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Formulation and scrutinization of a polyherbal combination containing *Berberis aristata, Andrographis paniculata* and *Thevetia peruviana* for its *in vitro* antioxidant and anti-cancer potential

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ABSTRACT: Herbal medicines are known for their active potential with high safety profile in humans for various disorders. This study was based on the anticancer potential of three widely used plants in the form of a polyherbal combination, which was designed to assess the antioxidant and cytotoxic potential of a polyherbal combination (2:1:1) and its prepared chitosan nanoparticles. Polyherbal combination was prepared at different ratios of plant extracts. Chitosan nanoparticles were prepared using ionic gelation method. Polyherbal combination (2:1:1) was characterized qualitatively and quantitatively by ultraviolet spectroscopy. The characterization of developed nanoparticles was done by scanning electron microscopy and X-ray diffraction. HepG2 cells were used to assess the cytotoxicity of different polyherbal combinations as well as prepared chitosan nanoparticles while 2, 2-diphenyl-1-picrylhydrazyl assay was performed to assess the antioxidant potential of selected polyherbal combination and its chitosan nanoparticles. Content of berberine, andrographolide and rosmarinic acid in polyherbal combination (2:1:1) was found to be 2.188, 0.472 and 0.264%w/w. Cytotoxicity of different polyherbal combinations (2:1:1, 1:1:1, 1:1:2 and 1:2:1) and its prepared chitosan nanoparticles (2:1:1) against HepG2 cells were found to be at an IC_{50} of 13.96, 16.47, 15.20, 16.21 and 15.67µg/ml respectively. 2, 2-diphenyl-1-picrylhydrazyl scavenging activity of Polyhebral combination (2:1:1) and its prepared chitosan nanoparticles (2:1:1) showed potent antioxidant potential. The developed Polyherbal combination (2:1:1) and its prepared chitosan nanoparticles was found to possess antioxidant and anticancer potential.

KEYWORDS: Berberine; Andrographolide; Rosmarinic acid; Cytotoxic activity; Chitosan nanoparticles.

1.INTRODUCTION

Nanotechnology is an emerging field of research and plant-based researches are not devoid. The species *Andrographis paniculata, Berberis aristata* and *Thevetia peruviana* contain andrographolide, berberine and rosmarinic acid as pharmacologically active components respectively and are individually reported to possess the antioxidant and cytotoxic potential [1-3]. The extract combinations of these plants are supposed to produce synergistic effect and can be beneficial as an adjuvant therapy in hepatic carcinoma. The current piece of research is to evaluate the cytotoxic and antioxidant potential and to develop the nanoparticle formulation of best combined effect in cytotoxic activity.

After extraction of three plants four combination was made and were assessed for their cytotoxic activity, the combination with best cytotoxic result were carried further for preparation of chitosan nanoparticles, qualitative and quantitative analysis for the presence of bioactive constituents followed by antioxidant activity and cytotoxic activity of the prepared chitosan nanoparticles.

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2. RESULTS

2.1 Percentage yield

Percentage yield of plant '*a*', '*b*' and '*c*' were found to be 9.04± 1.32%, 11.87± 1.07% and 10.09± 1.23% w/w respectively after extraction with ethanol.

2.2 Qualitative and quantitative determination

Qualitative UV spectrophotometric analysis of PHC (2:1:1) showed the presence of berberine, andrographolide and rosmarinic acid at wavelength 348, 226 and 325nm respectively as shown in Figure. 1.

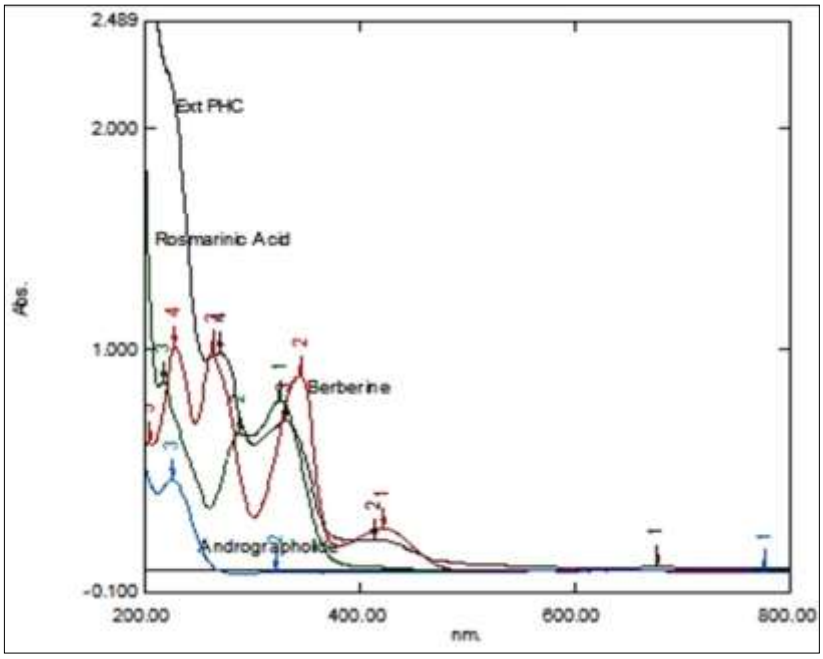

Figure 1. UV spectra of Berberine, Andrographolide, Rosmarinic acid and PHC (2:1:1).

Quantitative analysis was done using calibration curve of each standard drug and was plotted, trendline was generated and regression coefficient was determined. Calibration range, equation, regression coefficient and % drug content were shown in Table 1.

Table 1. Showing Calibration range, equation, Regression coefficient and % drug content.

Standard drug	Concentration Range	Calibration equation	Regression Coefficient (R^2)	% (w/w) drug content in PHC (2:1:1)
Berberine	2-10µg/ml	y = 0.122x-0.015	0.997	2.188
Andrographolide	10-50µg/ml	y = 0.022x+0.023	0.999	0.472
Rosmarinic acid	4-20µg/ml	y = 0.059x-0.037	0.995	0.264

2.3 Characterization of Nanoformulation

2.3.1. Scanning Electron Microscopy (SEM)

The structural characterization of Blank Chitosan nanoparticles (CNP-BL) and PHC (2:1:1) loaded Chitosan nanoparticles (CNP-EL) were studied using SEM micrograph at four different magnifications (5,000X, 10,000X, 15,000X and 27,000X) shown in Figure.2. The micrograph CNP-BL shows a smooth as well

as homogeneous surface along with clustering of even particles that were found to be rod-like in shape. Whereas, the CNP-EL micrograph also shows a non-smooth as well as non-homogeneous surface along with clustering of rod like as well as disc-like structures.

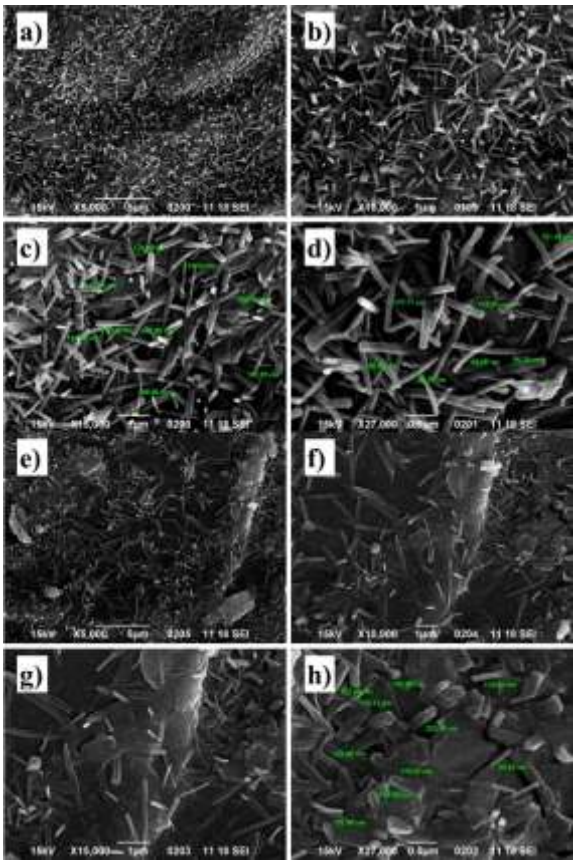

Figure 2. SEM images of Blank Chitosan nanoparticles (CNP-BL) from a) to d) and PHC (2:1:1) loaded Chitosan nanoparticles (CNP-EL) from e) to h) at different magnifications.

2.3.2 X-ray Diffractive (XRD) analysis

In the XRD pattern a broadening peak with lower intensity at about 2θ between 15° and 25° was observed in CNP-BL, indicating the decrease of the crystallinity of Chitosan structure and the presence of Chitosan in amorphous forms in the nanoparticles as shown in Figure. 3. The lower intensity exhibited by CNP-EL diffraction peaks shows that they are amorphous in nature.

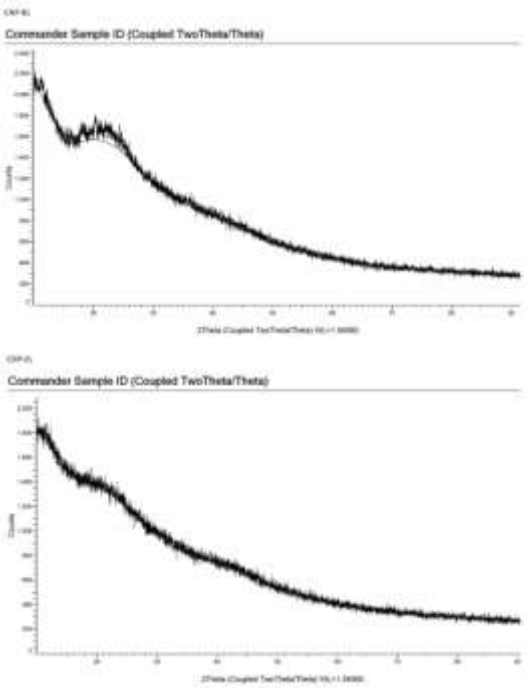

Figure 3. XRD Patterns of Blank Chitosan nanoparticles (CNP-BL) and PHC (2:1:1) loaded Chitosan nanoparticles (CNP-EL).

2.4 In vitro cytotoxic activity by MTT

In vitro cytotoxic activity of different Polyherbal combinations 2:1:1, 1:1:1, 1:1:2, 1:2:1 and developed PHC loaded chitosan nanoparticles was found to be 13.96, 16.47, 15.20, 16.21 and 15.67µg/ml respectively. However, it has been assessed that the increasing sample concentrations illustrated cytotoxic activity as indicated in Figure. 4. At 21µg/ml concentration of the PHC (2:1:1) 70.72±3.01% of cell death was found. While at the same concentration of CNP-EL only 66.15±3.11% of cells were inhibited as indicated in Table 3, PHC (2:1:1) indicating maximum cell death in the HepG2 cancer cell line. The result suggested that PHC (2:1:1) was more toxic to HepG2 cell line than CNP-EL. Treatment of HepG2 cells against different PHCs and developed chitosan nanoparticles are shown in Figure. 4.

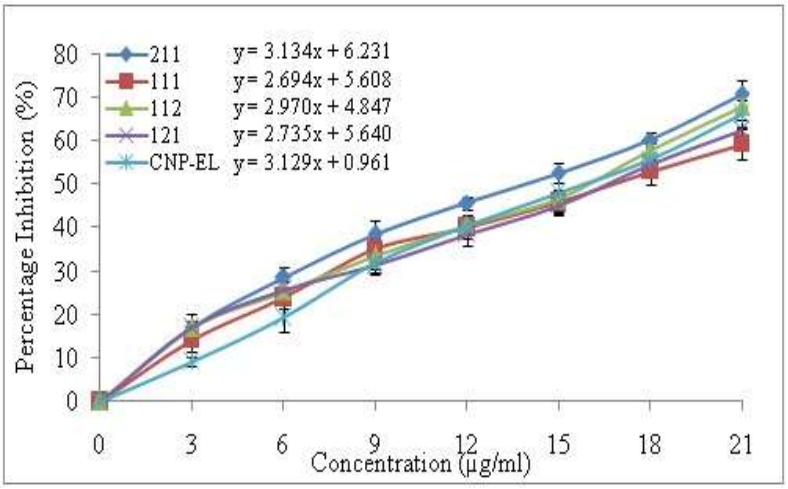

Figure 4. Showing percentage inhibition (%) of different PHCs and developed CNP-EL at different concentrations using MTT assay against HepG2 cells. All values were performed in triplicates and represented as mean ± SD.

2.5 DPPH radical scavenging activity

In the present study, it can be observed (Figure 5.) that the PHC (2:1:1) and CNP-EL at high concentrations demonstrated significant reduction in the absorption of DPPH radicals. Highest scavenging of radical was observed in case of PHC (2:1:1) followed by CNP-EL. Even though the scavenging property of PHC (2:1:1) was less than that of ascorbic acid. PHC (2:1:1) has found to possess highest scavenging of radicals followed by CNP-EL.

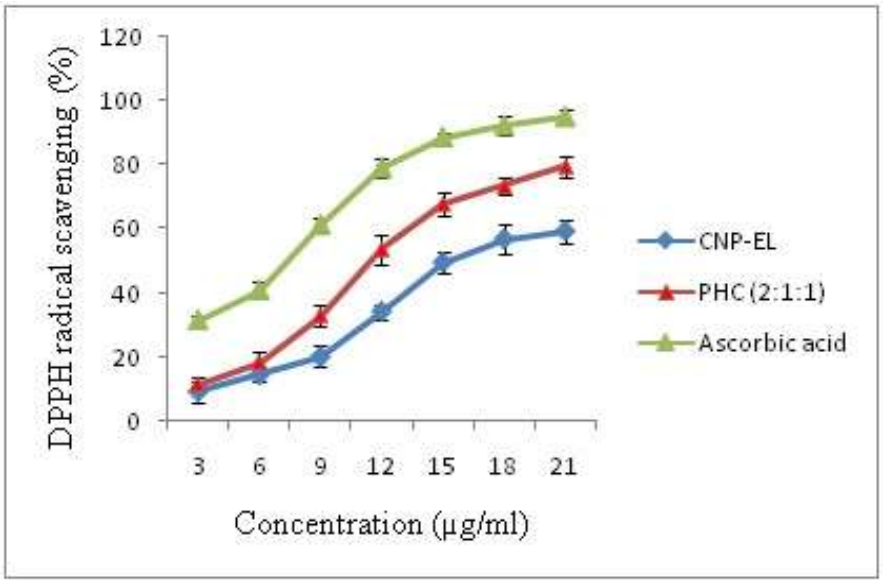

Figure 5. Showing percentage inhibition of PHC (2:1:1) and CNP-EL at different concentrations using DPPH assay. All values were performed in triplicates and represented as mean ±SD.

3. DISCUSSION

Biologically active compounds usually occur in low concentration in plants. An extraction technique is that which is able to obtain extracts with high yield and with minimal changes to the functional properties of the extract required [4]. Ethanolic extraction provides better yield of the plant extract. In the present study, the presence of alkaloids, phenolics and diterpenes in PHC (2:1:1) was confirmed by the qualitative as well as quantitative analysis. Phenolic compounds are usually found in plants that have already been accounted to possess a broad range of biological properties together with antioxidant properties. Studies showed that phenolic compounds are one of the most efficient antioxidants [5]. Flavonoids are phenolic compounds and these plant phenolics are a chief collection of compounds that proceed as principal antioxidants. Likewise, terpenoids act as a regulator of metabolism as well as play a defensive role as antioxidants. Alkaloids that are found in plant extracts are also used for extensive range of pharmacological properties including anti-cancer and so on [6]. Chitosan nanoparticles have gained attention in the field of biomedical engineering, nanomedicine and the progression of novel therapeutic drug release systems with enhanced bioavailability augmented specificity and sensitivity, also reduced pharmacological toxicity [7]. Morphologically, Chitosan nanoparticles prepared in this study were found to be rod-like in shape as observed by [8, 9]. In XRD pattern of CNP-EL, the absence of any other diffraction peaks, corresponding to impurities is observed, which indicates their purity. The rate of diffraction peaks is reduced in the case of chitosan nanoparticles, as a result of transforming crystallized chitosan into amorphous form after being cross-linked with TPP [10]. However, according to kinetic studies, the decrease in the crystallinity of polymer results in an improvement of metal ion sorption and capacity. The decrease in crystallinity possibly will be due to that chitosan nanoparticles are composed of a dense network structure of interpreting counter ions of TPP, where the polymer chains cross-link with each other by TPP. Thus, the XRD patterns of chitosan nanoparticles are characteristic of an amorphous polymer [11]. MTT assay is used to investigate the response of drug in malignancies. It is quick and suitable colorimetric assay for cellular growth as well as survival *in vitro*. The lesser the value, the more cytotoxic is the drug substance [12]. According to U.S. National Cancer Institute, the extracts with IC_{50} lower than 20µg/ml are considered to be highly active [13]. This study showed that the PHC (2:1:1) and its prepared chitosan nanoparticles (CNP-EL) exhibited strong cytotoxic effect against HepG2 cells. The action of

antioxidants on DPPH might be due to their hydrogen donating capability. Radical scavenging actions are really essential to prevent the harmful role of free radicals in various diseases, including cancer. DPPH free radical scavenging is an established method for screening the antioxidant property of plant extracts [14].

4. CONCLUSION

This research suggested that one out of four polyherbal combination with ratio 2:1:1 and its prepared chitosan nanoparticles investigated displayed significant cytotoxic activity and antioxidant potential.

4. MATERIAL AND METHODS

5.1 Materials

5.1.1. Procurement and authentication of plant materials

The bark and leaves of selected three herbal plants *viz. Berberis aristata, Andrographis paniculata* and *Thevetia peruviana* were procured from the local market and herbal garden of Lucknow, U. P. India. Authentication of these plant materials were assured from National Botanical Research Institute, Lucknow, U. P. 226001 under Voucher Specimen Reference Number: NBRI/CIF/665/2018.

5.1.2. Cells, Chemicals and reagents

HepG2 human hepatocarcinoma cells were obtained as a gift from CDRI, Lucknow, U. P., India. All cells were cultured in supplemented Dulbecco's modified Eagle medium (DMEM, Invitrogen) at 37^{0}C in a humidified atmosphere in an incubator of carbon dioxide and air (5:95). DMEM was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100µg/ml Streptomycin, and 100U/ml Penicillin (all from Invitrogen). The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was purchased from Invitrogen. Standard Berberine was purchased from Tokyo Chemical Industry, Tokyo, Japan. Andrographolide and Rosmarinic acid were procured from GLR Innovations, New Delhi. Chitosan was procured from Yarrow Chemicals, Lucknow and Sodium tripolyphosphate (TPP) was purchased from Sisco Research Laboratories Chemicals, Maharashtra, India.

5.2 Methods

5.2.1. Extraction of plant materials

Plant materials were extracted using conventional method. Briefly, *Berberis aristata* barks were extracted with 70% aqueous ethanolic solvent using cold maceration process for 24 hours [3]. Filtered through whattman filter paper number 1 and dried by a rotary evaporator at <50°C. Coarsely powdered sun dried *Andrographis paniculata* and *Thevetia peruviana* leaves were extracted with 70% aqueous ethanolic solvent using Soxhlet apparatus at 70^{o}C for 48 hours [1, 15]. Percentage yield was calculated using given formula:

$$\text{Percentage Yield (\%)} = \frac{\text{Weight (g) of Extract Obtained}}{\text{Weight (g) of Material taken}} \times 100$$

5.2.2. PHC (2:1:1) standardization using Ultraviolet (UV) Spectrophotometer

PHC (2:1:1) was standardized by UV spectrophotometer (SHIMADZU-UV1800) for the presence of their pharmacologically active component in each plant i.e., Androgrpholide in *Andrographis paniculata*, Berberine in *Berberis aristata* and Rosmarinic acid in *Thevetia peruviana*. UV spectrum was generated by individually scanning standard compounds and PHC by dissolving (~10mg) in suitable solvents (~10ml) and dilution was made in water.

5.2.3. Drug Content

Drug content of Andrographolide, Berberine and Rosmarinic acid were assayed in PHC (2:1:1) by generating calibration curve of each using UV spectrophotometer. Briefly, 10, 20, 30, 40 and 50µg/ml solution of Standard Andrographolide were prepared and absorbance was recorded at wavelength 226nm. 4, 8, 12, 16 and 20µg/ml solution of Rosmarinic acid were prepared and absorbance were recorded at wavelength 325nm [16, 17]. 2, 4, 6, 8 and 10µg/ml solution of Standard Berberine were prepared and absorbance was recorded at 348nm [18]. 50mg of PHC (2:1:1) was dissolved in 10ml solvent (prepared using 2ml methanol with 8ml water in a 10ml volumetric flask) and further dilution was made with water. UV absorbance was recorded at each wavelength i.e. 226nm, 325nm and 348nm for Andrographolide, Rosmarinic acid and Berberine respectively. Percent drug content was calculated using formula:

$$\% \text{ Drug Content} = \frac{Drug\ Content}{Weight\ of\ PHC\ taken} \times 100$$

5.2.4. Preparation of different Polyherbal Combinations

According to the above-mentioned extraction methods all the desired plant extracts (*viz. a=Berberis aristata, b=Andrographis paniculata and c=Thevetia peruviana)* were mixed together in a particular ratio to develop a fixed dose Polyherbal combination. LD_{50} of >5000mg/kg, 2000mg/kg and >3000mg/kg of plants '*a*', '*b*' and '*c*' respectively has been reported [2, 3, 19]. For the development of PHC, extract weights were taken below their reported LD_{50}. So, a dose of 250mg/kg was assumed 1 for '*a*', 200mg/kg was assumed 1 for '*b*' and '*c*' respectively. Therefore, the different Polyherbal combinations were developed in different ratios (PHC 1^a:1^b:1^c, PHC 1^a:1^b:2^c, PHC 1^a:2^b:1^c and PHC 2^a:1^b:1^c).

5.2.5. Preparation of Blank and PHC (2:1:1) loaded Chitosan nanoparticles

Chitosan nanoparticles (CNP-BL) were prepared by ionic gelation method. Briefly, 0.2% w/v Chitosan (Low molecular weight) was prepared in 1%v/v acetic acid solution by stirring overnight followed by filtration. Separately 0.1% TPP solution was prepared and added to chitosan solution dropwise while stirring at 1000rpm and kept on stirring for next 10 minutes. Chitosan TPP ratio was maintained at 3:1. PHC (2:1:1) loaded chitosan nanoparticles (CNP-EL) were prepared by dissolving PHC (2:1:1) 0.2% w/v in chitosan solution and rest was followed as blank nanoparticles preparation [20].

5.2.6. Characterization of nanoparticles

Scanning Electron Microscopy (SEM)

The microstructure was examined using Scanning Electron Microscope (Model: JOEL-JSM6490LV, Japan) under an accelerated potential of 15kV at various magnifications.

X-ray Diffraction (XRD)

The degree of crystallinity of CNP-BL and CNP-EL were detected using X-ray Diffractometer (Model: D8 Advance Eco, Bruker, Germany) with a Nickel monochromator filtering at 45kV and 20mA, that results in diffraction pattern within the 2θ range of 10^o to 90^o with a scanning speed of 0.02^o per minutes.

5.2.7. In vitro Cytotoxic activity by MTT

The effect of different PHCs and developed CNP-EL (Chitosan nanoparticles of PHC 2:1:1 or say PHC (2:1:1) loaded chitosan nanoparticles) on cell viability was measured using a standard colorimetric MTT reduction assay to assess the cell viability of PHCs and developed CNP-EL according to the previously reported protocol [21]. HepG2 cells were cultured in 96-wells plates at a density of $1x10^4$cells/well. After 24 hours, cells were treated with increasing concentrations (3, 6, 9, 12, 15, 18 and 21µg/ml) of the four Polyherbal combinations (1:1:1, 1:1:2, 2:1:1 and 1:2:1) as well as developed CNP-EL or 0.2% dimethyl sulfoxide (DMSO) (vehicle control) for 24 hours. After incubation 10µL of 5mg/ml MTT stock solution was added to each well and the cells were incubated for 3 hours at 37^0C. After incubation, the supernatants were aspirated, and 100µL DMSO was added to dissolve formazan crystals for 10 minutes at 37^0C. Optical density was measured at 540nm using a microplate reader (Biorad). Cell viability (%) has been shown as a ratio of absorbance (A_{540}) in treated cells to absorbance in control cells (A_{540}) and it was measured to evaluate the IC_{50} (50% inhibitory concentration) of different PHC and developed PHC loaded chitosan nanoparticles by the given formula:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \text{ x } 100$$

5.2.8. DPPH radical scavenging activity

The free radical scavenging ability of PHC (2:1:1) and developed CNP-EL were tested by utilizing DPPH (1, 1-diphenyl-2-picryl-hydrazyl) radical scavenging assay. In brief, 1ml of different concentrations of PHC (2:1:1) and developed CNP-EL (3, 6, 9, 12, 15, 18 and 21µg/ml of methanol) were mixed with 3ml of DPPH solution (0.004% in methanol) in separate tubes. Further, the tubes were incubated in dark at room temperature for 30 minutes and the optical density was measured at 517nm using UV-Vis spectrophotometer. The absorbance of the DPPH control was also noted. Ascorbic acid was used as reference standard. The radical scavenging activity of PHC (2:1:1), developed CNP-EL and ascorbic acid was calculated using the formula: Scavenging activity (%) = [(Ao – Ae) / Ao] x 100, where Ao is absorbance of DPPH control and Ae is absorbance of DPPH and PHC (2:1:1), CNP-EL/standard combination [22].

5.2.9. Statistical analysis

The data were expressed as the means of three replications with standard deviation (±SD) and were analysed on Microsoft Office Excel 2007.

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