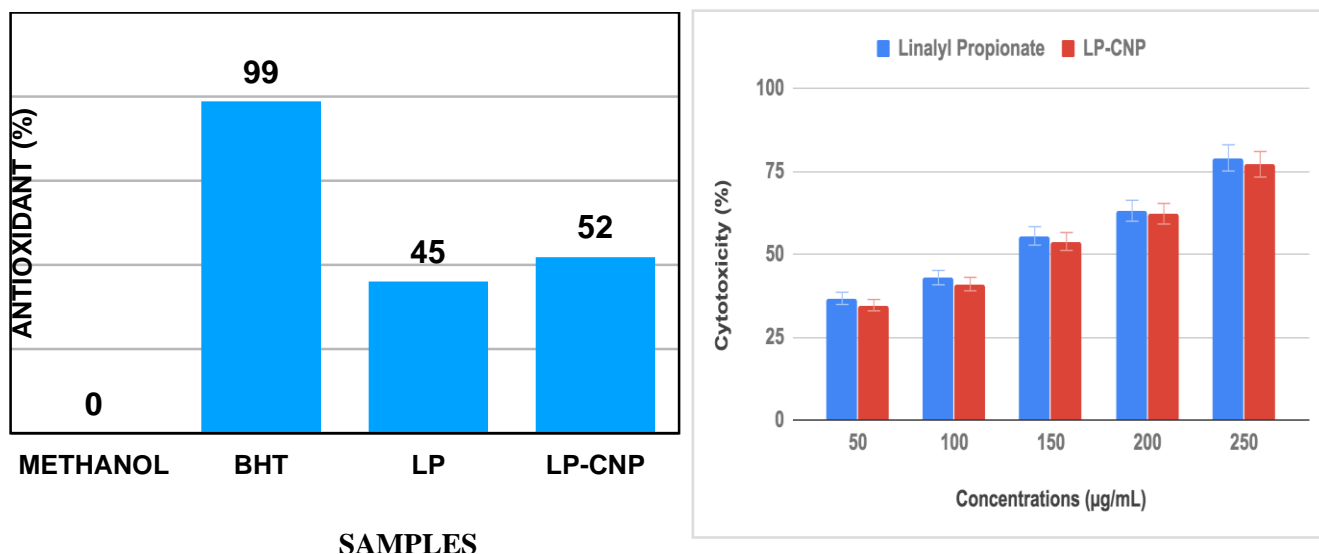




**Figure 5. Antibacterial activity using diffusion technique of (A) CNP, (B) LP-CNP at 25  $\mu\text{g/mL}$ , (C) Positive control Streptomycin (D) LP-CNP at 50  $\mu\text{g/mL}$ .**

The antioxidant potential of the samples was determined by the standard DPPH assay using quercetin as positive control and the results are presented in Figure 6A. An antioxidant changes the color of the DPPH solution from purple to pale yellow. The CNPs did not have the antioxidant property (<5%) whereas the LP and LP-CNP showed incremental but moderate antioxidant activity with respect to the concentration of the bioactive compound. Once the conjugate has entered the system, the bioactive compound shall be detached from its carrier by hydrolysis to show its biological activity. The free form of the carrier (CNP) should not induce any oxidative stress and hence a very minimal or nil antioxidant activity was desired from the carrier molecule [13]. From the results, it is clear that CNPs does not induce any antioxidant activity and it may be considered as a suitable carrier for a drug delivery system. In the case of active moiety, a moderate radical scavenging activity was desired since a strong antioxidant tend to

change the normal cells to cancerous cells. Moderate activity will synergistically enhance the chemotherapeutic effect of the drug moiety [15].



**Figure 6. (A) Antioxidant activity of LP and LP-CNP with BHT as positive control and methanol as negative control. (B) Results of MTT assay showing incremental cytotoxic activity of LP and LP-CNP.**

The MTT assay was performed to calculate the rate of the cytotoxic effects of the LP extract and LP-CNP against the HeLa cell lines (Figure 6B). The OD measured for a different concentration range (50-250 µg) showed that the extract and the conjugate showed a very gradual increase in cellular toxicity. Cellular toxicity of 79.14% and 77.23% (at 250µg/mL, S1) and an IC<sub>50</sub> value of 123.76 µg and 132.55 µg were achieved by the LP and LP-CNPs, respectively.

Our earlier studies on antibacterial activity by the chloroform extract of *C. sativum* showed strong zone of inhibition against gram-negative bacteria than our current results [17]. It may be due to the presence of all the three linalool associated compounds in the extract. The linalool and its associated compounds are natural terpenes that show antibacterial, anti-oxidant, anti-proliferative and anti-inflammatory property. But to find the level of activity of each bioactive compound it is essential to test its activity individually. The linalyl propionate which is generally used as a flavoring agent showed antibacterial, antioxidant and anti-proliferative property in this study. There is not much evidence which shows the medicinal property of this compound either individually or in combination with a drug delivery component. So this study is one of the unique attempts to forecast the utilization of this compound in chemotherapy. Both the conjugate and bioactive compound showed similar activity which suggests that the loading of LP on CNPs did not affect its efficiency.

## **Experimentals**

### **Chemicals**

Xylose, chloroform, ethyl acetate, petroleum ether, ethanol, orthophosphoric acid, urea, 1, 1-diphenyl 2-picrylhydrazyl (DPPH), methanol, ascorbic acid,

### **Instrumentation**

FTIR spectrum was measured on a FT-IR Nicolet 6700 model between 500 and 4000  $\text{cm}^{-1}$  to confirm the formation of the amide linkage between the molecules. The UV absorption spectra were measured on a Jasco spectrophotometer (JascoV-650 Series)

between 200-500 nm. JEOL 3010 was used for Transmission Electron Microscope (TEM) analysis by drop casting 5  $\mu$ L of the sample on a carbon coated copper grids and air drying.

### **Isolation of linalyl acetate from *Coriander Sativum***

The EO was extracted from *Coriander Sativum* using chloroform as described earlier [17]. It was concentrated by steam distillation and was analysed for the presence of characteristic peak by FTIR spectroscopy. The concentrated sample was purified by LC-MS separation method using 3% ethyl acetate in petroleum ether to isolate linalyl propionate. The solvent from the eluate was evaporated using rotary evaporator and was left without disturbance overnight. The final sample was stored in an air tight brown container at 4°C until further use.

### **Synthesis and functionalization of CNPs**

The carbon nanoparticles were synthesized using acid assisted carbonization of carbohydrates methods [18] with little modifications. 1 g of xylose was mixed with an acid catalyst mixture (0.5% orthophosphoric acid in water) and was melted in a microwave oven (2 - 4 minutes) to undergo charring. The charred sample was taken up in water and was precipitated using ice cold ethanol followed by centrifugation (10000 rpm for 10 min) and dialysis (3.5 kDa MW cutoff) for 24 hr. To amino functionalize CNPs, the sample was sonicated with urea (30% v/v) for 10 minutes followed by dialysis and lyophilization.

### **Synthesis of LP loaded CNP**

To load the LP, a 1% solution of CNPs was stirred with the isolated sample (1 mg/mL) in a borate buffer (pH 9) at 37°C. After 8 hours, the solution was dialysed and was ly-



ophilized to obtain LP loaded CNPs. The nano size of the freeze dried samples was characterized using TEM and PS distribution. The loading of LP on CNPs by the formation of the amide linkage were confirmed using FTIR spectrum. The percentage of LP loaded on CNP was predicted by extrapolating the UV-Vis reading against a standard graph.

### **Antibacterial activity**

The antimicrobial activity of the extract, CNP and LA loaded CNP was done using diffusion techniques [14, 17]. Two bacterial species were selected from gram positive and gram negative strains each. The bacterial stock culture was grown in the nutrient broth for 24 h at 37°C. The MHA plates were swabbed with respective bacterial culture and were divided into 4 portions with a hole to accommodate the samples. After incubation overnight, the zone of inhibition formed for each sample was measured.

### **Antioxidant activity**

The radical scavenging activity was estimated by DPPH assay to determine the antioxidant capacity of the extract, CNPs and LP-CNPs using the procedure discussed earlier [20]. The percentage of inhibition was calculated using the following formula

$$\text{Percentage of Radical Inhibition (\%)} = 100 (\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}) / \text{Abs}_{\text{Control}}$$

### **MTT assay using HeLa cell line**

Based on the ability of live but not dead cells to decrease a yellow tetrazolium dye into a purple formazan product, a MTT assay was performed [21]. Cells were maintained in DMEM medium, 10% Fetal Bovine Serum was used as a supplement, in humidified atmosphere at 37°C with 5% CO<sub>2</sub>. The cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2x 10<sup>4</sup> cells/well and allowed to attach over-

night at 37°C. The medium was then discarded and cells were incubated with different concentrations of the test sample (50-250 µg/mL) for 24 hours. Medium was discarded and 100µl fresh medium was added with 10µl of MTT (5mg/ml), after incubation period. After an incubation of 4 hours, the medium was discarded and the formazan crystals were dissolved by adding 100µl of DMSO. Then, in a microtitre plate reader the absorbance was read at 570 nm. Cyclophosphamide (25 µg) was used as positive control. Cell survival was calculated by the following formula:

$$\text{Viability \%} = (\text{Test OD} / \text{Control OD}) * 100$$

$$\text{Cytotoxicity \%} = 100 - \text{Viability\%}$$

## Supporting Information

Supporting information text

Supporting Information File 1:

File Name: S1.pdf

File Format: PDF

Title: XRD data of Carbon nanoparticle

Supporting Information File 2:

File Name: S1.pdf

File Format: PDF

Title: FTIR analysis of CNP and amino functionalized CNP formation

Supporting Information File 3:

File Name: S1.pdf

File Format: PDF

Title: MS spectrum of hydroxy linalool and linalyl acetate

Supporting Information File 4:

File Name: S1.pdf

File Format: PDF

Title: MTT plates of HeLa cell lines showing maximum inhibition

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