

## Antibacterial Properties of Biologically Synthesized Chitosan Nanoparticles Along With Leaves Extract of *Tinospora Cordifolia*

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**Abstract:** Chitosan nanoparticles have gained more attention as drug delivery carriers because of their better stability, low toxicity, simple and mild preparation method. Chitosan nanoparticles showed to be good adjuvant for vaccine. In the present study Chitosan nanoparticles were prepared by Ion tropic gelation method and the ethanol extract of *Tinospora cordifolia* leaves was prepared and used in combination with Chitosan nanoparticles which was obtained from crab shells. Characterization was by UV Spectroscopy and SEM. Ethanolic extract of *Tinospora cordifolia* extract showed better antibacterial activity. UV Spectroscopy SEM analysis revealed the presence of polyphenolic; proteins and alkaloids compounds act as effective agents for converting chitosan to CNPs. Moreover, the synthesized nanoparticles showed potent antibacterial activity against *Propionibacterium acnes*, *S. typhi*, *S. pyogenes* and *Pityosporum ovale*. The results reveal that natural sources of materials such as plants could be used for preparation of CNPs instead of use of chemical substances.

**Keywords:** Chitosan nanoparticles, leaves extract and antibacterial activity

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### I. Introduction

Chitosan (CS) is a polysaccharide obtained from deacetylation of chitin. CS is composed of deacetylated  $\beta(1-4)$  2-amino-2-deoxy- $\beta$ -D-glucan monomers and monomers with N-acetyl groups in place of amino groups that are linked by glycosidic bonds. Chitosan has received great attention in both the medical and pharmaceutical fields. Chitosan, a biodegradable and biocompatible polymer, is a modified natural carbohydrate and the second most abundant polysaccharide in nature. Chitosan is available in a wide range of molecular weights and deacetylation degrees. Due to its characteristics, chitosan has gained increasing attention in the pharmaceutical field. In addition, chitosan presents mucoadhesive, immune stimulating, antimicrobial and wound-healing properties. Moreover, it has been regarded as a promising polymer for the formulation of vaccine delivery systems. On the other hand, the evaluation of chitosan as an adjuvant for parenteral vaccination studies was reported together with the results of intranasal or oral vaccination studies, making the possible value of chitosan as an adjuvant for parenteral routes less noticeable in the scientific literature.

*Tinospora cardifolia* is a large deciduous climbing shrub found throughout India. The ayurvedic name of the plant is Guduchi, Giloy or Amrita. In India, the extract of the plant is used as a remedy for many diseases including diabetes, hepatitis etc., The plant finds a special mention for its use in tribal or folk medicine in different parts of the country. The drug has been subjected to extensive phytochemical, pharmacological and clinical investigations and many interesting findings have been reported (Nadkarni, 2005).

The nanoparticles are obtained under biological system, very mild conditions without the need of high temperature, surfactant and some other special experimental technology (b) The nanoparticles have small particle size and positive surface charges, which may improve their stability in the presence of biological actions [24] and is favorable for some drugs due to the interaction with negatively charged biological membranes and site – specific targeting in vivo [25,26]. The biomolecules of the plant extract are the possible compounds in the biosynthesis of nanoparticles [27]. However, aromatic and medicinal plants contain many biologically active compounds [28]. On the other hand, to the best of our knowledge, there is no report of CNPs biosynthesis by utilizing of aqueous leaf extracts of *Tinospora cordifolia*. So, this study aims at biosynthesis of CNPs using *Tinospora cordifolia* leaf extracts and investigation of their antibacterial effects for the first time

## II. Materials and methods

**Collection of sample:** The crab shell was collected from Mira road (E), fishery shop. Washed thoroughly 2-3 times under running water and once with sterile distilled to remove extraneous matter present on the surface. Shells were autoclaved and air dried under sunlight then grinded in small particles.

**Preparation of chitosan:** Chitin extraction from crab shells was carried out as described previously for other crustacean shells by an alkali-acid treatment with minor modifications of the treatment conditions. DPMCA (deproteinization + demineralization + decolorization + deacetylation) was taken as the traditional processing method. This nanoparticles obtained was used for further analysis.

**Collection of plant material:** The plant was collected from Bhavan's college campus Andheri (W). The leaves were washed thoroughly 2-3 times with running water and once with sterile distilled water to remove dirt particles and other extraneous matter present on the surface. The leaves were air dried under sunlight.

**Extraction and biosynthesis of CNPs:** All chemicals were purchased from Merck or Aldrich. Low molecular weight chitosan was dissolved at 2% (w/v) with 0.5% (v/v) acetic acid and then raised to pH 5 with 1N NaOH under magnetic stirring for 24 hours and brought to volume in a 200 ml volumetric flask. Frozen collected leaves were used for the preparation of *Tinospora cordifolia* leaf extract. 10 gram of finely cut leaves was taken and boiled in 100 ml of distilled water for 5 minutes. After cooling, the obtained extract was filtered through Whatman No. 1 filter paper and filtered extract was stored at 4 °C. CNPs formed spontaneously upon addition of 10 ml of the *Tinospora cordifolia* leaf extract to 40 ml of chitosan solution (1mg/ml) under magnetic stirring at 60 °C and 110 rpm to obtain an opalescent solution.

**Characterization of Chitosan Nanoparticles:** Chitosan nanoparticles were characterized by SEM (Scanning Electron Microscopy ) by Philips XLD 3D model, CIRCOT, Matunga East, Mumbai, to examine the particle size and surface morphology. Where CSNPs are coated with gold metals film and magnified under 15000X.

### *Antimicrobial Activity of Chitosan and chitosan Nanoparticles*

The antimicrobial activity of chitosan and chitosan nanoparticles is studied using agar diffusion method. For this petriplates containing 20 ml Muller Hinton medium are seeded with 24 hrs. old culture of bacterial strains (viz., *E. coli*, *C. albicans*, *S. aureus*, *P. aeruginosa*, *S. typhi*, *Pityosporum ovale* *Propionibacterium acnes*) wells are cut and 100 microliter of above solutions are added. The plates are often incubated at at 37 C for 24 hours. The antibacterial activity is assayed by measuring the diameter of the inhibition zone formed around the well (Lifeng *et al.*, 2004)

**Culture maintenance:** *E. coli*, *C. albicans*, *S. aureus*, *P. aeruginosa*, *S. typhi*, *Pityosporum ovale* *Propionibacterium acnes* were obtained from Bhavan's college laboratory. The microbial cultures were standardized at 0.5 OD

**Agar well diffusion assay:** Antimicrobial activity of plant extracts was evaluated using Agar well diffusion assay. 24 hr old microbial inoculums OD adjusted at 0.5 was aseptically spreaded using cotton swab on the surface of well solidified Nutrient Agar plates. A well of about 8mm was aseptically punctured with sterile cock borer. 80 µl of each extract prepared was poured into the well formed. DMSO was used as a negative control. Ciprofloxacin (5µg/disc) antibacterial discs, respectively were used as a positive control. Plates were kept in refrigerator for 15 minutes for pre diffusion of extract to occur and then incubated at 37°C for 24 hours.

**Phytochemical screening:** Preliminary qualitative phytochemical screening was carried out with the following methods (Khandelwal, 2001).

**Test for Tannins:** To 0.5 ml of extract solution, 1 ml of distilled water and 1 to 2 drops of ferric chloride solution was added, observed for blue or green black coloration.

**Test for Saponins:** Two ml of distilled water was added to 2 ml of the test solution shaken well and observed for frothing.

**Test for Flavonoids:** A volume of 1.5 ml of 50 % methanol was added to 4 ml of the extracts. The solution and magnesium metal was added and warmed. Then, 5 to 6 drops of concentrated hydrochloric acid was added to the solution and observed for red coloration.

**Test for Steroids** (Salkowski's test): Five drops of concentrated sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) was added to 2 ml of each extract and observed for red coloration.

**Test for Glycosides:** To 4 ml of extract solution and add few drops of glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid and observed for a reddish brown coloration at the junction of 2 layers and bluish green colour in upper layer.

**Test for Alkaloids:** To 4 ml of extract filtrate, a drop of Mayer's reagent was added along the sides of test tube. Creamy yellow or white precipitate indicates that the test is positive.

**Test for Anthraquinones:** One gram of powdered plant material was taken and extracted with 10 ml of hot water for five minutes and filtered. Filtrate was extracted with 10 ml of CCl<sub>4</sub> then CCl<sub>4</sub> layer was taken off. Five ml water and 5 ml dilute ammonia solution was added. No free anthraquinones were revealed as absence of appearance of pink to cherry red colour. One gram of second sample of the same plant material was extracted with 10 ml of ferric chloride solution and 5 ml of hydrochloric acid then it was heated on water bath for 10 minutes and filtered. Filtrate was cooled and treated as mentioned above.

**Test for phenolic compounds:** Two ml of extract was diluted to 5 ml with distilled water. To this a few drops of neutral 5 % ferric chloride solution was added. A dark green colour indicates the presence of phenolic compounds

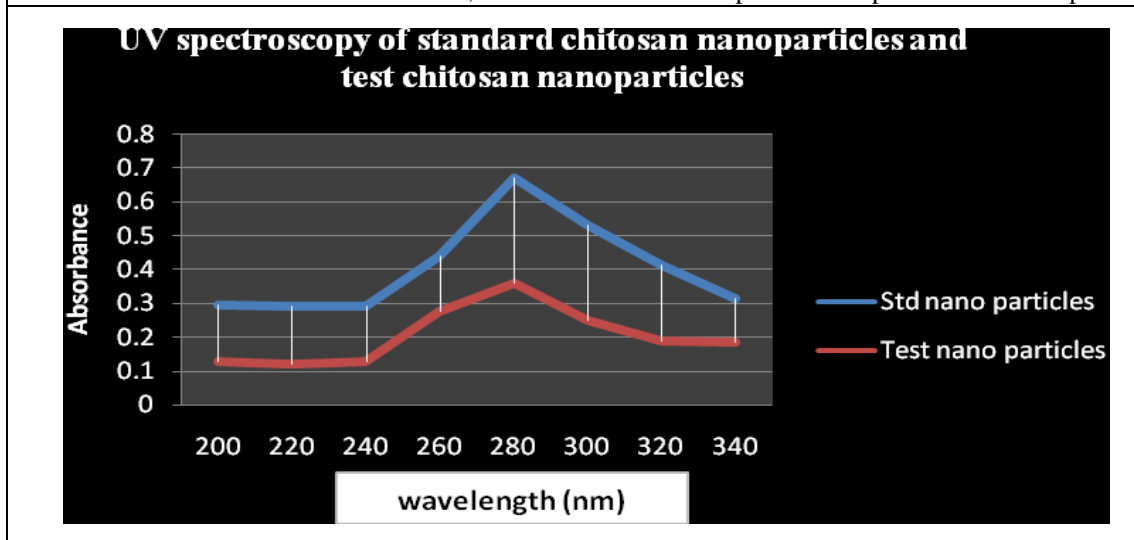
### III. Results and Discussions

In this study chitosan has been successfully prepared from *crab cells*. The synthesis of chitosan involves various chemical steps. Pretreatment methods were done using 1N NaOH and 2% acetic acid. The alkali and acid treatments remove proteins and minerals from chitin respectively and deacetylates simultaneously. These methods give advantages for obtaining of higher quality chitosan. Chitin is not soluble but chitosan, the deacetylated product of chitin, is soluble in very dilute acids like acetic acid, lactic acid, formic acid etc. the deacetylation experiment using 2 N NaOH was done to reduce acetyl group from molecular structure, because the presence of acetyl group prevents to make the solution of chitosan. 10 gm (dry weight) of *Saccharomyces cerevisiae* gives 0.9gm chitosan and percentage yield of chitosan is 0.81%.

#### *Preparation and characterization of chitosan nanoparticles*

Chitosan nano particles can be prepared using many methods such as ionic gelation method, emulsion cross- linking, and spray drying. In this study, ionic gelation method was applied because the method is easy and fast to be carried out. This simple technique involves electrostatic interaction between positively charged amino group of chitosan and negatively charged polyanions. Formation of nanoparticles occurs spontaneously through the formation of intra- and intermolecular cross- linkages under a constant stirring at room temperature (Liang *et al*, 2012).

**Fig3:-** UV spectroscopy of standard chitosan nanoparticles and test chitosan nanoparticles extracted from crab shells. Peak observed at 280nm, indicates Chitosan nanoparticles are present in test sample.



The chitosan nanoparticles prepared in the experiment exhibit a white powdered shape and are soluble in deionized water. The synthesized chitosan nanoparticles were characterized by scanning electron microscopy. SEM was used for the determination of the particle size and the morphological structure of the prepared polymer matrix. The coating of chitosan nanoparticles were done by gold metal and magnified under 15000X. It was observed that chitosan/ TPP has average particle size of 60- 110 nm. Fig.1 shows the size of chitosan nanoparticles. Particle size of chitosan nanoparticles is depend on concentration of chitosan and TPP, their mass ratios, and drying methods. Fig.1 shows the SEM picture of chitosan nanoparticles.

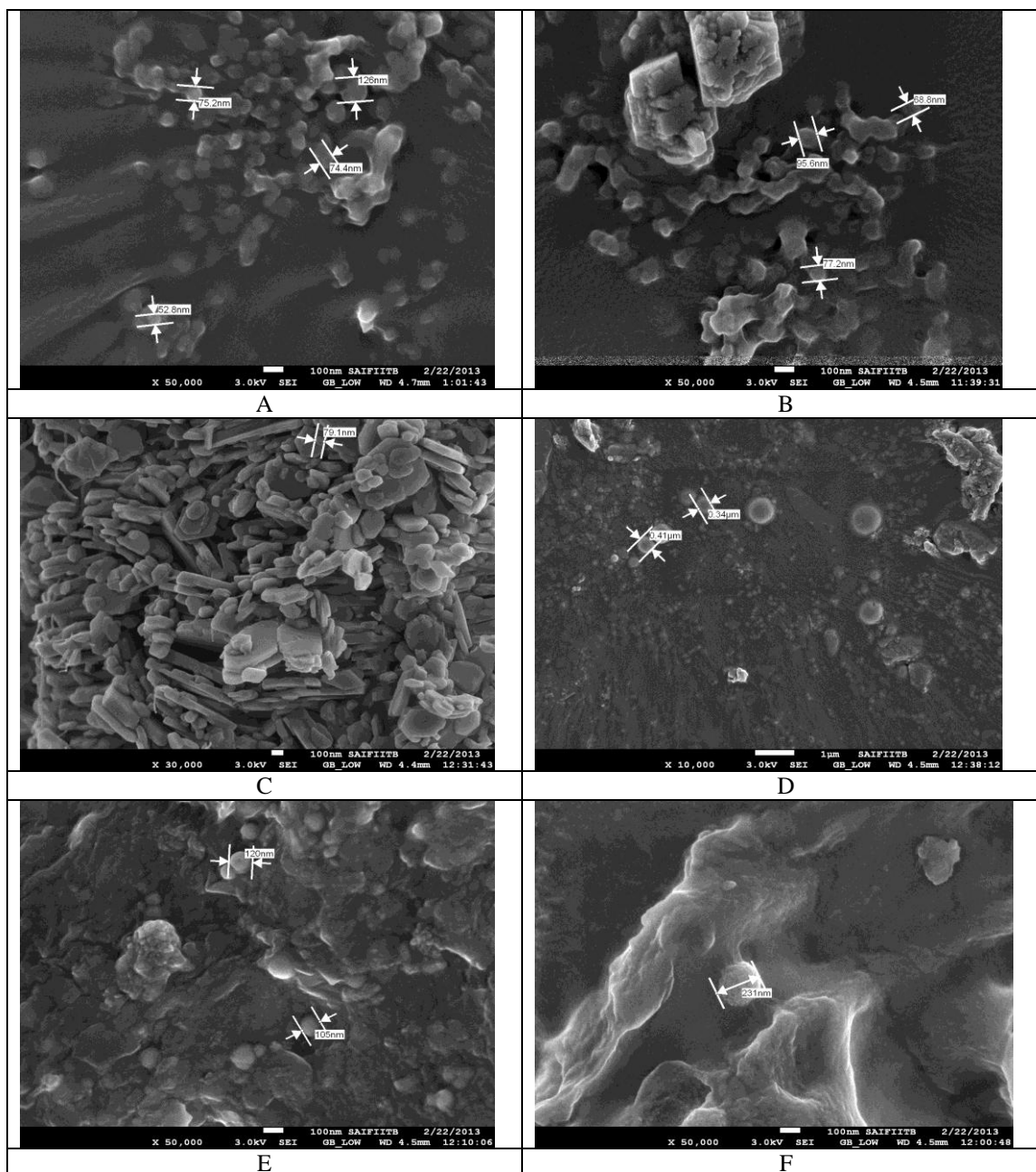


Fig:-Scanning electron Microscopy of Ethanolic extract of *Tinospora cordifolia* leaves and chitosan nanoparticles.(A), (B) (C) extract, (D),(E) standard chitosan nanoparticles.(F) chitosan nanoparticles obtained from crab shells.

It was observed that chitosan nanoparticles were present in the test samples when compared with the standard. It was also found that when both extract and nanoparticles were tested in combination the stability of Nanoparticles were increased Since all the nanoparticles obtained was of similar size and shape where as in case of standard sample varying size of nanoparticles were observed. Where as in case of test chitosan nanoparticles it was also observed that the ratio of Tpp (3:1) used in the preparation of chitosan nanoparticles had also affected the size of chitosan nanoparticles i.e increase in size (315nm) when compared with the standard nanoparticles.

Sr.No	Phytochemical Constitutes	Aqueous extract	Ethanol extract	Methanol extract	Acetone Extract
1	Alkaloid	+	+	+	+

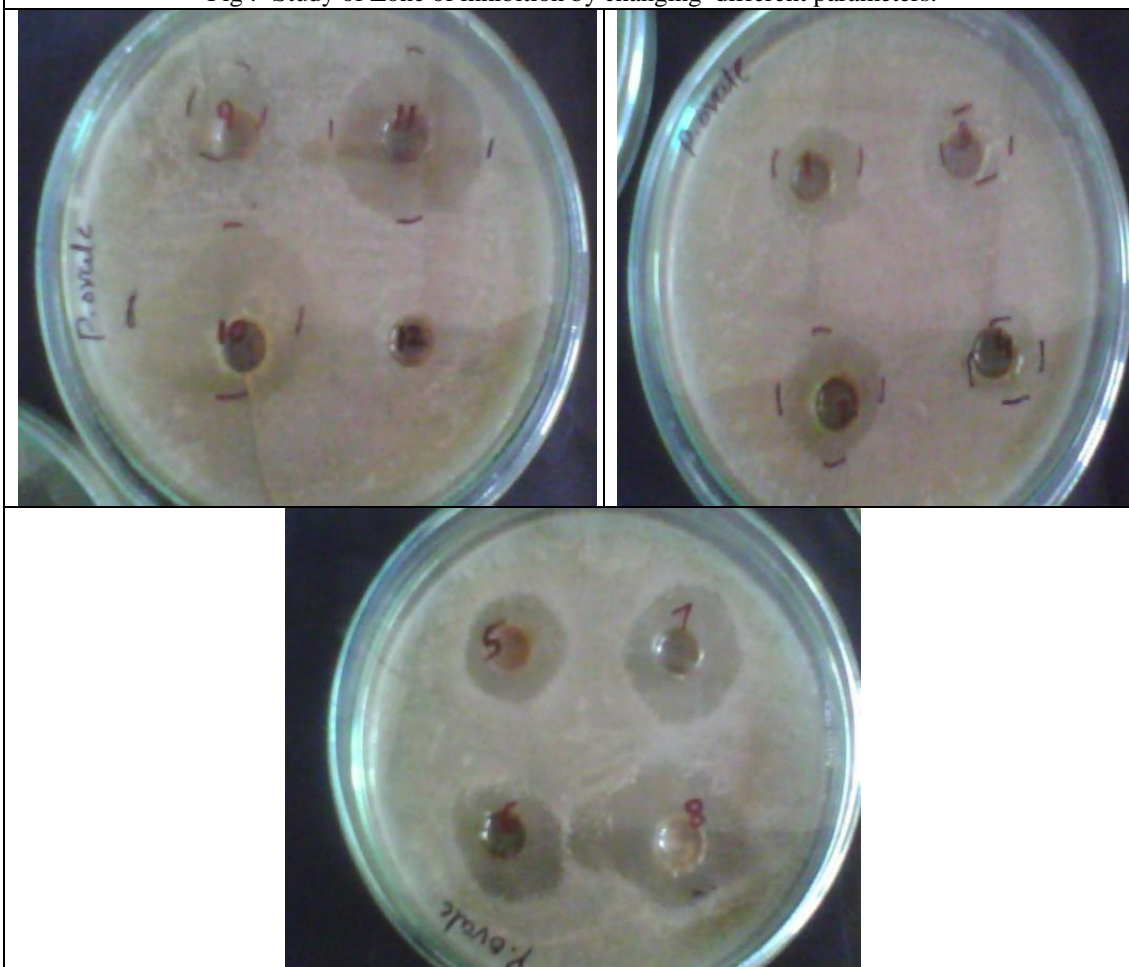
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2	Flavonoids	+	++	++	+++
3	Glycosides	+	+	+	+
4	Saponins	-	++	++	+
5	Steroids	-	+	+	+
6	Tannins	+	++	+++	+++
7	Anthroquinones	-	+	+	+
8	Phenolic compounds	-	+++	+++	+++

**Table 1.** Antibacterial activity of Chitosan Nanoparticles + leaves extract of *Tinospora cordifolia* (%)

Microorganisms	Zone of inhibition (mm)								
	Concentration Of Chitosan Nanoparticles + leaves extract of <i>Tinospora cordifolia</i> (%)			Concentration Of Chitosan Nanoparticles			Concentration Of Chitosan (%)		
	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
<i>E. coli</i>	25	28	31	14	18	22	-	-	12
<i>S. aureus</i>	31	32	35	16	26	28	-	14	14
<i>S. typhi</i>	24	26	30	20	24	26	-	-	-
<i>P. aeruginosa</i>	22	25	27	15	17	25	-	12	14
<i>C. albicans</i>	24	26	28	16	18	22	12	14	16
<i>Pityosporum ovale</i>	15	17	22	13	14	17	-	-	14
<i>Propionibacterium acnes</i>	22	24	26	18	20	22	-	-	12

Fig :- Study of Zone of inhibition by changing different parameters.



It has been well documented that the antimicrobial compounds are abundantly present in medicinal plants. These compounds are thought to be involved in the defence of the plant against microbial pathogens. According to the findings of the present study, the aqueous and organic solvent extracts showed considerable antibacterial and antifungal activity against the tested microorganisms. Antimicrobial activity of *T. Cordifolia* and chitosan nanoparticles was evaluated by agar well diffusion method.(1,2,3,4) The present study indicated that the tested plant extracts and chitosan nanoparticles possess the potential of antibacterial activity and antifungal activity against *E. coli*, *C. albicans*, *S. aureus*, *P. aeruginosa*, *S. typhi*, *Pityosporum ovale* and *Propionibacterium acne*, showed maximum antimicrobial activity. It was observed that the ethanolic extract of *Tinospora cordifolia* was found to be most effective. The results of this study revealed that the *S. typhi*, *S. pyogenes*, *Propionibacterium acne* and *Pityosporum ovale* showed maximum antimicrobial activity with ethanol extract. The chitosan nanoparticles obtained from crab shells were also tested for their antimicrobial activity and results revealed considerable antimicrobial activity as compared with the herbal extracts. It was found that the combined activity of chitosan nanoparticles and *Tinospora cordifolia* ethanolic was most effective as compared to the individual activity of both the samples. Ciprofloxacin (5µg/disc) was used as a positive control against all bacterial culture. Characterization of chitosan nanoparticles obtained from crab shells and standard chitosan nanoparticles was done by UV spectroscopy and SEM. It was observed that the peak was observed at 280nm by UV spectroscopy which indicates that the chitosan nanoparticles are present in the test sample (22). SEM results proved that the chitosan nanoparticles were present in the test samples. Characterization of chitosan nanoparticles was done by Scanning electron Microscopy. Where 4 samples were tested by SEM:- 1) Chitosan Nanoparticles obtained from crab shells (Test), 2) Ethanolic Extract of *Tinospora cordifolia* leaves, 3) Standard Chitosan Nanoparticles (Control), 4) Combination of Chitosan nanoparticles + Ethanolic Extract of *Tinospora cordifolia* leaves.

#### IV. Conclusion

Biosynthesis of silver nanoparticles was carried out using by using the aqueous extracts of medicinal plants with the bio-reduction of silver ions in short period and tested for their antimicrobial activity. The CNPs of *T. cordifolia* have shown good antimicrobial efficacy and hence has a potential to be used as antimicrobial agent against wide range of microbes over conventional antibiotics.

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