Anti-inflammatory Activity of *Ocimum tenuiflorum L.* Leaf Extract Loaded Chitosan Nanoparticle as a Drug Carrier – An In Vitro Study

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ABSTRACT

Chitosan is the second most abundant natural polycationic linear polysaccharide next to cellulose derived from crustacean chitin shells. Chitosan has a variety of commercial and potential biomedical applications. Chitosan nanoparticles (CSNPs) have recently gained popularity due to their bio-nature, biocompatibility, biodegradability, quantum size effects, large surface-to-volume ratios, and ease and low cost of production. This study evaluated the potentiality of *Ocimum tenuiflorum* (OT) leaf extract-loaded CSNPs as an effective alternative for targeted drug delivery. Tulsi has powerful anti-inflammatory, analgesic, anti-bacterial, and anti-viral properties. Even today, people consume tulsi, decoction, and juice to treat a variety of ailments. When compared to indomethacin, fresh tulsi leaves (TL) in paste form exhibit significant anti-inflammatory activity with minimal side effects. Fixed oil and linolenic acid present in tulsi have

the ability to block cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism. Therefore, they show anti-inflammatory activities against PGE2. As a result, fresh tulsi leaves could be used as an adjuvant in conjunction with CSNPs, conventional anti-inflammatory agents (TLCSNPs) to treat inflammation.

Keywords: Chitosan, Nanoparticles, Drug delivery, Tulsi leaf, Anti-inflammation activity, TLCSNPs

INTRODUCTION

Nanotechnology deals with the understanding and control of matter at dimensions ranging from 1 to 100 nanometers, where unique phenomena allow for novel applications. In a nanometric scale, nanoparticles are generally classified as organic, inorganic, and carbon-based particles with improved properties when compared to larger sizes of respective materials. Because of their small size, nanoparticles have enhanced properties such as high reactivity, strength, surface area, sensitivity, stability, and so on. Nanoparticles are synthesized using a variety of methods for research and commercial applications, which are classified into three major types: physical, chemical, and mechanical processes that have seen significant advancements over time. Nanoparticles are increasingly important in a wide range of applications. Nanotechnology has the potential to create novel food ingredients and products with significant health benefits. Nanomaterials, also known as nanoparticles, open up new avenues of research in material science and biology. These nanomaterials have emerged as key players in modern medicine in recent years, with applications ranging from contrast agents in medical imaging to carriers for gene delivery into individual cells (Agarwal et al., 2018; Ali et al 2017).

Chitosan (CS) is a partially deacetylated chitin derived primarily from crustacean exoskeletons. Chitosan is a natural polymer made up of β -(1-4)-linked D-Glucosamine molecules that are randomly distributed. Chitin monomers and chitosan monomers are the two types of monomers found in them. It's made by treating shrimp and other crustaceans' chitin shells with an alkaline substance like sodium hydroxide. It is also used as a chelating agent due to its ability to bind with cholesterol, lipids, proteins, and metal ions. It stands out among other biomaterials due to its abundance, adaptability, and unique properties such as biodegradability, biocompatibility, non-toxicity, hydrophilicity, anti-bacterial and anti-fungal capabilities, and wound-healing activities. Surgical thread is used in both wound healing and tissue engineering (Gomes et al., 2017; Kalabharathi et al., 2011).

Chitosan nanoparticles (CSNPs) have recently gained popularity due to their biological properties, biocompatibility, biodegradability, quantum size effect, large surface area to volume ratio, and ease and low cost of production. These are unique properties of chitosan nanoparticles. Chitosan can be easily processed into foils, threads, tablets, membranes, and microparticles/nanoparticles. CSNPs have been reported to be successfully used in drug delivery for the treatment of a variety of diseases, including per-oral drug delivery, nasal and ocular drug delivery, pulmonary drug delivery, mucosal drug delivery, gene delivery, vaccine delivery, and cancer therapy. Inflammation is a complex biological reaction of vascular tissues to potentially

damaging stimuli. Inflammation is also linked to pain, and it causes a rise in protein denaturation, vascular permeability, and membrane change, among other things. The release of chemical mediators from wounded cells or tissues and migrating cells speeds up the body's response to inactivate or eradicate invading stimuli or organisms, remove irritants, and lay the way for tissue repair. The release of cytokines and the migration of leukocytes from the venous systems to the site of damage are known to play important roles in the inflammatory response. These substances cause blood capillaries to enlarge and capillary permeability to increase. This will result in more blood flowing to the affected area. (Gunathilake et al., 2018)

Tulsi known as the Queen of herbs has main chemical constituents are: oleanolic acid, ursolic acid, rosmarinic acid, eugenol, carvacrol, linalool, and -caryophyllene, and have been used extensively for many years in food products, perfumery, and dental and oral products, and plant extract continues the numerous searches for more effective drugs of plant origin that are less toxic and available for low socio-economic population in the treatment of diseases. Ocimumosides A and B are compounds found in tulsi. These compounds reduce stress and help the brain's neurotransmitters serotonin and dopamine balance. Recent research suggests that Tulsi, like many modern painkillers, may be a COX-2 inhibitor due to its high concentration of eugenol. The purpose of this study was to assess the phytochemical screening of aqueous extracts of OT leaves. This medicinal herb can be used as a pharmaceutical adjuvant in the formulation of various dosage forms, according to research. Tulsi's anti-inflammatory properties lower inflammation and blood pressure. (Sandip et al., 2016; Rampino et al., 2013).

MATERIALS AND METHODS

SAMPLE IDENTIFICATION AND COLLECTION

The prawns were bought from a local market in Tiruchirappalli, Tamil Nadu. The prawns were identified and authenticated by Dr. P. Mohideen Askar Nawas, Assistant Professor, Department of Zoology, Thanthai Periyar Government Arts & Science College, Tiruchirappalli, Tamil Nadu, India. The prawns were thoroughly washed, and the shells were removed and allowed to air dry completely.

ISOLATION AND EXTRACTION OF CHITOSAN

The shells were rinsed thrice with tap water twice with distilled water. Washed shells were dried for 2-3 days. After the shells dried, the shells are crushed into small pieces using mortar and pestle.

DEPROTEINIZATION

The dried shells of exoskeletons were soaked in boiling 4% Sodium Hydroxide (NaOH) for 1 hr in order to dissolve the proteins and sugars. After boiling with sodium hydroxide the shells are removed from NaOH and allowed to cool for 30 minutes at room temperature. Then the exoskeletons of prawn waste (shells) were crushed to small pieces of about 0.5- 5.0 mm (Yateendra Shanmukha et al., 2012).

DEMINERALIZATION

Demineralization was carried out using 1% hydrochloric acid (HCl) with four times its quantity. In order to remove the minerals (mainly calcium carbonate) the samples were allowed to remain in HCl for 24 hrs. (Trung et al., 2006). After done with demineralization the shells were treated for 1 hr with 50 ml of 2% NaOH. The remaining shells were washed with deionized water and then drained off.

DEACETYLATION

By this process the chitin is further converted into chitosan, deacetylation is processed by adding 50% NaOH and then boiled for 2 hr at 100°C on hot plate. Then the samples are allowed to cool at room temperature for 30 min. Hereafter the samples were washed continuously with 50% NaOH and it is filtered in order to retain the solid matter (which is chitosan). The samples were left uncovered and dried. (Paul et al., 2014)

PURIFICATION OF CHITOSAN

REMOVAL OF INSOLUBLE WITH FILTRATION

1 mg/ml chitosan acetic acid 1% (v/v) solution is prepared by a magnetic stirrer until a homogenous solution is obtained. The insoluble was removed by filtration through Whatman filter paper 22µm.

REPRECIPITATION OF CHITOSAN WITH 1N NaOH

Titration with 1 N NaOH until pH 8.5 is being used to precipitate chitosan from filtered chitosan solution. The acquired chitosan is rinsed many times with distilled water before being centrifuged at 8,000 to 10,000 xg. In terms of giving more uniformity and reproducibility amongst chitosan batches for biomedical applications, these processes must be performed in the presence of the reducing agent Dithiothreitol (DTT) (Islam et al., 2017)

DEMETALLIZATION OF RETRIEVED CHITOSAN

Reprecipitation is carried out in relation to Dementalizations by adding 1 ml of a 10% w/v Aqueous solution of sodium dodecyl sulphate (SDS) and stirring for 30 minutes to dissolve any residual protein. After stirring the solution at room temperature up overnight, 3.3 ml of 5% w/v ethylene diamine tetra acetic acid (EDTA) were added and stirred at room temperature for 2 hours for heavy metal precipitation with EDTA. The water-insoluble chitosan precipitate was obtained using REMI and centrifuged at 5000xg for half an hour before being rinsed numerous times with distilled water by resuspending and centrifugation for 30 minutes. To avoid physical damage to the chain structure, the residue is gently dried in a hot air oven at 60 ° C. (Bangun et al., 2018)

PHYSIOCHEMICAL PARAMETER

SOLUBILITY AND pH

Chitosan solubility was tested in a variety of solutions including distilled water, acetone, acetic acid. The chitosan obtained here has been entirely soluble in acetic acid. A pH meter was used to measure the pH of the chitosan solution. (Azab et al., 2016)

DEGREE OF DEACETYLATION

The degree of deacetylation is estimated by potentiometric titration and refers to the

removal of the acetyl group from the chain. Chitosan homogeneous solution were prepared using Diluted HCl containing 0.010 mol/L and titrated against 0.1M NaOH. The inflections of the pH values represent the end point. Two significant inflections were observed, the first corresponding to HCl neutralization and the second to ammonium ion neutralization for the chitosan chain. The difference in two points represents the number of amino groups in the chitosan chain. (Bobbarala, Varaprasad 2015).

CHARACTERIZATION OF PURIFIED CHITOSAN

The purified chitosan was characterized by Gas Chromatography-Mass Spectrometry analysis (GCMS). (Prabhu et al., 2022)

PLANT SAMPLE COLLECTION AND IDENTIFICATION

The plant was identified and authenticated by Dr. Soosairaj, Assistant Professor, from the Department of Botany, St.Joseph College, Tiruchirappalli. The leaf samples were collected from the tulsi plant in my residential area at Thilagar street, Trichy, Tamil Nadu, India.

ETHANOLIC EXTRACT OF TULSI LEAF

The leaf samples were washed in tap water, dried, and placed into a blender to be grounded into powder. For ethanolic extraction, ethanol (>99.5%) is used for the maceration extraction process. The leaf powder was added to ethanol to make a 25% concentration (25g) in the ratio of 1:10 (25g in 250ml of ethanol). The mixtures were made in sterile bottles wrapped with parafilm in order to avoid evaporation and exposure to light for 5 days at room temperature. The bottle was placed on an orbital shaker. After 5 days of soaking in solvent, the extracts were filtered using Whatman filter paper and the extract is stored for further use. (sudharsan et al., 2022)

PREPARATION OF CHITOSAN NANOPARTICLE (IONIC GELATION METHOD)

Ionic gelation method has been used to synthesize CSNPs. 10ml of 0.1 % chitosan solution was prepared in 1% acetic acid with varying amounts of the cross-linking agent STPP (0.5, 1%, 1.5, and 2%). At room temperature, 5ml of STPP was added drop by drop to the chitosan acetic acid solution, that was magnetically stirred. Continuous stirring for 60 minutes after an opalescent color was seen (Othman et al., 2018)

PREPARATION OF TLCSNPs

To make TLCSNPs, different concentrations of plant extract (5%, 10%, 15%, & 20%) were added to 10 ml chitosan solution by magnetic stirring prior to adding the 5 ml STPP dropwise. This solution was stirred for another 2 hours before being centrifuged at 10000g for 10 minutes and the TLCSNPs were rinsed three times with distilled water. The pH of the nanoparticles was kept at 4.8, and 1–2 drops of 1% Tween-80 were added to prevent agglomeration. (Gu et al., 2013)

ANTI INFLAMMATORY ACTIVITY OF TLCSNP

Erythrocytes suspension was prepared by adding 100µL of erythrocytes in 900µL 1XPBS. Each 100µL erythrocytes suspension was mixed with 100µL of test samples (1000µg/mL, 900µg/mL, 800µg/mL, 700µg/mL, 600µg/mL, 500µg/mL, 400µg/mL, 300µg/mL,

200µg/mL and 100µg/mL) and 100µL of 1XPBS was used as negative control and 100µL of 1% SDS as positive controls. Reaction mixture was incubated at 37oC water bath for 60 min. The volume of reaction mixture was made up to 300µl by adding 1X PBS. Finally, it was centrifuged at 1500 rpm for 5 min and the resulting hemoglobin in supernatant was measured at 540 nm by spectrophotometer to determine the concentration of hemoglobin. The average value was calculated from triplicate assays. The hemolysis percentage for each sample was calculated by dividing sample's absorbance on positive control absorbance (complete hemolysis) multiplied by 100. The level of hemolysis was calculated using the following equation.

% inhibition of hemolysis = $100 \times (1 - A2/A1)$ where A1 = absorption of the control, and A2 = absorption of the test sample mixture. (Gunathilake et al., 2018).

INHIBITION OF PROTEIN DENATURATION ASSAY

200µL of 1% egg albumin was added to 100µL of test sample later it was make up to 1000µL with phosphate buffer saline. This mixture was kept at room temperature for 37°C for 15 minutes, followed by heating at 70°C for 5minutes. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm. Acetyl salicylic acid was taken as a positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using:

% Inhibition=$100 - ((A1-A2)/A0)*100)$

Where A1 is the absorbance of the sample, A2 is the absorbance of the product control and A0 is the absorbance of the positive control.

A dose-response curve was plotted to determine the IC50 values. IC50 is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged.

ANTI – OXIDANT ACTIVITY BY FRAP ASSAY

240µl of FRAP reagent was added with 10µl of sample in the wells. 240 µl of FRAP reagent was added with 10µl of standard in the wells. 75 % ethanol was used as the blank. The plate was incubated for 5mints at 37 ^{0} C. The absorbance was read at 620 nm in ELISA plate reader

CHARACTERIZATION OF CSNPs

X-Ray diffraction, UV - Vis spectroscopy, Scanning Electron Microscopy, and FTIR (Shimadzu (8400S) spectrometry have been used to characterize the obtained chitosan nanoparticles (De et al., 2017).

RESULT

ISOLATION AND EXTRACTION OF CHITOSAN

The synthesis of chitosan involves many chemical processes, beginning with the removal of proteins from the crude shells, followed by demineralization to remove the carbon as well as other salt present in the crude form, which is preceded by deacetylation of the chitin, which results in chitosan. Following the above process yields typical chitosan, but a polymer pharmaceutical-grade must remain within the range of its specified quality attributes, and a first stage of deproteinization typically synthesizes commercial chitins following the second process

of demineralization.

Figure 1: Chemically treated shells and powdered chitosan from shells

PURIFICATION OF CHITOSAN

During the purification process, insoluble contaminations were removed, and deproteinization and Demetallization reagents, Sodium dodecyl sulphate and EDTA, are added in the presence of the reducing agent, DTT. As a result, water-insoluble chitosan precipitates or flocculants formed, and also water-soluble supernatants containing the deproteinization agent and also any proteins that had complexed with it, as well as the Demetallization agent and the extracted metals. By dissociating any disulfide links in the proteins, the reducing agent increased the water solubilization of protein contaminants. To avoid the generation of chitosan-metal chelate conjugates, the Demetallization agent was combined with the chitosan solutions at basic pH conditions.

PHYSIOCHEMICAL PARAMETER

The overall parameters were reported in Table 1 & their attributes are discussed. After purifying the whole exoskeleton, the chitosan yield was found to be 64 %. Chitosan solubility was tested using three different solvents: water, acetone, and acetic acid. When comparing acetic acid to acetone & water, it was much more soluble in acetic acid. Solubility was reported to be 90%. Chitosan has a pH value range varies from 6.2 to 8.0.

Physiochemical Parameter	Chitosan
Yield	64%
pH	6.4
Solubility	Acetic acid
Degree of Deacetylation	87%

Table 1: Physiochemical parameter of chitosan

The degree of deacetylation (DD) of chitosan is an important parameter in evaluating its

specialized applications. The compound's degree of deacetylation is 87% percent. However, such higher DD values are really the result of a large amount of protein, obtaining high-quality of chitosan ideal for the pharmaceutical application. The degree of deacetylation tends to affect the chemical, biological and physical properties of chitosan, such as adsorption, covalent linking, encapsulation.

ETHANOLIC EXTRACT OF TULSI LEAF

The maceration extraction method for ethanolic extraction involves ethanol (>99.5%). In a 1:10 ratio, the leaf powder was mixed with ethanol to make a 25% concentration (25g in 250ml of ethanol). The extracts were filtered by Whatman no.1 filter paper after five days of soaking in solvent, then the extract subjected for further use.

PREPARATION OF CSNPs and TLCSNPs

CS nanoparticles were synthesized by gelating a Chitosan solution with sodium tripolyphosphate (STPP). The interaction between positively charged amino groups and negatively charged TPP promotes ionotropic gelation. Chitosan was dissolved in 1 % acetic acid aqueous solutions for 2 hours at room temperature with magnetic stirring until a solution is obtained. Chitosan concentrations ranging from 0.5-2 % were prepared. Surfactant tween 80 [% (v/v)] was introduced to chitosan solution with leaf extract to prevent agglomeration of nanoparticles, and afterward chitosan solutions were increased up to pH 4.6–4.8 using 1N NaOH. Different concentrations of sodium tripolyphosphate solutions were prepared.

STPP solution was added to chitosan solution dropwise with a dropper with magnetic stirring at 800 rpm at room temperature. The samples were visually examined and categorized: clear solution, opalescent suspension, and aggregate. The opalescent suspension zone refers to very small particles. The resultant chitosan particle suspension was centrifuged for 30 minutes at 12000g. The pellet had been resuspended in water. Before any further use or analysis, the chitosan nanoparticles suspension was freeze-dried.

ANTI-INFLAMMATORY ACTIVITY OF TLCSNP

INHIBITION OF HEMOLYSIS ASSAY

The hemolysis percentage for each sample was calculated by dividing the sample's absorbance by positive control absorbance (complete hemolysis) multiplied by 100. The level of hemolysis was calculated using the following equation based on Charles O Okoli et al.

$$\% \text{ inhibition of hemolysis} = 100 \times (1 - A2/A1)$$

Various concentrations such as 50 µg/mL,100 µg/mL,150 µg/mL,200 µg/mL,250 µg/mL of TLCSNPs were taken for the study, which showed 13.36%, 12.52%, 4.38%, 0.04%, -6.24% of hemolysis inhibition respectively and it was described in the Table 2. The graph (figure 2) represents the percentage of hemolysis inhibition. 50µg/mL concentration of TLCSNPs showed 13.36% of hemolysis inhibition which is higher than rest other concentrations.

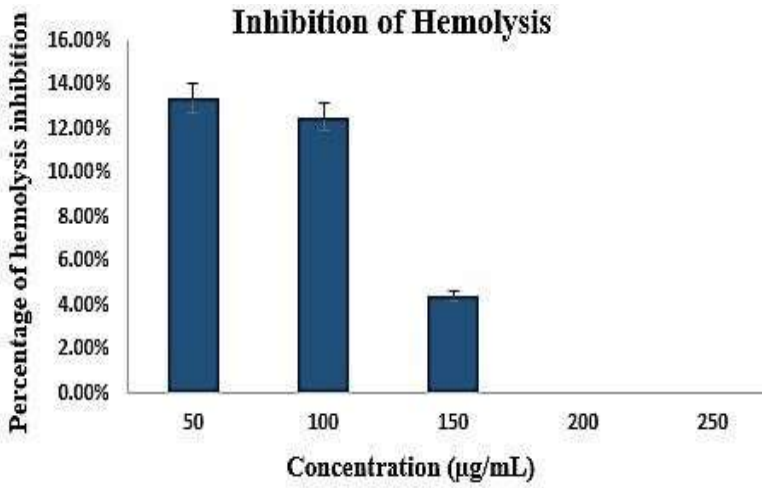

Figure 2. The graph indicating the percentage of hemolysis inhibition

Concentration (µg/mL)	OD1	OD2	OD3	Average	Percentage
50	0.576	0.603	0.612	0.597	13.36%
100	0.634	0.781	0.8	0.7383333333	12.52%
150	0.748	0.809	0.864	0.807	4.38%
200	0.798	0.843	0.89	0.8436666667	0.04%
250	0.82	0.881	0.989	0.8966666667	-6.24%
Control	0.788	0.821	0.923	0.844	0.00%

Table 2: Inhibition of hemolysis assay

INHIBITION OF PROTEIN DENATURATION ASSAY

The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using:

$$\% \text{ Inhibition} = 100 - ((A1-A2)/A0) * 100)$$

In this assay various concentrations such as 50 µg/mL,100 µg/mL,150 µg/mL,200 µg/mL,250 µg/mL of TLCSNPs were taken for the study which showed 59.27%, 57.84%, 42.60%, 21.70%, 0.00% of inhibition of protein denaturation. It was represented in figure 3 and tabulated (Table 3). 50µg/mL concentration of TLCSNPs showed 59.27% of activity which is higher than rest of other concentrations. At lower concentration the activity increases effectively.

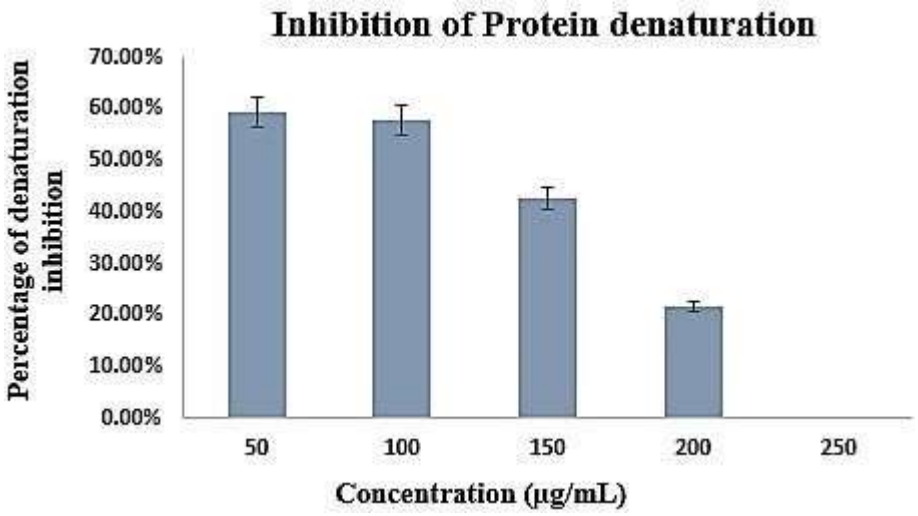

Figure 3. The graph indicates the percentage of protein denaturation inhibition

Concentration (µg/mL)	OD1	OD2	OD3	Average	Percentage
50	0.483	0.68	0.512	0.5583333333	59.27%
100	0.537	0.873	0.682	0.6973333333	57.84%
150	1.057	0.97	0.821	0.9493333333	42.60%
200	1.59	1.296	0.999	1.295	21.70%
250	1.791	1.647	1.582	1.673333333	0.00%
control	1.617	1.756	1.589	1.654	0.00%

Table 3: Inhibition of protein denaturation assay

ANTIOXIDANT ACTIVITY BY FRAP ASSAY

Various concentration such as 50 µg/mL,150 µg/mL,200 µg/mL,250 µg/mL showed 56%, 43.87%, 32.96%, 26.22%, 10.76% of antioxidant activity respectively which is represented in figure 4. Antioxidant Activity by FRAP Assay determined that 25 µg/mL concentration of

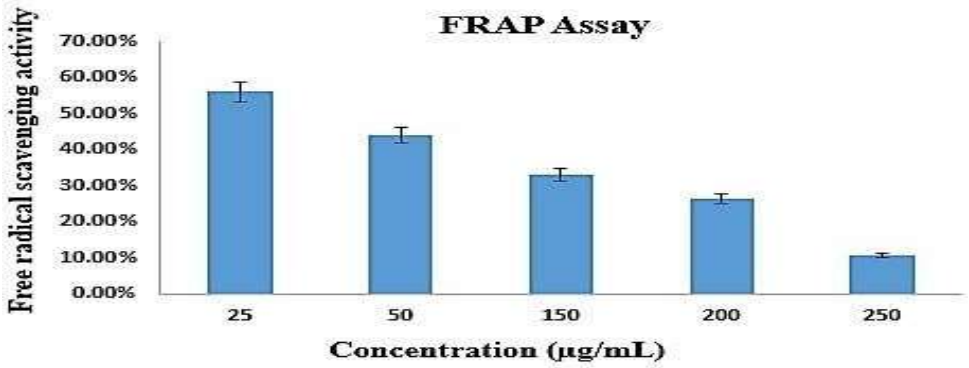

Figure 4. The graph indicates the free radical scavenging activity FRAP

TLCSNPs showed 56% antioxidant capacity which is higher than rest other concentrations (50 µg/mL,150 µg/mL,200 µg/mL,250 µg/mL).

CHARACTERIZATION OF CSNPs

FTIR ANALYSIS

The FTIR spectrum of synthesized chitosan nanoparticles gives information about the functional groups involved in chitosan nanoparticles (figure 5). The following peaks are observed in the chitosan nanoparticle CSNPs (Table 4): 3447.86, 2923.32, 2851.88, 2138.23, 1644.08, 1412.57, 1385.10, 1114.11, 993.00 and 626.07 cm-1 respectively corresponding to C-I Stretching, C-O Stretching, C-N Aliphatic amine, C-H vibration, O-H Bending, C=O Stretching of Amide I bond, C-N Stretching vibration, Aliphatic C-H group, Aliphatic C-H group, N-H Stretch of amines. Analysis of these spectra strongly suggested the presence of flavonoids and phenols, which were mainly responsible for the formation of chitosan nanoparticles.

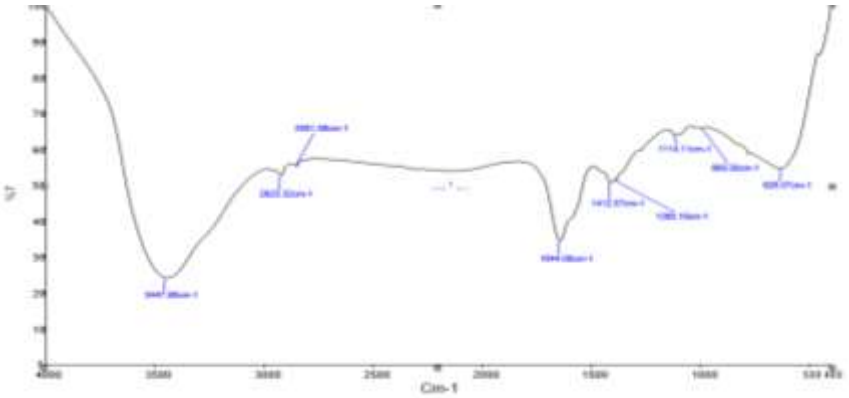

Figure 5. FITR analysis of CSNPs

Functional group	Wavelength (cm-1)
C-I Stretching	626.07
C-O Stretching	993.00
C-N Aliphatic amine	1114.11
C-H Vibration	1385.10
O-H Bending	1412.57
C=O Stretching of Amide I bond	1644.08
C-N Stretching Vibration	2138.23
Aliphatic C-H group	2851.88
Aliphatic C-H group	2923.32
N-H Stretch of amines	3477.86

Table 4: FTIR Spectrum of CSNPs

UV- VIS ANALYSIS

The presence of chitosan nanoparticle was viewed by using UV-V is spectral technique at room temperature. The UV visible absorption spectrum was noted at the range of 823-992 nm.

Sample	No	Peak (nm)	Peak (AU)
CSNPs	1	823.95	0.018
CSNPs	2	932.25	0.014
CSNPs	3	992.00	0.020

Table 5: UV-Vis peak values and absorption

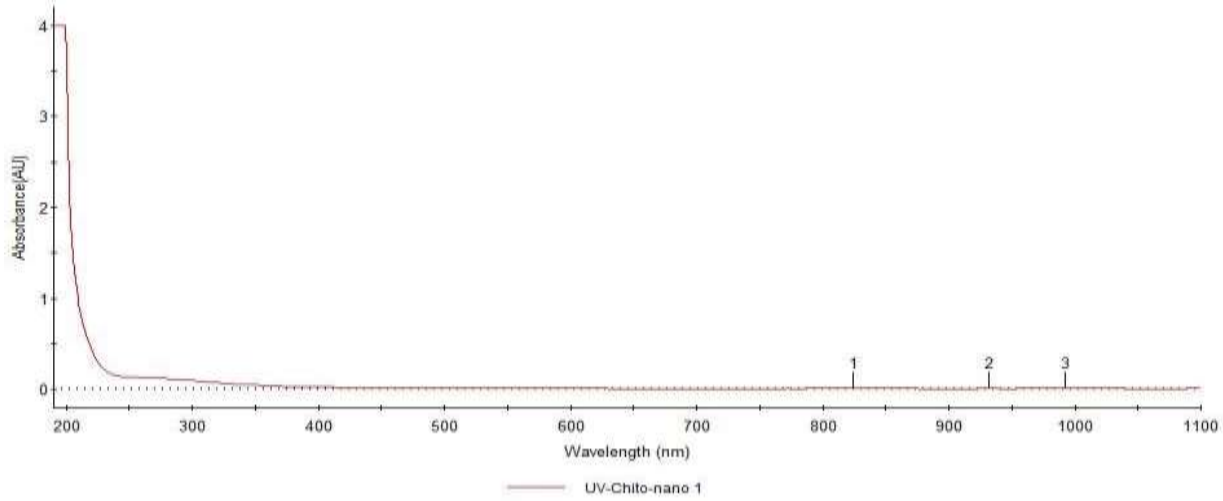

FIGURE 6: UV-Vis Spectrum of synthesized CSNPs

SEM ANALYSIS

SEM image reveals most of the synthesized chitosan nanoparticles were spherical in shape and well dispersed (figure 7). From the SEM image it was concluded that synthesized chitosan nanoparticle synthesized from prawn shells using ionic gelation method were almost uniform in shape and size. SEM image of synthesized chitosan nanoparticles lie between 89.61-121.3 nm (figure 7 and the average size of the nanoparticle is ~ 200nm, whereas the shapes were spherical.

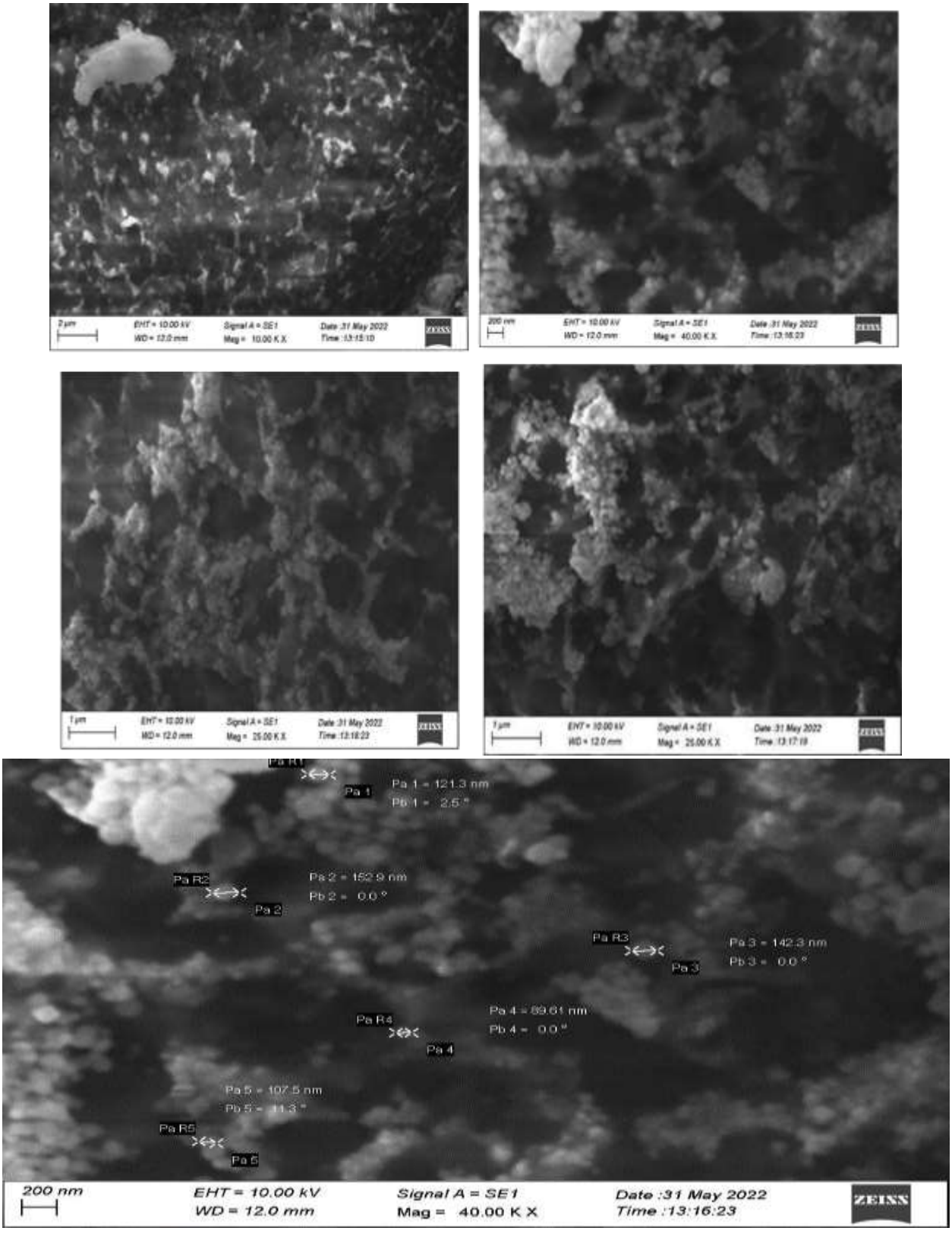

FIGURE 7: SEM images of synthesized CSNPs

XRD ANALYSIS

In X-ray crystallography the crystalline nature of chitosan nano particles was confirmed. The XRD pattern of synthesized chitosan nanoparticles pattern showed broad diffraction peaks at 2-theta range of 19.85, 20.22 which was identified as CSNPs represented in (Figure 8). The lower intensity exhibited by the diffraction peaks of CSNPs revealed that they are amorphous in nature. The ionic interaction between TPP and –NH3+ of chitosan molecules has resulted in the formation of CSNPs. In case of CSNPs, the intensity of diffraction peaks was increased as a consequence of transforming amorphous chitosan into crystallized form after reaction with TPP.

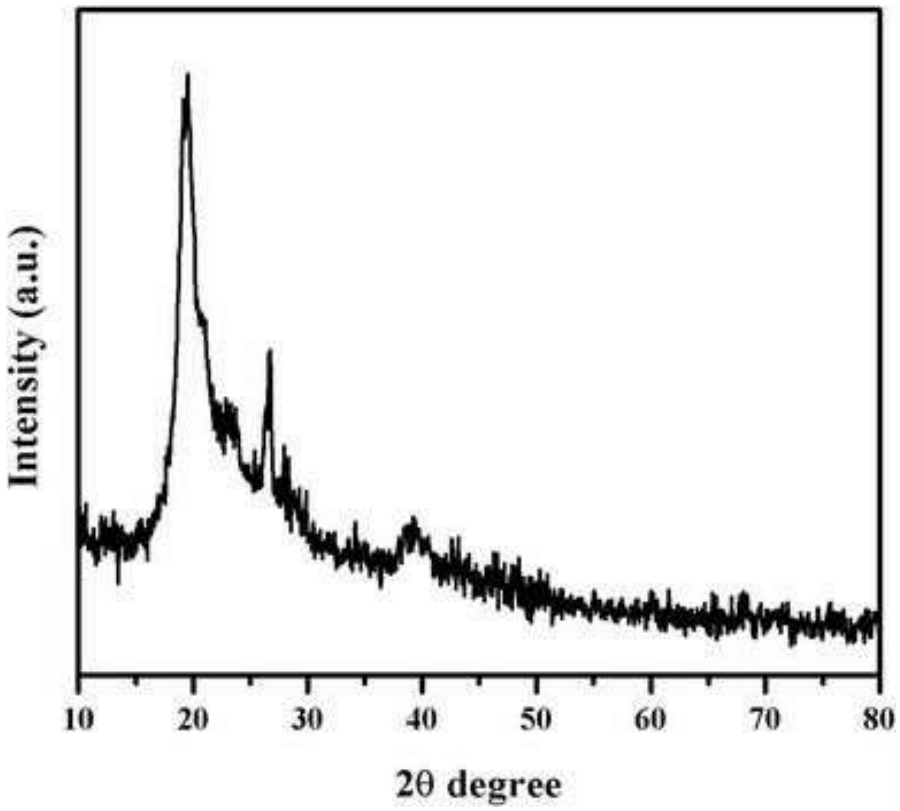

Figure 8: XRD analysis of CSNPs

GCMS ANALYSIS

GCMS is an important tool for the identification of bioactive compounds present in the chitosan obtained from the exoskeleton of crustaceans (Prawn shells) and also used to analyze their retention time, molecular formulae, molecular weight, and structure of the active constituents present in the chitosan (Table 6). So, in the present study, nearly 60 bioactive compounds were identified from the chitosan obtained from prawn shells.

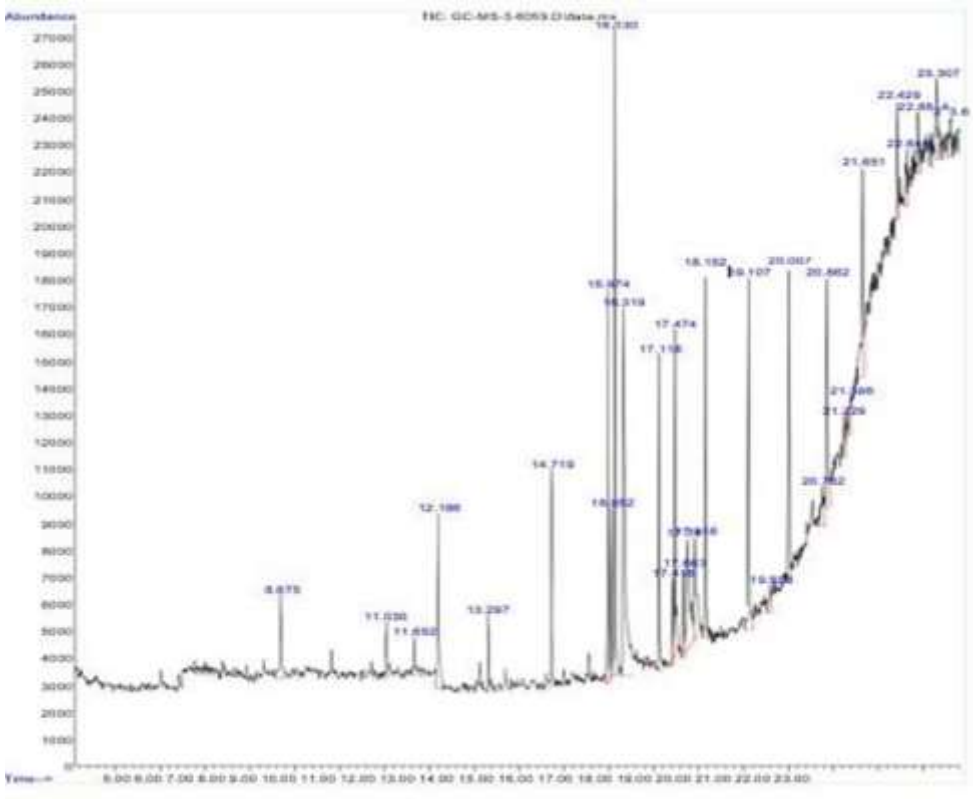

Figure 9: GC-MS Chromatogram of chitosan obtained from prawn shell

Table 6: Bioactive compounds identified in Chitosan obtained from prawn shell

S. No	Peak No	R. Time	Area %	Compound Name
1.	1	8.675	2.00	3-ethyl-2-methylpentane
2.	1	8.675	2.00	1-Iodoundecane
3.	1	8.675	2.00	2,10-Dimethylundecane
4.	2	11.030	1.14	Hexatriacontane
5.	2	11.030	1.14	Hentriacontane
6.	2	11.030	1.14	Octacosane
7.	3	11.652	0.89	Tetradecamethyl cycloheptasiloxane
8.	3	11.652	0.89	Tetradecamethyl cycloheptasiloxane
9.	3	11.652	0.89	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5tris(trimethylsiloxy)tetrasiloxane
10.	4	12.186	3.34	2,4-Di-tert-butylphenol
11.	4	12.186	3.34	2,4-Di-tert-butylphenol
12.	4	12.186	3.34	2,4-Di-tert-butylphenol
13.	5	13.297	1.15	N-Trifluoroacetylprolyl chloride

14.	5	13.297	1.15	Benzene acetic acid, alpha.,3,4-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester
15.	5	13.297	1.15	Benzoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester
16.	6	14.719	3.09	Octadecamethyl cyclononasiloxane
17.	6	14.719	3.09	Octadecamethyl cyclononasiloxane
18.	6	14.719	3.09	Octadecamethyl cyclononasiloxane
19.	7	15.974	6.15	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetra siloxane
20.	7	15.974	6.15	Tetrakis(trimethylsilyloxy)silane
21.	7	15.974	6.15	Dodecamethyl Penta siloxane
22.	8	16.052	2.58	Hexadecenoic acid, methyl ester
23.	8	16.052	2.58	Hexadecenoic acid, methyl ester
24.	8	16.052	2.58	Pentadecanoic acid, methyl ester
25.	9	16.130	8.91	Benzene propanoic acid, 3,5-bis(1,1-dimethylethyl)-4hydroxy-, 1-(2-(3-(3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl)-1-oxopropoxy) ethyl)-2,2,6,6-tetramethyl-4-piperidinyl ester
26.	9	16.130	8.91	Sarcosine, N-(3-phenylpropionyl)-, propyl ester)
27.	9	16.130	8.91	Silane, dimethyl (2,4-dichlorophenoxy)
28.	10	16.319	11.86	n-Hexadecenoic acid
29.	10	16.319	11.86	n-Hexadecenoic acid
30.	10	16.319	11.86	Pentadecanoic acid
31.	11	17.118	4.26	Silane, (pregn-5-ene-3. beta.,11. beta.,17,20. alpha. tetrayltetraoxy) tetrakis[trimethyl]
32.	11	17.118	4.26	Benzoic acid, 2,3-bis[(trimethylsiloxy)-methyl ester
33.	11	17.118	4.26	Cyclodecasiloxane, eicosamethyl
34.	12	17.418	1.28	Methyl linolelaidate
35.	12	17.418	1.28	Methyl linolelaidate
36.	12	17.418	1.28	Methyl 6-cis,9-cis,11-trans-octadecatrienoate
37.	13	17.474	5.23	13-Octadecenoic acid, methyl ester
38.	13	17.474	5.23	12-Octadecenoic acid, methyl ester
39.	13	17.474	5.23	9-Octadecenoic acid, methyl ester
40.	14	17.663	1.47	16-methyl-10-oxo-heptadecanoic acid
41.	14	17.663	1.47	Octadecanoic acid, methyl ester
42.	14	17.663	1.47	Octadecanoic acid, methyl ester
43.	15	17.741	4.91	1,9-Tetradecadiene

44.	15	17.741	4.91	1-Tridecene
45.	15	17.741	4.91	Z-(13,14-Epoxy) tetradec-11-en-1-ol acetate
46.	16	17.918	3.36	Octadecanoic acid
47.	16	17.918	3.36	2,2-Diphenyl-2H-1-benzopyran-6-ol
48.	16	17.918	3.36	Octadecanoic acid
49.	17	18.152	4.96	Octadecamethylcyclononasiloxane
50.	17	18.152	4.96	Tetradecamethyl hexasiloxane
51.	17	18.152	4.96	Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2phenylene]bis(oxy)]bis[trimethyl
52.	18	19.109	5.63	Cyclodecasiloxane, eicosamethyl
53.	18	19.109	5.63	Tetradecamethyl hexasiloxane
54.	18	19.109	5.63	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-
55.	19	19.596	0.77	5-Methyl-2-phenylindolizine
56.	19	19.596	0.77	1,2-Benzenediol, 3,5-di(1,1-dimethylethyl
57.	19	19.569	0.77	Trimethyl[4-(2-methyl-4-oxo-2-pentyl) phenoxy] silane
58.	20	20.007	4.79	Octadecamethyl cyclononasiloxane
59.	20	20.007	4.79	Formic acid, 1-(4,7-dihydro-2-methyl-7-oxopyrazolo[1,5a] pyrimidin-5-yl)-, methyl ester
60.	20	20.007	4.79	N-Benzyl-N-ethyl-p-isopropyl benzamide
61.	21	20.762	1.73	Acetamide, N-[4-[(trimethylsilyl)oxy] phenyl]
62.	21	20.762	1.73	Trimethyl[4-(2-methyl-4-oxo-2-pentyl) phenoxy] silane...
63.	21	20.762	1.73	4-Methyl-2-trimethylsilyloxy-acetophenone
64.	22	20.862	4.63	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15Hexadecamethyloctasiloxane
65.	22	20.862	4.63	1,4-Bis(trimethylsilyl)benzene
66.	22	20.862	4.63	2-Hydroxy-5-(phenylazo)-2,4,6-cycloheptatrien-1-one methacrylate
67.	23	21.229	0.83	4-Nitrophthalhydrazide
68.	23	21.229	0.83	2,4-Dimethylbenzo[h]quinoline
69.	23	21.229	0.83	2-Hydroxy-5-(phenylazo)-2,4,6-cycloheptatrien-1-one methacrylate
70.	24	21.396	1.23	2-Hydroxy-5-(phenylazo)-2,4,6-cycloheptatrien-1-one methacrylate

71.	24	21.396	1.23	Hexamethyl Cyclotrisiloxane
72.	24	21.396	1.23	Hexamethyl Cyclotrisiloxane
73.	25	21.651	5.27	Hexamethyl Cyclotrisiloxane
74.	25	21.651	5.27	1,2-Bis(trimethylsilyl)benzene
75.	25	21.651	5.27	2-Hydroxy-5-(phenylazo)-2,4,6-cycloheptatrien-1-one methacrylate
76.	26	22.429	2.18	Hexamethyl Cyclotrisiloxane
77.	26	22.429	2.18	2,4-Dimethylbenzo[h]quinoline
78.	26	22.429	2.18	Hexamethyl Cyclotrisiloxane
79.	27	22.640	1.44	Hexamethyl Cyclotrisiloxane
80.	27	22.640	1.44	1,2-Benzenediol, 3,5-di (1,1-dimethylethyl
81.	27	22.640	1.44	Bis-(trimethylsilyl)diethyl silicate
82.	28	22.884	1.43	Decamethyltetrasiloxane
83.	28	22.884	1.43	1,2-Bis(trimethylsilyl)benzene
84.	28	22.884	1.43	1,1,1,3,5,5,5-Heptamethyltrisiloxane
85.	29	23.307	2.09	2-methyl-7-phenyl-1H-indole
86.	29	23.307	2.09	Hexamethyl Cyclotrisiloxane
87.	29	23.307	2.09	Hexamethyl Cyclotrisiloxane
88.	30	23.618	1.42	Hexamethyl Cyclotrisiloxane
89.	30	23.618	1.42	Trimethyl-[5-methyl-2-(1-methylcyclohexyl) sulfonyl phenyl] silane
90.	30	23.618	1.42	Decamethyltetrasiloxane

DISCUSSION

The extraction of Chitosan from prawn shells which has many chemical processes such as deproteinization, demineralization, and deacetylation. This results in the synthesis of crude chitosan. In the standardization and purification process of chitosan, Murshida khan et al., described that these process were carried out by trial and error by setting up the parameters like prewashing with different concentration of low acids, extraction with increased concentration of NaOH (Charles et al., 2008), changing with fresh NaOH solution for several times and the extraction of different high temperatures. The method used by Yateendra Shanmukha et al., (2012) which helped out in obtaining the purified chitosan by following the steps such as removal of insoluble with filtration, reprecipitation of chitosan with 1N NaOH and demetallization of retrieved chitosan. During the process of purification to remove the insoluble particles by filtration through Whatman filter paper 22µm and then it was reprecipitated with 1N NaOH at the time of this process DTT were used to produce more consistent and reproducibility for biomedical applications. At basic pH conditions was

maintained while combining the demetallization agent to avoid the chitosan metal conjugates. (Dinesh et al., 2000) The physiochemical parameters reported in Table 1 states that the yield of chitosan is 64% while comparing to the previous study which states the yield of chitosan was 34% which is comparatively lower. The pH was found to be 6.4 and DD was 87%. The chitosan were completely solubilized in Acetic acid. The previous study by Paul et al., 2014 showed that the yield of the chitosan is 57.69%, pH was 6.7, and DD was found. TLCSNPs were prepared by ionic gelation method which was then subjected to Anti – Inflammatory Activity. (Koilparambil et al., 2017) Inhibition of hemolysis assay, inhibition of protein denaturation assay, and Antioxidant activity was performed. The previous study stated that the anti-inflammatory response of 500 mg/kg of the tulsi paste was found to be 88.15% as that of the response observed with 100 mg/kg of indomethacin (Singh and Jaggi 2003). The fresh tulsi leaf in its paste form also shows considerable anti-inflammatory activity in comparison to Indomethacin, with minimal side effects. Inhibition of protein denaturation assay various concentrations such as 50 µg/mL,100 µg/mL,150 µg/mL,200 µg/mL,250 µg/mL of TLCSNPs were taken for the study, in that 50µg/mL concentration of TLCSNPs showed 59.27% of activity which is higher than other concentrations. Antioxidant Activity by FRAP Assay determined that 25 µg/mL concentration of TLCSNPs showed 56% antioxidant capacity which is higher than rest other concentrations (50 µg/mL,150 µg/mL,200 µg/mL,250 µg/mL). The FTIR spectrum of synthesized chitosan nanoparticles gives information about the functional group involved in the CSNPs (Figure 5). The following peaks are observed in the chitosan nanoparticle CSNPs (Table 4): 3447.86 cm-1 corresponding to C-I Stretching, 2923.32 cm-1 corresponding to C-O Stretching, 2851.88 cm-1 corresponding to C-N Aliphatic amine, 2138.23 cm1 corresponding to C-H vibration, 1644.08 cm-1 corresponding to O-H Bending, 1412.57 cm-1 corresponding to C=O Stretching of Amide I bond, 1385.10 cm-1 corresponding to C-N Stretching vibration, 1114.11 cm-1 corresponding to Aliphatic C-H group, 993.00 cm-1 corresponding to Aliphatic C-H group and 626.07 cm-1 corresponding to N-H Stretch of amines. Analysis of these spectra strongly suggested the presence of flavonoids and phenols, which were mainly responsible for the formation of chitosan nanoparticles. The previous studies showed the FTIR spectrum of chitosan nanoparticles with the following peaks 3440. 16, 1647. 26, 1399. 4, 1124. 54, 1399. 40, 906. 57 and 1007. 24 cm-1 respectively representing O-H stretching of alcoholic and phenolic groups; N-H bending vibrations; C-H bending in a ring, CN stretching, C-C stretching (in ring), N-H wagging of primary and secondary amines; and C-O stretching of alcohols and carboxylic acids. These functional groups were expected as they are the major components of chitosan (Ayodele et al., 2018). In X-ray crystallography, the crystalline nature of chitosan nanoparticles was confirmed. The XRD pattern of synthesized chitosan nanoparticles showed broad diffraction peaks at 2-theta range of 19.85, 20.22 which was identified as CSNPs represented in (Figure 8). The lower intensity exhibited by the diffraction peaks of CS NPs revealed that they are amorphous in nature. The ionic interaction between TPP and –NH3+ of chitosan molecules has resulted in

the formation of CS NPs. In case of CS NPs, the intensity of diffraction peaks was increased as a consequence of transforming amorphous chitosan into crystallized form after reaction with TPP. (Zeng, Zhaowu 2011). X-ray diffractogram of the chitosan nanoparticles is shown first two major peaks appeared at 2Θ = 17° and 24°. This presents a shift of the peaks for normal chitosan (2Θ = 10° and 20°), as reported and also reported that XRD of chitosan nanoparticles showed two peaks at 2Θ = 17o and 25°. The crystallinity of chitosan is a key parameter affecting accessibility to internal sites for water or metal ions (Jaworski et al., 2003). There is however a broad band between 2Θ = 50° and 90° in the spectrum, connoting amorphous parts of the chitosan nanoparticles. The presence of chitosan nanoparticles was viewed by using UV-Vis spectral technique at room temperature. The UV visible absorption spectrum was noted at the range of 823.95 nm, 932.25 nm, 992.00 nm with peak absorption 0.018 AU, 0.014 AU, 0.020 AU respectively. SEM image reveals most of the synthesized chitosan nanoparticles were spherical in shape and well dispersed (Figure 7). From the SEM image it was concluded that synthesized chitosan nanoparticles synthesized from prawn shells using ionic gelation method were almost uniform in shape and size. The SEM image of a synthesized chitosan nanoparticle lies between 89.61-121.3 nm (Figure 7) and the average size of the nanoparticle is ~ 200nm, whereas the shapes were spherical. The previous studies reported that the SEM analysis showed the synthesized chitosan nanoparticles micrographs of chitosan nanoparticles. (Thakur et al., 2009) The SEM image presents chitosan nanoparticles aggregated to form a solid lump with a coarse surface. The coarse nature of the chitosan surface depends on the degree of deacetylation. The graph depicts very smooth and fine surfaces, as observed in Figure 7 The fine surfaces could be as a result of the addition of tripolyphosphate (TPP) to the chitosan solution, which converted the chitosan to nanoscale particles

CONCLUSION:

CSNPs are promising and effective for drug carriers, which enhance the activity of the drug rapidly and thus result in the welfare of the public recovering speedily from inflammation. *Ocimumtenuiflorum L.* leaf extract loaded with chitosan nanoparticles as a drug carrier showed anti- inflammatory activity. The CSNPs as the drug carrier that exhibits the anti-inflammatory activity showed in the study. To conclude the result of this study *Ocimum tenuiflorum L.* leaf extract loaded with chitosan nanoparticles which has potential anti-inflammatory activities and antioxidant activity. Normally, tulsi is widely used in curing various disease and when combined with it CSNPs(TLCSNPs) is effective against inflammation. Furthermore, more research is required for discovering and purifying the chemicals that are responsible for therapeutical efficacy.

REFERENCE

1. Agarwal M, Agarwal MK, Shrivastav N, et al. (2018: Preparation of chitosan nanoparticles and their in-vitro characterization. Int J Life Sci Scienti Res.; 4(2): 1713–1720

2. Olajide Ayodele*, Afamefuna Elvis Okoronkwo, Olugbenga Oludayo Oluwasina, and Taiwo Olusola Abe (2018) Utilization of blue crab shells for the synthesis of chitosan nanoparticles and their characterization, Songklanakarin J. Sci. Technol. 40 (5), 10431047, Sep. - Oct. 2018
3. Ali, Akbar; Ahmed, Shakeel (2017). *A Review on Chitosan and its Nanocomposites in Drug Delivery. International Journal of Biological Macromolecules, (), S0141813017338928–*. doi:10.1016/j.ijbiomac.2017.12.078
4. Gomes LP, Paschoalin VMF, Del Aguila EM (2017) : Chitosan nanoparticles: production, physicochemical characteristics and nutraceutical applications. Rev Virtual Quim. 2017; 9(1): 387–409.
5. Gunathilake, K.; Ranaweera, K.; Rupasinghe, H. (2018). In Vitro Anti-Inflammatory Properties of Selected Green Leafy Vegetables. Biomedicines, 6(4), 107 doi:10.3390/biomedicines6040107
6. Sandip I. Vidhani1 , Vijay G. Vyas1 , Heena J. Parmar1 , Viren M. Bhalani1 , Mohammad M. Hassan2,* , Ahmed Gaber2 , Baljibhai A. Golakiya1 Evaluation of Some Chemical Composition, Minerals Fatty Acid Profiles, antioxidant and Antimicrobial Activities of Tulsi (Ocimum sanctum) from India, American Journal of Food Science and Technology, 2016, Vol. 4, No. 2, 52-57
7. Yateendra Shanmukha Puvvada , Saikishore Vankayalapati , Sudheshnababu Sukhavasi(2012) Extraction of chitin from chitosan from exoskeleton of shrimp for application in the pharmaceutical industry, International Current Pharmaceutical Journal 2012, 1(9): 258263.
8. Trang Si Trung; Wah Wah Thein-Han; Nguyen Thi Qui; Chuen-How Ng; Willem F. Stevens (2006). Functional characteristics of shrimp chitosan and its membranes as affected by the degree of deacetylation. , 97(4), 659–663.
9. Paul, S., A. Jayan, C. S. Sasikumar, and S. M. Cherian (2014). "Extraction and Purification of Chitosan from Chitin Isolated from Sea Prawn (Fenneropenaeus Indicus)". Asian Journal of Pharmaceutical and Clinical Research, vol. 7, no. 4, Sept. 2014, pp. 201-4,
10. Islam, SZ; Khan, M; Alam, AKM Nowsad (2017). Production of chitin and chitosan from shrimp shell wastes. Journal of the Bangladesh Agricultural University, 14(2), 253– . doi:10.3329/jbau.v14i2.32701
11. Bangun H, Tandiono S, Arianto A., (2018) Preparation and evaluation of chitosan–tripolyphosphate nanoparticles suspension as an antibacterial agent. J App Pharm Sci,; 8(12): 147–156.
12. Azab, Abdullatif; Nassar, Ahmad; Azab, Abed (2016). Anti-Inflammatory Activity of Natural Products. Molecules, 21(10), 1321–. doi:10.3390/molecules21101321
13. Bobbarala, Varaprasad (2015). Concepts, Compounds and the Alternatives of Antibacterials || Chitosan as a Biomaterial — Structure, Properties, and

Electrospun Nanofibers., 10.5772/59522(Chapter 4), –. doi:10.5772/61300

14. Sudharsan, P., Siva, D., Prabhu, K., & Janani, C. (2022). The Influence of Portulaca Oleracea L. Leaves Extracts on the Histoarchitecture of Culex Quinquefasciatus and Anopheles Stephensi Larvae. Clinical Complementary Medicine and Pharmacology, 2(3), 100053.
15. Prabhu, K., Sudharsan, P., Kumar, P. G., Chitra, B., & Janani, C. (2022). Impact of Piper betle L. bioactive compounds in larvicidal activity against Culex quinquefasciatus. Journal of Natural Pesticide Research, 2, 100013.
16. Charles O Okoli; Peter A Akah; Nkemjika J Onuoha; Theophine C Okoye; Anthonia C Nwoye; Chukwuemeka S Nworu (2008). Acanthus montanus: An experimental evaluation of the antimicrobial, anti-inflammatory and immunological properties of a traditional remedy for furuncles. , 8(1), 27–0. doi:10.1186/1472-6882-8-27
17. De Queiroz Antonino RSCM, Lia Fook BRP, de Oliveira Lima VA, de Farias Rached RÍ, Lima EPN, da Silva Lima RJ, Peniche Covas CA, Lia Fook MV (2017), Preparation and Characterization of Chitosan Obtained from Shells of Shrimp (Litopenaeus vannamei Boone). Mar Drugs. 2017 May 15;15(5):141. doi: 10.3390/md15050141. PMID: 28505132; PMCID: PMC5450547.
18. Singh B. and R.K Jaggi K.R (2003) Anti-inflammatory Effect Of Ocimum Sanctum Linn And Its Cultures. Indian J Pharmaceutical Sci. 65:425-428.
19. Dinesh K. Singh & Alok R. Ray (2000) Biomedical Applications of Chitin, Chitosan, and Their Derivatives, Journal of Macromolecular Science, Part C: Polymer Reviews, 40:1, 69-89
20. H.L. Kalabharathi, R.N. Suresha, B. Pragathi, Pushpa Vh, (2011) Anti-inflammatory activity of fresh tulsi leaves (Ocimum Sanctum) in albino rats October 2011International Journal of Pharma and Bio Sciences 2(4):45-50
21. Gu, Z., Xie, H., Huang, C., Li, L., & Yu, X. (2013). Preparation of chitosan/silk fibroin blending membrane fixed with alginate dialdehyde for wound dressing. International Journal of Biological Macromolecules, 58, 121–126. doi:10.1016/j.ijbiomac.2013.03.05
22. Thakur K. and K.S. Pitre S.K.(2009). AntiInflammatory activity of extracted eugenol from Ocimum sanctum L. Leaves. Rasayana J. Chem.2: 472-474.
23. Koilparambil, Divya & Shanavas, Jisha. (2017). Chitosan nanoparticles preparation and applications. Environmental Chemistry Letters. 16. 10.1007/s10311-017-0670-y.
24. Rampino, Antonio; Borgogna, Massimiliano; Blasi, Paolo; Bellich, Barbara; Cesàro, Attilio (2013). Chitosan nanoparticles: Preparation, size evolution and stability. International Journal of Pharmaceutics, 455(1-2), 219–228. doi:10.1016/j.ijpharm.2013.07.034
25. Othman, Nurhanisah; Masarudin, Mas; Kuen, Cha; Dasuan, Nurul; Abdullah,

Luqman; Md. Jamil, Siti (2018). Synthesis and Optimization of Chitosan Nanoparticles Loaded with L-Ascorbic Acid and Thymoquinone. Nanomaterials, 8(11), 920–. doi:10.3390/nano8110920

26. Zeng, Zhaowu (2011). Recent advances of chitosan nanoparticles as drug carriers. International Journal of Nanomedicine, (), 765–. doi:10.2147/IJN.S17296