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Synthesis and Characterization of Chitosan Linked Nanopolymer Using *Rosmarinus Officinalis* Leaf Extract

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ABSTRACT

Nanotechnology has numerous applications in science and technology. It has useful applications in medicine and the environment. Physical, chemical, and biological processes are used to create nanomaterials. Green synthesis of nanoparticles is a cutting-edge method that is simple to implement and typically results in the development of more stable materials. Chitosan is a biodegradable, biocompatible, and non-toxic linear polysaccharide produced by de-acetylation of chitin with numerous applications in the medical field. *Rosmarinus officinalis,* commonly known as Rosemary is proved to possess various beneficial anti-properties due to the presence of various bioactive compounds. In our study we have synthesized chitosan nanoparticles using Rosmarinus officinalis leaf extract by the ionic gelation technique. The synthesized nanopolymer was characterized using UV spectrophotometer and FTIR. The FTIR spectra of Rosemary leaf extract derived chitosan nanoparticles revealed two major absorption peaks at 3381 cm^{-1} and 1666 cm^{-1} that represented O-H bond stretching and C=C stretch respectively.

KEY WORDS: Chitosan, *Rosmarinus officinalis*, FTIR, Nanoparticles, Ultra-Visible spectrophotometer.

1. INTRODUCTON

Nanobiotechnology is the application of nanotechnology in biological fields. Nanotechnology is a multidisciplinary field that utilises both conventional and advanced engineering, physics, chemistry, and biology technologies and facilities (Fakruddin, 2012). Nanotechnology-based tools are heading to emerge as promising platforms for quantitative and accurate analysis of live cells and tissues (Kim, 2013). Green synthesis is a new area of bio-nanotechnology that, environment friendly ‘green' processes in chemistry and chemical technologies are becoming more popular (Thuesombat, 2014). Chitosan is a polycation which is soluble in acidic medium (pKa 6.5) in which the negatively charged TPP interacts with the positively charged groups of amino acids of Chitosan which forms inter or intramolecular structure (Dash, 2011). It is famous for anti-properties like antimicrobial, antioxidant, and chelating effects, as well as its nontoxic and biocompatible existence (Gedda, 2016). Nanoparticles (NP) made from chitosan and chitosan derivatives have a positive surface charge and mucoadhesive properties, which allow them to adhere to mucus membranes and slowly release the drug payload (Mohammed, 2017).

Hydrophilic medications, proteins and peptides, nucleic acids and polysaccharides are dangerous because of their low permeability through the nasal epithelium. Chitosan is biodegradable, biocompatible, low-toxic, adheres to mucus, and opens nasal membranous junctions (Casettari and Illum, 2014). For example, the bioavailability of leuprolide, which is used to treat prostate cancer and hormone-dependent diseases, was found to be increased when it was formulated as thiolated-chitosan NPs (Illum, 2003).

Mucoadhesive nanoparticles/microparticles will cling to mucus membranes (of the vaginal, urethral, or pulmonary sites) and slowly release the medication. Chitosan's mucoadhesive property is due to its high positive charge, which aids in the formation of a bond with negatively charged mucus, since it is mucoadhesive, permeation enhancer, and creates a protective shield for the medication, chitosan is an excellent carrier for many drugs. (George and Abaham, 2006). The small particle size of chitosan also enhances the penetration of the drug through the mucus layer.

Rosmarinus officinalis, more commonly known as rosemary, is a Mediterranean herb that belongs to the Lamiaceae family of mints. It might, however, be found all over the world. It's an aromatic annual with shrub-like branches brimming with leaves. *R. officinalis* is used in cooking as a spice, as a natural preservative in the food industry, and as an ornamental and medicinal plant (Gonzalez-Trujano, 2007; Perez-Fons, 2010; Brewer, 2011; Raskovic, 2014).

The present investigation aims at the synthesis and characterization of Chitosan linked nanopolymer using *Rosmarinus officinalis* leaf extract.

2. MATERIALS AND METHODS

Chemicals: Ethanol, $FeCl_3$, NaOH and HCL, glacial acetic acid, Chloroform, H_2SO_4, Dragendroff’s reagent, Potassium acetate, $AlCl_3$, obtained from Fischer Scientific. Quercetin, Chitosan, TPP were analytical grade from HiMedia, Karnataka.

Plant source: The Fresh Rose Mary (Rosmarinus officinalis) plant was collected from Lal Bagh Botanical garden at Kaval Bysandra, Bangalore through random selection. Rosemary plant called as 'Gulmehendi' in India was selected for our project as it is grown and available in multiple varieties all over the world, easily available and easily harvested.

Preparation of plant extract: The plant extracts were prepared from the roots, stems and leaves of Rose Mary (Rosmarinus officinalis) plant. The plant was washed properly under tap water to remove the dust particles and other impurities from the plant. These separated parts of the plant were then oven dried overnight at 60° and it was later made into a coarse powder. A 5% aqueous and ethanolic (50% ethanol) extracts of the root, stem and leaves of the Rosemary plant was prepared. The weighed amount of the coarse powder was mixed with the solvent system and placed on a magnetic stirrer for 30 minutes. After which the solution was centrifuged at 8,000 rpm for 18 mins at 25°C. The supernatant was then separately collected and used for further experimental analysis.

Phytochemical analysis: Test for Alkaloids, Flavonoids, Tannins, Saponins, terpenoids, cardiac glycosides and phenols tested using known volume of extract was carried out allowing it to react with specific reagents as described by the standard protocol (Harborne JB, 1991; Khandelwal KR, 2009).

Quantification of flavonoids: The amount of flavonoids present in the plant sample was estimated using the protocol by Jia Zhishen (1999). In this assay, 0.2 to 1 ml aliquots of standard Quercetin (100µg/ml) solution were added in test tubes except in the blank tube. 0.1 ml of the plant extracts were also pipetted into different test tubes. The volume in the tubes were made to 2 ml using methanol. 0.1 ml of 10% $AlCl_3$ was added in each tube followed by 0.1 ml of 1M Potassium acetate (CH_3CO_2K). 2.8 ml of distilled water was added into all the tubes so that the total volume of contents in each tube would finally be 5ml. The tubes were incubated for 30 mins at room temperature. The absorbance was read at 450nm using colorimeter against a suitable blank.

Identification of the potent molecule by high performance liquid chromatography: The sample was further characterized for alkaloids. The analysis was made on C18 column (symmetry, 4.6mm X 250mm) in isocratic mode with the mobile phase acetonitrile (COUTO, 2011) and water in the ratio 7:3 with the RP-HPLC C-18 column at a flow rate of 1mL/min and 20µL of the sample was injected and the elution was monitored at 230nm.

Synthesis of chitosan nanopolymer: The ionic –gelation method was employed for the synthesis of chitosan nanoparticle using sodium tripolyphosphate (TPP) as a cross linking agent. About 1%(w/v) of the plant extract was mixed with 0.5%(w/v) of TPP and the solution was added drop wise into the chitosan solution containing 0.5%(w/v) chitosan and 1%(v/v) acetic acid under gentle magnetic stiring of REMI 2-MLH.The solution was incubated for 20 mins and used for characterization (Sujima Anbu, 2016).

Characterization of nanopolymers:

Ultra-Visible spectrophotometer: The chitosan and the target compound interaction were monitored by measuring the UV –Vis spectrum of the nano-polymer suspension .The absorbance spectrum of the nano-polymer suspension was recorded immediately after the synthesis and a reference of de-ionized water was recorded before the actual sample analysis. To check the stability of nano-polymer, the absorption spectrum was recorded for pure chitosan, Rosemary extract, TPP, TPP –Rosemary extract, Chitosan –TPP, Chitosan-Rosemary extract and Chitosan-TPP-Rosemary extract .The spectrophotometer used was UV Scan 2600 (Thermo Fisher) and the software was spectrum TM version 6.87. Absorbance spectra were recorded over the range of 220-400nm. Wavelength of peak absorbance and λ max was calculated.

FTIR: FTIR spectral analysis: The biomolecules stacked chitosan nanoparticles were freeze dried and the powdered test was used for FTIR spectroscopy studies. The FTIR examination of chitosan nanoparticles sample was performed with a technologies portable attenuated total reflectance (ATR) Fourier transform infrared spectroscopy (ATR-FTIR). Sample spectra were recorded within the range of 4000 cm-1 to 400 cm-1 with a resolution of 4cm in the absorbance mode for 10 scans at room temperature. FTIR spectra of chitosan nanoparticles were obtained by placing 1mg of test on the sensor of the instrument and spectrum was then compared with the spectrum of Chitosan standard.

3. RESULTS AND DISCUSSION

In this current study, Rosemary and its plant parts were studied. It has wide range of phytochemicals and possess enormous pharmacological uses, ethnobotanical uses and other miscellaneous uses. The plant is easily available in the market. Rosemary plant have enormous benefits in medical field. Thus, chitosan linked nanopolymer will be more effective than its original form.

Phytochemical Screening:

Qualitative Analysis: The Aqueous and ethanolic extracts of Rosemary were screened for phytochemicals. It was performed according to Harborne JB, 1991; Khandelwal KR, 2009 which determined the presence of various constituents like flavonoids, tannins, saponins, terpenoids, cardiac glycosides, alkaloids and phenols. The result of phytochemical screening of aqueous and ethanolic extracts has been tabulated in Table.1. As illustrated, Rosemary leaf and root indicated the presence of higher quantity of alkaloid in both aqueous and ethanolic extract,

furthermore Rosemary leaf showed the presence of higher quantity of flavonoids in both aqueous and ethanolic extract, but Rosemary root proved the presence of higher quantity of flavonoids in aqueous extract. Thus, the study on phytochemical screening of Rosemary plant extract was correlated with the qualitative analysis of Salvia officinalis. Mekhaldi Abdelkader (2014), revealed that the Salvia officinalis extract indicated the presence of tannins, flavonoids, and terpenoids whereas in the Rosemary plant extract tannins, flavonoids, terpenoids, alkaloids, and saponins were detected.

Table.1. Qualitative analysis of phytochemical in the aqueous extract and ethanolic extract of Rosemary Plant

Phytochemicals	**Stem**		**Root**		**Leaf**	
	Aqueous	Ethanolic	Aqueous	Ethanolic	Aqueous	Ethanolic
Tannins	-	+	+	+	+	+
Saponins	+	+	+	+	+	+
Flavonoids	+	+	++	+	++	++
Terpenoids	+	+	+	+	+	+
Cardiac glycosides	-	-	-	-	+	+
Alkaloids	+	+	++	++	+++	++
Phenols	-	-	+	+	+	+

Note: '+' indicated a positive result and '-' indicated a negative result (What about ++ and +++)

Quantification of flavonoids: As illustrated in Figure.1, the total flavonoids content of ethanolic extract of Rosemary root (0.1689g) and leaf (0.1829g) was higher than the aqueous extract of root (0.1161g) and leaf (0.1508), but in aqueous system, the stem (0.0528g) content was higher than the ethanolic extract (0.0290).

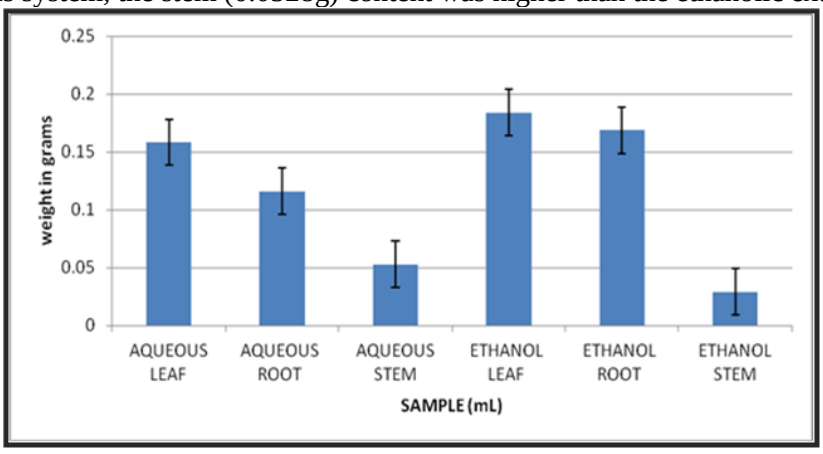

Figure.1. Estimation of flavonoids in plant extract

Purification by HPLC: High performance liquid chromatography was performed to identify the alkaloids present in Rosemary leaf. Partially purified samples were subjected to HPLC for further purification and identification. The sample showed 6 peaks, in which one major peak was 2.8000 and 5 minor peaks at 0.1500, 3.5000, 4.2833, 4.6500, 6.4333. With previous studies it was found that codeine was found to be present at 2.8 minutes, Caffeine showed a peak at a retention time of 3.5 minutes, oxymatrine was found to be present in Corydalis yanhusuo showing a peak at a retention time of 2.8 minutes and harmaline was found to be in Passion fruit showed a peak at a retention time of 6.6 minutes.

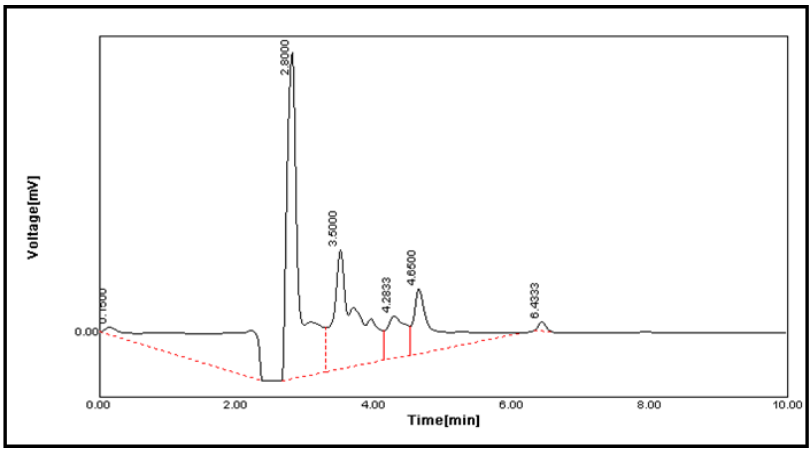

Figure.2. Chromatogram of the purification of ethanolic extract of Rosemary leaf by HPLC

Table.2. HPLC profile of purified ethanolic leaf extract of Rosmarinus officinalis

No.	RT [min]	Area [mV*s]	Height [mV]
1	0.1500	682.9336	9.8608
2	2.8000	846.5366	67.6208
3	3.5000	580.7429	24.7682
4	4.2833	166.3126	8.7567
5	4.6500	316.5809	13.3570
6	6.4333	13.7282	1.8331
Sum		2606.8352	126.1966

Characterisation by Nanoploymers:

UV Spectrophotometer analysis: The preliminary characterisation of the prepared Chitosan-Rosemary nanopolymer was determined in a UV-Vis spectrophotometer. The maximum absorbance of the Chitosan-Rosemary nanopolymer was found to be at 0.587 O.D at 240 nm. As revealed by Vaezifer S et al (2013), at 220-322nm chitosan nanoparticles showed broad absorption bands in UV-Vis spectrophotometer analysis. An increase in optical density of chitosan- Rosemary nanopolymer was observed on comparing it with the OD of pure Chitosan. This revealed the formation of chitosan plant nanopolymer in the preliminary aspect. This increase in the OD of the chitosan-Rosemary nanopolymer was due to bonding of the pure chitosan with the Rosemary plant extract.

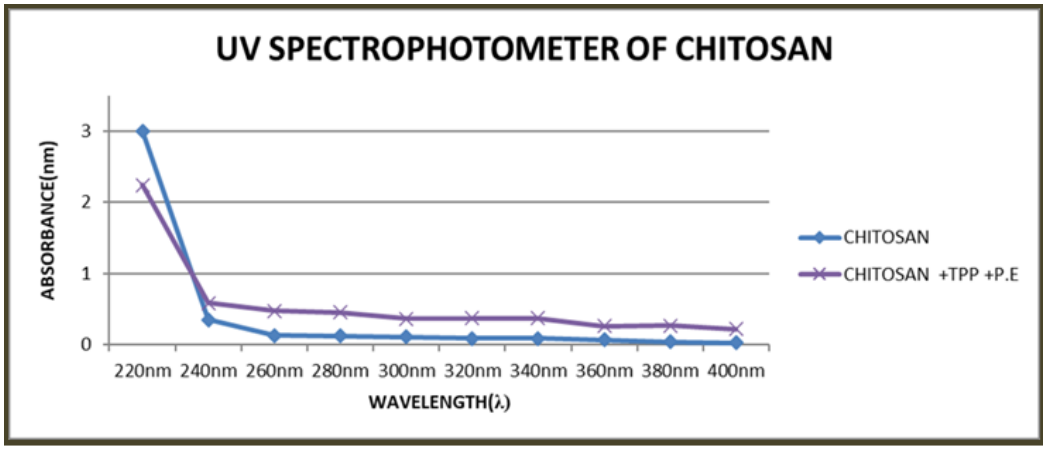

Figure.3. UV-Vis spectrum of pure chitosan solution; Solution of 0.5% chitosan+ TPP+ P.E.

FTIR Analysis: The capability of the ionic gelation process to form biomolecules loaded chitosan nanoparticles was assessed by FTIR for the determination of plant-chitosan interactions. The spectral analysis of chitosan nanoparticles and bioactive molecules loaded chitosan nanoparticles are depicted in the figure 4(a) and figure 4(b). The IR studies of chitosan nanoparticles (CNPs) revealed the presence of two major peaks at 3333 $cm^{-1}$and 1637 cm^{-1} while the FTIR spectra of Rosemary leaf extract derived chitosan nanoparticles revealed two major absorption peaks at 3381 cm^{-1} and 1666 cm^{-1}. The spectra observed at 3381 cm^{-1} for the synthesized nanoparticle indicated the presence of O-H bonds. The strong band noticed at 1666 cm^{-1} indicated the presence of phosphorous groups confirming TPP with ammonium group of chitosan. The isolated alkene groups of the C=C stretch was found at 1666 cm^{-1} for the biomolecules loaded chitosan nanoparticle (AbdElhady, 2012).

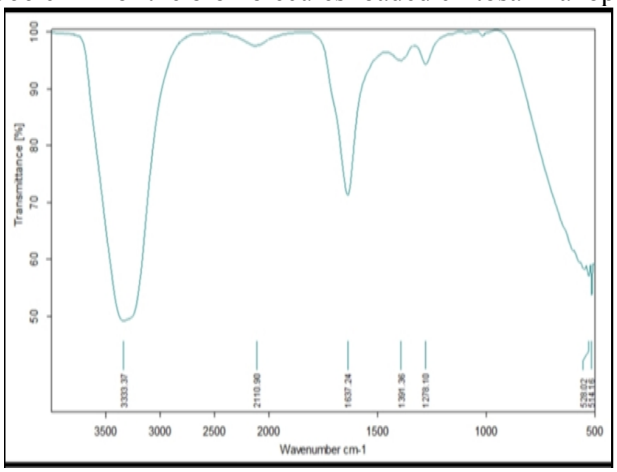

Figure.4(a). FTIR spectral analysis of chitosan nanoparticles

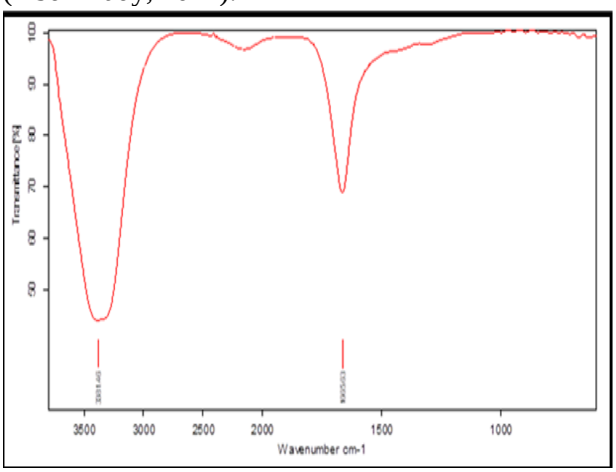

Figure.4(b). FTIR analysis of Rosemary leaf extract cross linked chitosan nanopolymer

4. CONCLUSION

Nanotechnology, in general, is concerned with structures with at least one dimension varying from 1 to 100 nanometers, and it involves modifying or producing materials of that small size. As a result, the material becomes much lighter, stronger, faster, smaller, and more durable. The presence of tannins, flavonoids, terpenoids, alkaloids, and saponins in the Rosmarinus officinalis plant was discovered via phytochemical screening in this research. The presence of alkaloid in both the extracts was higher in rosemary leaf and root, and the detection of flavonoids in both aqueous and ethanolic extracts was higher in rosemary leaf. However, Rosemary root had a higher concentration of flavonoids in its aqueous extract, which was confirmed using thin layer chromatography profiling. Following that, the Folin ciocalteau colorimetric approach was used to quantify flavonoids in root, stem, and leaf extracts, with Quercetin as the standard. The total flavonoids content of ethanolic extract of Rosemary root (0.1689g) and leaf (0.1829g) was higher than the aqueous extract of root (0.1161g) and leaf (0.1508), but in aqueous the stem (0.0528g) content was higher than the ethanolic extract (0.0290). Later, alkaloid were purified using HPLC. In samples chromatogram, 6 peaks were observed in which one major peak was 2.8000 min and 5 minor peaks at 0.1500 min, 3.5000 min, 4.2833 min, 4.6500 min, 6.4333 min. This proved alkaloid were present in the ethanolic extracts of Rosemary leaves.

Chitosan- Rosemary nanopolymer of leaf (NPL) was synthesized which was later characterised using UV Spectrophotometer and FTIR. UV Spectrophotometer spectrum showed the highest absorbance value of 0.587 O.D at 240 nm. This was later confirmed by FTIR analysis.

5. ACKNOWLEDGEMENT

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