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SYNTHESIS, CHARACTERIZATION AND *IN-VITRO* ANTICANCER SCREENING OF *VITIS VINIFERA* SEED ETHANOLIC EXTRACT LOADED CHITOSAN NANOPARTICLES IN H-29 COLON CANCER CELL LINE

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ABSTRACT: Nanotechnology can be applied to deliver and protect antioxidants in order to control the oxidative stress phenomena in several chronic pathologies. Chitosan (CS) and its carboxymethyl derivatives are smart biopolymers that are non-toxic, biocompatible, and biodegradable, and, hence, suitable for various biomedical applications, such as drug delivery gene therapy and tissue engineering. *Vitis vinifera* (VV) seeds are the major chemotherapeutic agent with antioxidant, anti-inflammatory, antiproliferative, anticancer, and antimicrobial effects. However, the potential of these leaves as a chemotherapeutic agent is limited by their hydrophobicity and poor bioavailability. The chitosan tripolyphosphate (CS/TPP) nanoparticles have been used in this study as an alternative to encapsulate bioactive flavonoids from ethanolic extract of *Vitis vinifera* seeds. The Vitis *vinifera seeds* extract-loaded CS (CS-VVE) nanoparticles were characterized using FT-IR. The Zeta sizer and zeta potential confirmed that the particle has 216nm and 50.3mV. This study also found that the CS-VVE NPs shown anticancer activity in human colon cancer cell lines should be explored further for Cytotoxicity studies using MTT assay and tested drug-induced apoptotic process in the cell lines confirmed by the Dual staining. The CS-VVE nanoparticles showed specific toxicity towards cancer cells and non-toxicity to normal cells. It is suggested that the chitosan nanoparticles fabricated in our study may provide a suitable alternative to traditional adjuvant systems. Hence, it was observed from the present investigation that the anticancer activity of *Vitis vinifera* seed extract loaded chitosan nanoparticles provided a platform for treating cancer with biopolymer nanomaterials.

INTRODUCTION: In Indian systems of medicine, a large number of drugs of either herbal or mineral origin have been implicated in various diseases and other pathological conditions in humans. The importance of medicinal plants in drug development is known to us, and humans have used them for different diseases from the beginning of human history [1].

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The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed, and about 80% of the world's population relies on herbal medicines [2].

Plants contain a wide range of chemical compounds that can be used to treat chronic as well as infectious diseases [3]. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower, seed, *etc*. Some drugs are prepared from excretory plant products such as gum, resins, and latex. Even the Allopathic system of medicine has adopted a number of plant-derived drugs from an important segment of modern Pharmacopeia.

Some important chemical intermediates need for manufacturing modern drugs are also obtained from plants.

Cancer of the large intestine (colon) is one of the main causes of death due to cancer. While the numbers for colon cancer are somewhat equal in women (47,820) and men (47,700), it will be diagnosed in (16,190) men (23,720) more than women. Multiple factors are involved in the development of colorectal cancer, such as lack of physical activity [4], excessive alcohol consumption [5], old age [6], family history [7], high-fat diets with no fiber and red meat, diabetes [8], and inflammatory bowel diseases, including ulcerative colitis and Crohn's disease [9]. Nano-sized particles are capable of working as pharmaceutical carriers for various delivery systems, orally, transdermally, or intravenously. Studies show that NPs have been tremendously used in the biomedical sector to treat diseases like diabetes [10-12] and cancer [13–15].

Among all the existing NPs, a polymer that makes good NPs is chitosan (CS) due to its special properties. It has antimicrobial characteristics and is capable of healing wounds [16]. Additionally, CS is a biodegradable and biocompatible carbohydrate [10]. CS has also been widely used in the medical sector, for instance, in enhancing the delivery of a cancer drug, silibinin [17]. This paper mostly highlighted the cold extraction method, phytochemical screening analysis, and *in-vitro* anticancer screening of *Vitis vinifera* seed ethanolic extract loaded Chitosan nanoparticles in the H-29 colon cancer cell line.

MATERIALS AND METHODS:

Collection of Plant Materials: The disease-free fruit of *Vitis vinifera* were collected from Cheyyar, Tamil Nadu. *Vitis vinifera* was authenticated by Dr. V. Gangadevi, Assistant professor and Head, Department of Botany, Arignar Anna Government Arts College, Cheyyar. The fruit peels are removed and isolated the seeds. Seeds were shade-dried at low temperature (50-60 °C) and finely powdered by using pulverize and maintained in an air-tight container a**t** 4 ºC and used for further study.

Preparation of Extract:

Ethanol Extract by Cold Extraction Method: Weigh the 10g of dried powder and added into a 50ml conical flask with ethanol and allow keeping it at room temperature for thirty-minute shaking after every twenty-four hours for seven days. Finally, filter the extract using Whatman filter paper under vacuum and dry it at room temperature in a watch glass dish. Note down the weight of each dish prior to drying of the extracts and after drying too. Calculate the weight of the extract from the difference [18].

Phytochemical Screening: Phytochemical analysis was carried out using the standard procedure by Harborne, 1973 [18].

Materials for Preparation of Chitosan - *Vitis vinifera* (CS-VV) Nanoparticle: N-carboxymethyl chitosan (MW= 61 kDa, degree of deacetylation 83%) and TPP were purchased from Haidebei Marine Bioengineering Company (Jinan, Shandong, China). HT-29 cells were obtained from National Centre for Cell Science, Pune, India, and other reagents were of analytical grade.

Preparation of Chitosan - *Vitis vinifera* Nanoparticle (CS-VV NPS): Chitosan - *Vitis vinifera* (CS-VV) Nanoparticle were prepared by an ionic interaction method, performed according to the following procedure. The aqueous solution of carboxymethyl chitosan and chitosan hydrochloride were obtained at a concentration of 3.0mg/mL and 1.2 mg/mL, and solution pH about 8.5 and 3.3, respectively. A certain amount of Ethanol *VV* extract was added into chitosan solution. As a consequence of the addition of carboxymethyl chitosan solution (12ml) was dropped slowly into Ethanol *VV* extract solution (30 mL) with stirring at room temperature and continuous stirring for 30 min. The formation of nanoparticles started spontaneously *via* the ionic gelation mechanism. The nanoparticles suspensions were immediately subjected to further analysis and applications.

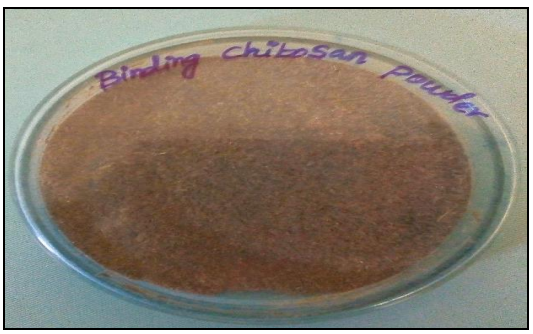


FIG. 1: PREPARATION OF CS-VV NPS

Fourier Transform Infrared Spectroscopy: Fourier Transform Infrared (FTIR) spectroscopy deals with the vibration of chemical bonds in a molecule at various frequencies depending on the elements and types of bonds. After absorbing electromagnetic radiation, the frequency of vibration of a bond increases leading to a transition between the ground state and several excited states. These absorption frequencies represent excitations of vibrations of the chemical bonds and thus are specific to the type of bond and the group of atoms involved in the vibration. The energy corresponding to these frequencies corresponds to the infrared region (4000–400 cm^{-1}) of the electromagnetic spectrum. The term Fourier transform (FT) refers to a recent development in the manner in which the data are collected and converted from an interference pattern to an infrared absorption spectrum that is like a molecular "fingerprint". The FTIR measurement can be utilized to study the presence of protein molecules in the solution, as the FTIR spectra in the 1400-1700 cm^{-1} region provide information about the presence of –CO- and –NH-groups.

Size Measurement and Determination of Zeta Potential: The particle size of the formulation was determined by photon correlation spectroscopy with a zeta master (Malvern Instruments, UK) equipped with the Malvern PCS software. The surface charge (Zeta potential) was determined by measuring the electrophoretic mobility of the nanoparticles using a Malvern zeta sizer (Malvern Instruments, UK). Samples were prepared by diluting with distilled water.

***In-vitro* Assay for Cytotoxicity Activity (MTT Assay) of CS-*VVE* NPS**

Preparation of HT-29 Cell Suspension: A subculture of HT-29 in Dulbecco's Modified Eagle's Medium (DMEM) was trypsinized separately, after discarding the culture medium. To the disaggregated cells in the flask, 25 mL of DMEM with 10% FCS was added. The cells suspended in the medium by a gentle passage with the pipette, and the cells were homogenized.

Seeding of Cells: One ml of the homogenized cell suspension was added to each well of a 24 well culture plate along with the different concentrations of samples C1 and C2 (0 to 400 mg/mL) and incubated at 37 °C in a humidified CO_2 incubator with 5% CO_2. After 48 h incubation, the cells were observed under an inverted tissue culture microscope. With 80% confluence of cells, cytotoxicity assay was carried out.

Cytotoxicity Assay: The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial Succinate dehydrogenase and reductase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. After 48 h incubation, the wells were added with MTT and left for 3 h in room temperature. All wells have removed the content using a pipette, and 100µl SDS in DMSO was added to dissolve the formazan crystals, absorbance's were read in Lark LIPR- 9608 microplate reader at 540 nm.

Acridine Orange/Ethidium Bromide (AO/EtBR) Staining: The acridine orange/ethidium bromide (AO/EB) double staining assay was carried out using the methodology. Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if interrelated into a double-stranded nucleic acid (DNA) or red fluorescence if bound to single-stranded nucleic acid (RNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA. We distinguished four types of cells according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei. Viable cells have uniform bright green nuclei with an organized structure. Early apoptotic cells (which still have intact membranes but have started to undergo DNA cleavage) have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments. Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. Necrotic cells have uniformly orange to red nuclei with a condensed structure. The amount of 20 µL of dye mixture (10 µL/mg AO and 10 µL/mg EB in distilled water) was mixed with 100 µL cell suspension (10,000 cells/mL) in a 96-well plate. After the incubation times with the IC_{50} of the drug, suspension was immediately examined and viewed under a Nikon inverted fluorescent microscope (Ti-Eclipse) at 400 × magnifications. We observed untreated cells as controls.

RESULTS AND DISCUSSION:

TABLE 1: PHYTOCHEMICAL ANALYSIS

S. no.	Test /Seed extract	Ethanol
1	**Test for Alkaloids**	
	a. Mayer's test:	+
	b. Wagner's test	+
	c. Dragendroff's test	+
2	**Test for Flavonoids**	
	a. Shinoda test	+
	b. Alkaline reagent	+
3	**Test for Carbohydrates**	
	a. Benedict's test	+
	b. Molisch's test	+
4	**Test for Glycosides**	
	a. Bontrager test	+
	b. Keller killiani test	+
5	**Test for Sapponins**	
	a. Froth test	-
	b. Lead acetate	+
6	**Test for Tannins**	
	a. Ferric chloride test	+
	b. Lead acetate test	+
7	**Test for Terpenoids**	
	a. Salkowski test	+
8	**Test for Protein**	
	a. Ninhydrin test	+
	b. Biuret test	+
9	**Test for Anthroquinone**	
	a. Ammonia test	+

Phytochemicals are a chemical compound that occurs naturally in plants. These phytochemicals are responsible for the medicinal properties and these phytochemicals are generally used to refer those chemicals that may have biological significance such as antioxidant, anti-inflammatory, antifungal, anti allergestic *etc*. It was estimated that there may be 1000 different phytochemicals which having potential role in curing many diseases. In this study the taken *Vitis vinifera seed* ethanolic extract shows most of the important phyto-chemicals. Such as Alkaloids, Tannins, Saponins, carbohydrates, Glycosides, Proteins, Terpenoids, Flavonoids and Anthroquinone [19-21]. This phyto-chemical having potential anti-cancer activity was shown in the above results is tabulated in **Table 1**.

FTIR Analysis of Chitosan - *Vitis Vinifera* Nanoparticle (CS-VV NP): Fig. 2a, 2b depicts the FTIR pattern of chitosan, and CS -*VV*E. FTIR spectra of pure chitosan, reaction solution of chitosan with seed broth of *VV* including CNPs, are shown in **Fig. 2a**.

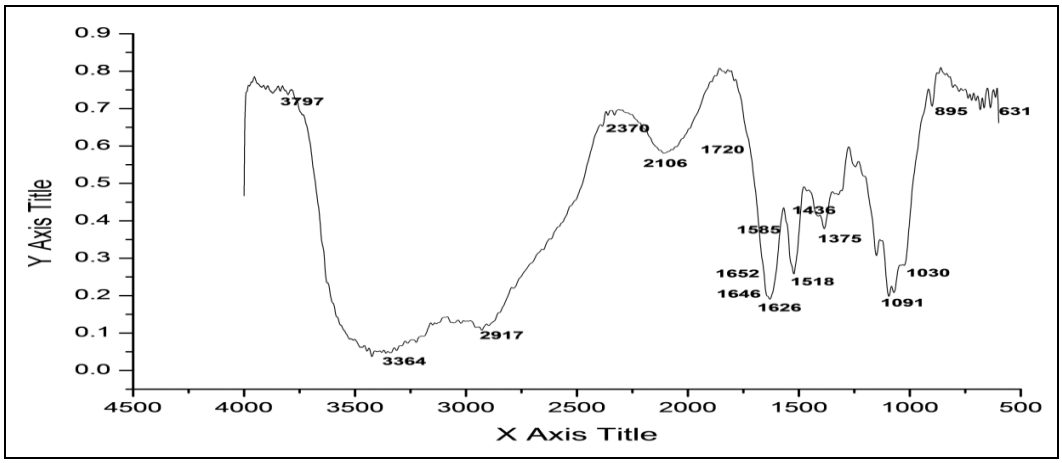


FIG. 2A: FTIR ANALYSIS OF PURE CHITOSAN

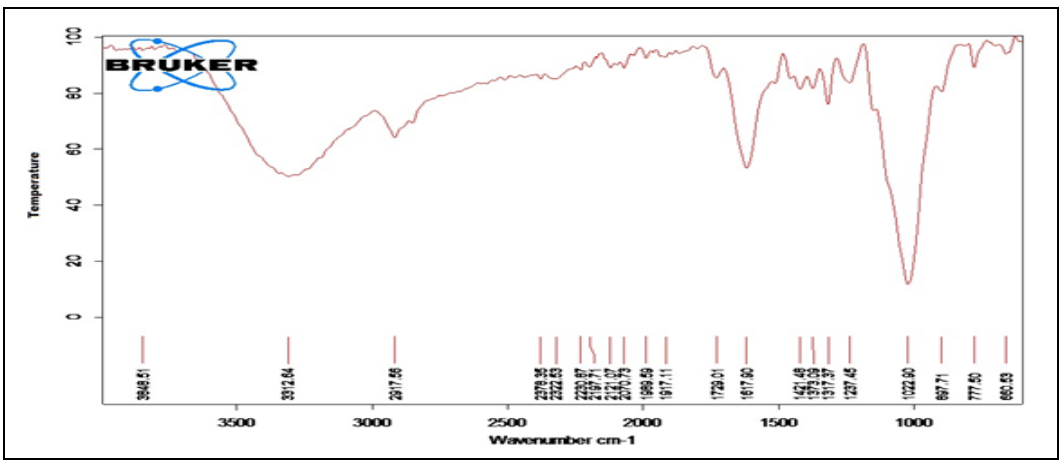


FIG. 2B: FTIR ANALYSIS OF CS-VV NP

The peak was observed at 3438.10, 2925.34, 1629.68, 1414.08, 1121.17, 673.35, and 602.82 $cm^{-1,}$ respectively, in the reaction solution of chitosan with seed broth of *VV*E. The band at 3448.61 cm^{-1} corresponds to the combined peak of the NH_2 and OH groups stretching vibration in chitosan. For reaction solution, the intensities of amide bands at 1560.77 cm^{-1}, which can be observed clearly in pure chitosan, decrease dramatically, and one new absorption band at 1629.68 cm^{-1}, which can be assigned to the absorption peak of the NH_3^+ absorption of chitosan is observed. The absorption peak at 1414.08 cm^{-1} in reaction solution could be assigned to symmetric stretching vibrations of -COO anion groups. This result indicates that carboxylic groups of the leaf broth compounds are dissociated into - COO groups which complex with protonated amino groups of chitosan through electrostatic interaction to form CS-*VV*ENPs. The peak at 3384.07 cm^{-1} indicates alcohol and phenolic OH groups along with the peak of 1605.78 cm^{-1} which represents $CONH_2$ group in seed broth. These peaks decrease dramatically in the reaction solution. *VV*E contains different compounds such as flavonoids, triterpenoids, Vitamin C, stilbene derivatives, and many others, *e.g.*, resveratrol, piceatannol, pallidolperthe-nocissin, and phyto-sterols. Out of which ascorbic acid, triterpene, β-sitosterol, ketosteroid, two asymmetrical tetracyclic triterpenoids, and calcium were identified as major constituents this plant. These chemical constituents are mainly responsible for various biological activities. It is postulated that anion groups of seed broth such as OH (alcoholic and polyphenolic compounds) and COO^-(amino acids residues, free carboxylate groups in proteins, carboxylic acids, alkaloids) interact with the ammonium groups of chitosan, which series to enhance both the inter and intramolecular interaction in CS *VV*E NPs [22-24].

Size Measurement and Zeta Potential Determination of *CS-VV NP*: The respective average diameters, measured by Zetasizer, The size of CS*VV* at selected concentration was 216 nm. The results obtained by Zetasizer revealed that the CS-*VV* nanoparticles are larger than the chitosan-TPP ones, possibly due to the high molecular weight and large size of loaded chitosan molecules, *vv* Extract surface adsorption during incubation time and negligible elevation of viscosity by CS-*VV* in the loading process. The Zeta potential of CS-*VV* nanoparticles can greatly influence their stability in suspension by means of electrostatic repulsion between the particles. Our results demonstrated the respective zeta potential of CS-*VV* nanoparticles of 50.3 mV. This result showed that the nanoparticles as zeta potential.

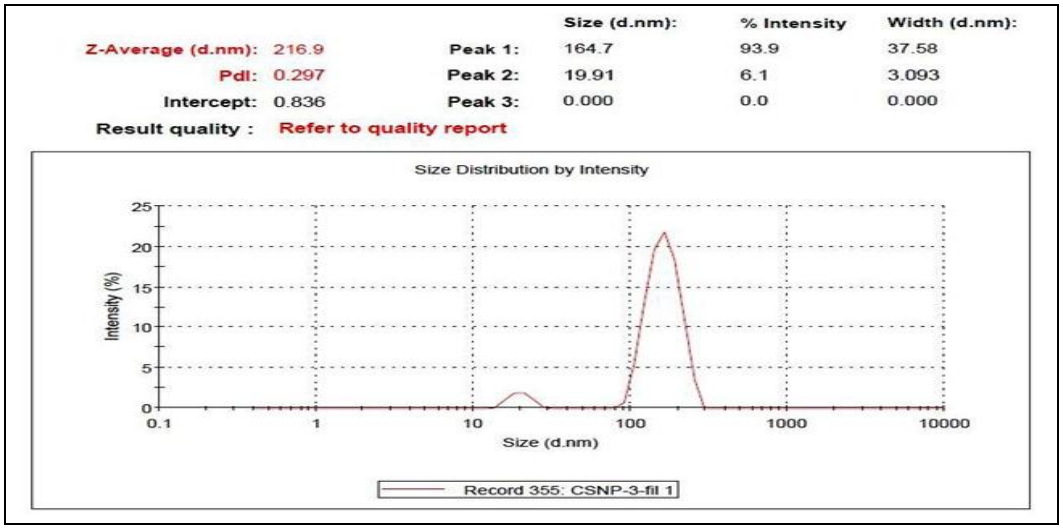


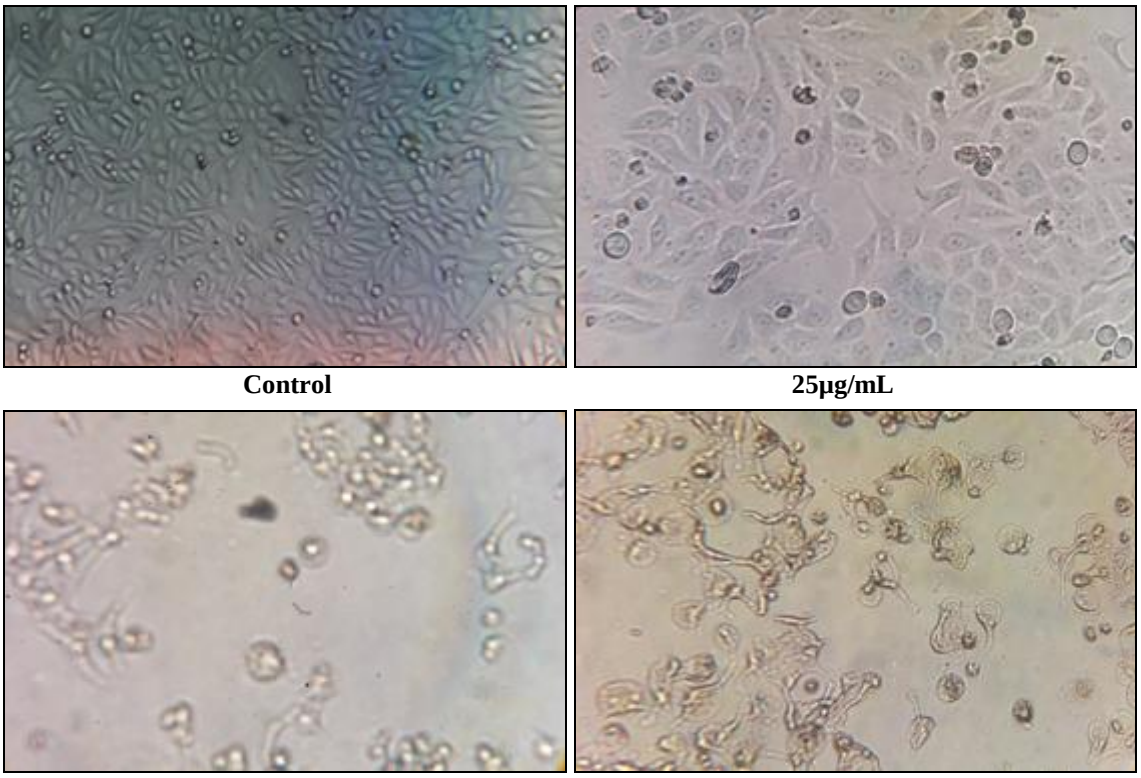
FIG. 3: ZETA SIZE ANALYSIS OF CS*VV* NPS

Effect of CS-*VV*E NPS on *in-vitro* Cytotoxicity: Cytotoxicity of the nanoparticles prepared was determined by MTT assay, which is a colorimetric test based on the selective ability of viable cells to reduce the tetrazolium component of MTT into purple-colored formazan crystals. The *in-vitro* cytotoxicity activity studies were proved that cancer cell lines were inhibited significantly with the increasing of sample concentration. It was observed in results **Table 4**.

In the HT-29 cell line, more cytotoxicity effect was observed in a sample in 48 h treatment; it also revealed that increased concentration of drug shown good toxicity over the cancer cell. This effect is due to the samples having a more specific activity to cancer cell lines. It was recorded IC_{50} 150 µg/ml and 250 µg/ml on tested samples CS-VVE NPS, respectively. The IC_{50} was calculated as 67.88 µg/ml against HT -29 cell lines **Fig. 4** & **5**.

TABLE 2: *IN-VITRO* CYTOTOXICITY EFFECT OF DRUG SAMPLE A AGAINST HT 29 CELL LINES

Sample Concentrations (µg/ml)	Cell Viability (%)
0	100.00
3.125	68.95
6.25	62.09
12.5	53.65
25	47.78
50	41.37
100	36.02
200	26.07



Control **25µg/mL**

100µg/mL **200µg/mL**

FIG. 4: CYTOTOXICITY ACTIVITY OF CS-VVE AGAINST HT-29 CELL LINES

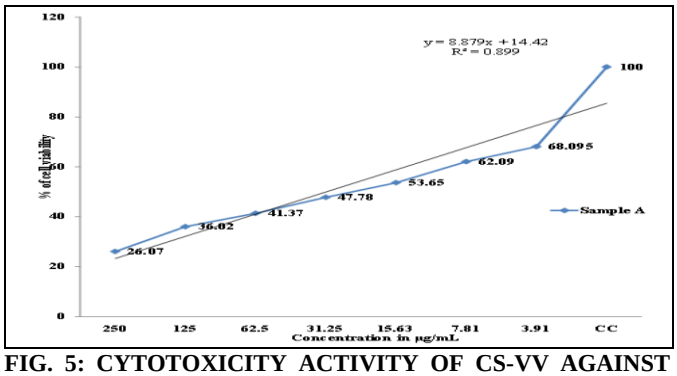


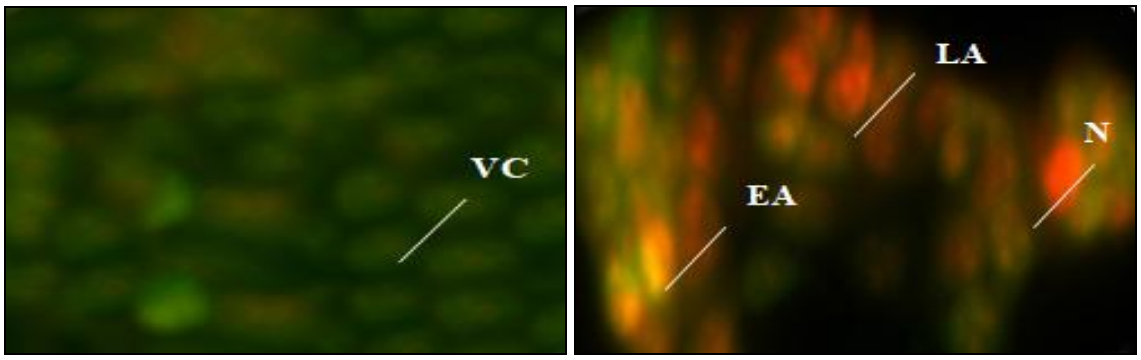
FIG. 5: CYTOTOXICITY ACTIVITY OF CS-VV AGAINST HT-29 CELL LINES

Drugs continually released from the matrix accelerated cell apoptosis, and the apoptosis tendency was more obvious when the incubation time continued. This phenomenon indirectly suggested that the nanoparticles can effectively prolong drug release. Cell apoptosis rate was increased from 30% after 24 h to 62% at the end of 72 h induced by CS-*VV*E NPs with the same concentration at 1mg/ml. It was also observed that cell apoptosis rates were increased at higher concentrations of nanoparticles. That is due to higher concentrations of drug-loaded into nano-particles. The cytotoxicity of different concentrations of N-succinyl- chitosan nanoparticles on H-29 cells also presented dose and time-dependence.

Surprisingly, when incubated with control nano-particles, the cell apoptosis rate also reached about 2-5%. This may be because free chitosan itself can induce cancer cell apoptosis in some extent. But a comparison with VVE polyphenols, the free chitosan has weaker antitumor activity in solution. Moreover, Reports showed that chitosan nano-particles with small particle size and positive

surface charge also could exhibit antitumor activity. As expected, the cytotoxicity was caused mainly by VVE polyphenols itself, and chitosan nanoparticles only played a limited role in cell apoptosis [25, 26].

Acridine Orange/Ethidium Bromide (AO/ETBR) Staining: The percentages of viable, apoptotic, and necrotic cells of the drug-treated with 48 h has dramatically induced apoptosis in the tested cell lines. About 41.55% early apoptotic cells were formed and 15.78% of the late apoptotic cells, and only 7.68% of necrotic cells were observed. The tested drug was inducing the apoptotic process in the tested cell lines.



A: CONTROL CELL **B: TREATED CELL**

FIG. 6: CONTROL CELL AND TREATED CELL MORPHOLOGY UPON DUAL STAINING WITH ACRIDINE ORANGE /ETHIDIUM BROMIDE. VC: Viable cells; EA: Early apoptosis; LA: Late apoptosis; N: Necrosi

Nanotechnology can be applied to deliver and protect antioxidants in order to control the oxidative stress phenomena in several chronic pathologies. Chitosan (CS) nanoparticles are bio-degradable carriers that may protect antioxidants with potent biological activity such as *VV* extracts for safe and innovative therapies. We have shown that biocompatible chitosan has been successfully modified by condensation reaction using natural compounds of *VV* seed extract as cross-linking agents to form nanoparticles. Opalescent stable colloid systems based on chitosan were fabricated in aqueous medium at room temperature.

Biosynthesis of CNPs using green sources like *VVE* is a better alternative to chemical synthesis, science this green synthesis is pollutant-free and eco-friendly. The results suggest that *VV* seed extract plays an important role in the biosynthesis and stabilization of CSNPs. This study also found that the CS-*VV*ENPS, Being of cationic character, chitosan is able to react with polyanions giving rise to polyelectrolyte complexes. Hence chitosan has become a promising natural polymer for the preparation of microspheres/nanospheres and microcapsules [27].

In addition, since the chitosan microspheres offer highly convenient and flexible systems for different applications, it is believed that the biosynthesized novel CNPs can be considered for different purposes particularly biomedical application. The core of this work was to underline the potential application of sage and savory for pharmaceutical of biomedical formulations, considering the benefits of natural extracts containing *VV* Bearing in mind that natural nanoparticles offer the most advanced treatment modality for crude extracts incorporation. This could be a fundamental alternative nanomedicine to enhanced antioxidant performance for oxidative stress conditions [27].

CONCLUSION: In this study, *Vitis vinifera* seed ethanolic extract loaded chitosan is safe to use as a carrier for the drug, which was observed from the present investigation. This could contribute to the discovery of a new method for the treatment of colon cancer that may overcome the difficulties in the currently available procedures or therapies. Further studies would be focused onto report the drug release efficiency under different physiological conditions and evaluate them in an animal model.

Hence, it was observed from the present investigation that, the anticancer activity of *Vitis vinifera* seed extract loaded chitosan nanoparticles against HT-29 cell line provided a platform for treating cancer with biopolymer nanomaterials.

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CONFLICTS OF INTEREST: Nil

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