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Research article

Chitosan and glyceryl monooleate nanostructures containing gallic acid isolated from amla fruit: targeted delivery system

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ABSTRACT

Gallic acid, active constituent of amla fruit its natural abundance with beneficial multi actions in body make them attractive for clinical applications. In present study, we focused on extracting, separating and characterizing gallic acid from amla and further formulated into chitosan nanoparticles, so bring it to increase its aqueous solubility and thereby bioactivity. Gallic acid nanoparticles were prepared by using poloxamer 407, chitosan and Glyceryl Monooleate (GMO) using probe sonicator and high pressure homogenization method. Prepared nanoparticles were characterized by particle size, zeta potential, DSC, XRD, SEM, entrapment efficiency, loading content, *in-vitro* release and stability study. They showed approximately 76.80% encapsulation of gallic acid with average size of 180.8 ± 0.21 nm, and zeta potential +24.2 mV. The cumulative in vitro drug release upto 24 hrs 77.16% was achieved suggesting that from all our findings, it can be concluded that work will facilitate extraction, design and fabrication of nanoparticles for protection and sustained release of gallic acid particularly to colonic region.

1. Introduction

Phyllanthus emblica Linn (*Emblica officinalis*) commonly known as Indian gooseberry or amla is an edible fruit and widely distributed in subtropical and tropical areas [1]. It is recognized that amla is a rich source of active constituents like gallic acid, ellagic acid, quercetin, rutin, cathecol, ethyl gallate, chebulagic acid, kaempferol, isocorilagin, chebulanin, and mallotusinin [2]. These bioactive molecules have extensively investigated for different biological activities as anti-tussive, antioxidant, cardio, gastro, neuroprotective, hypoglycemic, hypolipidemic, chemo-modulatory and anticancer [3, 4, 5, 6, 7]. Gallic acid (GA) is a naturally available phenolic compound present in amla fruit which is water insoluble and one of the major constituent of amla which might contribute to the health effects [8, 9, 10, 11].

However, the pharmacokinetic properties of GA, such as its particle's size is large, less absorption, poor solubility, poor bioavailability and quick elimination have negative effects on its application by the human body. Hence the use of gallic acid contributes major limitations in various formulations to treat different diseases; results were unsatisfactory because it is not stable at extreme temperatures. It is encouraging to modify for optimized the properties of the gallic acid by formulating into nanoparticles that serve to improve the solubility and bioavailability [12]. To strengthen its effectiveness, a delivery system of gallic acid loaded chitosan nanoparticles was synthesized in our study [13, 14].

Nanoparticle approaches like polymeric nanoparticles have been found promising to overcome traditional problems as low solubility, higher stability, sharper size distribution, sustained and controlled release profiles, higher encapsulation efficiency for weakly water soluble agents and can be effective in enhancing bioavailability with better targeting efficiency [15, 16]. Chitosan found to carry a variety of hydrophilic and hydrophobic drugs and poloxamer 407 is a nonionic US FDA approved stabilizer used in various formulations [17, 18, 19]. However, the surface properties of polymeric nanoparticles are altered by surface modification and/or to improve the pharmacokinetics of these colloidal carriers here chitosan is used as a polymer [16, 20]. The glyceryl monooleate (GMO) and chitosan (Chi) is a unique nanoparticulate system having ability to protect the encapsulated drug and increase its stability with efficient delivery to the target sites [21].

Novelty of our findings are several attempts have been done on extraction of gallic acid previously, extraction using Soxhlet extraction method with wide variety of solvent were screened for extraction procedure [22] likewise, this extract showed highest radical scavenging. In previous reported, antioxidant activity of Soxhlet extracts was found to be 1.8 and 3 times higher than ultrasound and maceration but slightly (1.2 times) higher than microwave [23].

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In continuity of previous findings, we planned to investigate the effect of solvent polarity on extraction efficiency of phytochemical compound in amla fruit with their antioxidant activity. However, by using Soxhlet extraction method the extracts in nonpolar solvents including petroleum ether and ethyl acetate were found to be comparatively high in Total Phenolic Content (TPC) as compare to water more polar solvent. Total Extractable Components was found to be comparatively high in water, a polar solvent, than that in nonpolar solvents. This indicates a negative correlation between Total Extractable Components and TPC of extracts suggesting that phenolic acids are extracted more in nonpolar solvents. The extraction of more polar components in polar solvents which possess good antioxidant and reducing capacities. Although being rich in phenolic acids, the extracts in nonpolar solvents were found to be poor in antioxidant and reducing properties. Hence from our entire conclusion here we summarized that ethyl acetate is a best solvent for extraction of amla fruit for Total Extractable Components, Total Phenolic Content and for higher scavenging ability against DPPH [24, 25, 26].

Present work involves extraction of gallic acid from amla fruit and formulated in chitosan nanoparticles [27]. Extraction followed by its thorough identification with qualitative and quantitative analysis has been carried out [28]. The isolation and purification of gallic acid from amla fruit was performed using gas chromatography, thin layer, flash and column chromatography [29]. Further extracted gallic acid was structurally elucidated using UV, FTIR spectroscopy, GC-MS and Nuclear magnetic resonance spectroscopy. Further quantitative estimation was done by using chromatographic techniques as HPLC and HPTLC [30, 31].

So far, no nano formulations have been reported based on GMO/chitosan system for preparation of GA-loaded Chi nanoparticles where gallic acid isolated from amla fruit. In present study we attempted to formulate extracted gallic acid in suitable nanoparticles approach as to form Gallic acid loaded chitosan nanoparticles (GA-loaded Chi nanoparticles). One another study reported that the green synthesized gallic acid silver nanoparticles not only inhibited the growth of E. coli, S. aureus, and C. albicans, but also represented selective cytotoxicity against cancerous cells but not normal cells are sufficient safe for clinical use. A large number of nanoparticles are being explored in many areas of industry technology, biotechnology and agriculture [32].

Here formulated GA-loaded Chi nanoparticles in the drug delivery system, quite often increase the solubility, stability and biodistribution enhancing their efficiency of gallic acid biomolecule. In presence of nanoparticles the absorption of gallic acid increases several times therefore, used as a drug delivery system. In addition to, Response Surface Methodogy (RSM) could effectively predict the effect of the synthesis variables on the particle size and zeta potential of the GA-loaded Chi nanoparticles [33]. RSM was successfully applied to develop empirical models for the prediction of the GA-loaded Chi nanoparticles synthesis conditions. Optimized formulation further characterized for different parameters as particle size, zeta potential, entrapment efficiency, loading efficiency, FT-IR, DSC, XRD and SEM. Finally, the release kinetic studies performed using method for conventional nanoparticle release behavior assessment.

2. Material and methods

2.1. Chemicals

Kolliphor® 407 (poloxamer 407) from BASF (Mumbai), Chitosan microcrystalline powder with 90% dda from Central Institute of Fisheries Technology Cochin, MONEGYL®-0100 (GMO) from Mohini organics Pvt. Ltd., (Mumbai), gallic acid, Foline-Ciocalteu reagent were purchased from Loba Chemie, Mumbai and 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich (Singapore). The sample of different parts of plant were collected from Kolhapur district, Maharashtra, authenticated and further voucher herbarium (PSP-1) has been deposited in the department of pharmacognosy, Bharati Vidyapeeth College of Pharmacy, Kolhapur.

2.2. Soxhlet extraction method [34]

Soxhlet extraction method is one of the most commonly used techniques even so, with the advent of new technology and emerging safety and environmental concerns because of its unattended and straightforward use carried out for extraction of amla fruit by using different solvents [35]. The effect of solvent polarity on extraction efficiency of phytochemical and antioxidant compounds of amla fruit was studied by consecutive extraction in a series of solvents with increasing polarity (n-hexane, petroleum ether, chloroform, ethyl acetate, ethyl acetate, ethanol, acetone and water) [36].

2.3. Phytochemical screening (quantitative test)

Folin-Ciocalteu's method was used for total phenolic content determination. Different solvent extract chloroform, ethanol and ethyl acetate of amla fruit used for determination of phenolic content. The reaction mixture consists of 1 mL of methanolic extracts, 2.5 mL of 10 % Folin-Ciocalteu's solution and 2.5 mL 7.5 % Sodium bicarbonate. Same procedure used for preparation of blank and gallic acid (Standard) and absorbance measured at λmax 765 nm [37].

2.4. Techniques of isolation and purification of bioactive molecule from amla fruit [38, 39]

2.4.1. Gas chromatography

The gas chromatography of the crude ethyl acetate extract was performed using a GC Agilent Gas Chromatography system 7890 B with Agilent DB 624 column with helium gas at 1 mL/min flow mode. GC temperature was set at 50 °C (hold for couple of min) to 250 °C at 20 °C/min. (hold upto 5 min).

2.4.2. Separation by using column chromatography

Separation was performed by means of silica gel filled column padded with cotton at base which was filled by elution solvent. Amla fruit extract powder of 5 g was introduced over silica gel (200 g). Ethyl acetate: Methanol: Tolune (8:2:1) used to elute twenty (A1 to A20) fractions (each of 25 mL) which were collected.

2.4.3. Fractionation of bioactive compound by flash chromatographic technique

Flash chromatography instrument consisting of TBP2H02 pump along with TBD2000 UV detector by mobile phase Ethyl acetate: methanol 100:0 to 0:100 with flow rate 4 mL/min. Column was loaded with 8.0 g slurry (3g extract + 5g silica gel) in 25 g of silica gel (200–400 mesh size from Loba chemicals). The Five fractions (FA001 to FA005) were isolated by linear gradient with peak tube volume was 14 mL and run time was 15 min. The fractionated extracts were concentrated on buchi roto evaporators (R-210 water bath B-491).

2.5. Structural clarification of the bioactive molecules [40, 41, 42]

The band of isolated compound (Fraction No.A16) from column chromatograph were filtered, dried and kept at 4 °C for characterization of UV-Visible Spectroscopy, FT-IR and ^{1}H-NMR techniques.

2.5.1. UV-Visible Spectroscopy

Isolated compound (Fraction A16 from) column chromatography was scanned by Shimadzu UV/Vis spectrophotometer. The given sample solution scanned and spectrum was recorded λ max at 270 nm.

2.5.2. FTIR spectroscopy

FTIR has proven to be a valuable tool for the characterization and identification of functional groups present in compound from plants extract. Infrared spectra was collected using IR (α-ATR Bruker Germany spectrometer) operated form 4000–600 cm^{-1} at resolution of 4 cm^{-1}. Data analyzed using Opus software.

2.5.3. NMR spectroscopy of the isolated compound

Only fraction A16 was additionally elucidated by ^{1}HNMR by using mix solvent D_6 + $CDCL_3$ MIX. The analysis was done at the BRUKER instrument of 400 MHz.

2.5.4. GC-MS spectrometry

The fraction no A16 (by column chromatography) was used for identification of biomolecules in amla fruit by using GC-MS. Sample was introduced into a GC-MS system (Perkin Elmer, model Clarus 600 gas chromatograph coupled 600C mass spectrometer. The temperature programmed as from 70° to 135 °C at 10 °C/min, from 135° to 220^{0}at 150 °C/min, from 220° to 270 °C at 100/min which was held upto 10 min. Helium gas was maintained at 1.9 mL/min [43].

2.5.5. HPLC of isolated compound

HPLC PU-2080 Plus (Systronics) with UV-2075 plus intelligent detector and HPLC C18 column (250 × 4.6mm, 5μm) was set at 270 nm for estimation of gallic acid. The mobile phase Acetonitrile and 2% Acetic Acid with ratio 40:60 used for elution. Flow of mobile phase and injection loop was set at 1.0 mL/min and 20μL respectively. Quantitative determination of gallic acid content and then diluted in order to have fraction concentrations (FA4 by flash chromatography) in the range 0.01–0.5 mg/mL.

2.5.6. HPTLC of the isolated compound [44]

A Camag HPTLC with Linomat V automatic sample applicator and TLC Scanner III) along with software Win CATS version 1.4.0 was used for analysis. The most suitable solvent system was found to be ethyl acetate: methanol: toluene (8:2:1) for quantitative analysis of gallic acid from amla fruit. Different concentrations of standard gallic acid solution (40–240 ng/spot) were spotted on the HPTLC plate. The sample solution (Fraction FA4 by flash chromatography) having concentration of 0.4 mg/mL is prepared and filtered using (0.22 μ membrane filter Millipore). This concentration is used for the estimation of gallic acid from the dried amla powder.

2.6. Antioxidant activity by DPPH method [45]

Depending on chemical nature of different phytochemicals are extracted in solvents of different polarity to extract phytochemicals and to determine their antioxidant activity of that plant material. Here serial Soxhlet extraction method used for successive extraction with solvents of increasing polarity from non-polar (n-hexane) to more polar solvent (water) solvent to determine their scavenging ability [46, 47].

Here comparative Free radical scavenging capacities of water extract and isolated flash chromatographic fraction were determined in terms of radical scavenging capacity. The scavenging action on 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical on amla fruit water extract and isolated fraction no F00A4 (from flash chromatography) by using procedures reported in the literature [48, 49]. The extract and isolated fraction of different concentrations was mixed with an aliquot of DPPH (1 mL, 0.004% w/v) and analyzed at 517 nm by using UV Visible spectrophotometer. Then the scavenging capacity was calculated using Eq. (1) as:

$$\text{Scavenging activity } (\%) = \frac{(\Delta A517 \text{ of control} - \Delta A517 \text{ of sample})}{\Delta A517 \text{ of control}} \times 100 \quad (1)$$

2.7. Formulation of nanoparticles

2.7.1. Design of experiments and data analysis

As compared to other statistical techniques, which those were based on classical one-variable-a-time, response surface methodology (RSM) has numerous advantages including generation of the various important data using minimum experiment runs and evaluation of the linear, quadratic and interaction effects of the independent parameters on the responses. Therefore, central composite design (CCD), used for estimation of independent factors with facilitation of less number of experiments using a block, with RSM were chosen to design of experiments. The effect of a change in concentration of chitosan and poloxamer 407 on physicochemical properties was executed using factorial design and evaluate the effects of the synthesis parameters namely; amount of chitosan (X1, 1.2 to 3.6 gm) and amount of poloxamer 407 (X2, 0.05 to 0.15 gm), on the response variables particle size (Y1) and zeta potential (Y2) of the formed GA-loaded Chi nanoparticles.

2.7.2. Preparation of nanoparticles

The nanoparticles of isolated gallic acid from amla extract with GMO/Chitosan system formulated by use of probe sonicator and high pressure homogenizer followed with freeze drying. As per factorial design batch number one used for formulation of nanoparticles. Gallic acid (100 mg) was dissolved in molten 1.75 mL GMO. A 12.5 mL of 0.1% of poloxamer 407 was added drop wise and simultaneously sonicated at 18 W for 3 min. To this primary emulsion, 2.4% chitosan low-molecular-weight (in acetic acid) solution was added. The emulsion was subjected to nine cycles of HPH at 15,000 psi and dried in rotary evaporator to give the final nanoemulsion. Purification was carried out by using dialysis membrane (molecular weight cutoff 12 kDa; Sigma Aldrich). The dialyzed product was freeze-dried for 48 h using 2% mannitol as the cryoprotectant to obtain a fine powder of nanoparticles. The whitish lyophilized product was then stored at 4 °C in a refrigerator [50, 51].

2.8. Characterization of nanoparticles

2.8.1. Particle size and charge measurements [52]

Dynamic light scattering and electrophoresis principles were used for determination of particle size and electrical charge on them.

2.8.2. By FTIR spectroscopy

FTIR has proven to be a valuable tool for identification of functional groups present in different constituents of plants. Attenuated total reflection/Fourier transform infrared spectroscopic (ATR/FTIR) spectra was collected at room temperature by coupling ATR accessory to an FTIR spectrometer (Perkin Elmer, Spectrum 100).

2.8.3. Differential scanning colorimetry (DSC) [53]

The phase behavior of gallic acid, poloxamer 407, chitosan, physical mixtures and GA-loaded Chi nanoparticles scanned using instrument (DSC Q10, TA Instruments, DE, USA) at 10 °C/min and 50 mL/min flow. Samples were analyzed in triplicate.

2.8.4. X-ray diffraction analysis (XRD) [54]

The XRD graphs of gallic acid, poloxamer 407, chitosan and GA-loaded Chi nanoparticles were recorded on a Bruker D8- Advance diffractometer (Bruker AXS Inc., Madison, WI, USA) with backgroundless sample holders and operation at 40 kV and 30 mA.

2.8.5. Scanning electron microscopy (SEM) [55]

To understand the surface form, nanoparticles were subjected to Scanning Electron Microscope (XL 30; Philips, Eindhoven, The Netherlands) after drying on aluminum disc at ambient temperature.

2.9. Determination of % entrapment efficiency and % drug loading [56]

The effect of a change in concentration of chitosan and poloxamer 407 on the EE and LC of the system was investigated by central composite design. (Data not shown here) GA-loaded Chi nanoparticles (20 mg) were completely dissolved in 10 mL of DMSO to dissolve nanoparticle coating. Residue washed after solvent evaporation and diluted suitably. Mixture is vortexed for 24 h at 37 °C and centrifuged at 16,000 g for 10 min. Absorbance of diluted supernatant was determined at 270 nm in UV spectrophotometer. The amount of gallic acid encapsulated in nanoparticles (% EE) and (% LC) calculated as equation number (2) and (3) follows,

$$\%\ \text{Efficiency of Entrapment} = \frac{[\text{Drug}]\text{total} - [\text{Drug}]\text{free}}{[\text{Drug}]\text{total}} \quad (2)$$

$$\%\ \text{loading capacity} = \frac{[\text{Drug}]\text{total} - [\text{Drug}]\text{free}}{[\text{Weight of nanoparticle}]\text{total}} \quad (3)$$

2.10. In vitro release studies [57]

The *in vitro* dialysis release profile of GA-loaded Chi nanoparticles was performed in different pH environments of gastrointestinal tract. The experiments relatively carried in pH 2.0 (to mimic fasted stomach) and phosphate buffer pH 4.5, 6.8, 7.4 (which mimic duodenum, jejunum, ileo-colon respectively). Nanoparticles equivalent to 4 mg were placed in dialysis bag (mole wt. cut-off 12000 Da; Sigma Aldrich) used for release studies which was then immersed in release medium [58]. Experiment was carried out in shaking incubator at speed of 90–100 rotations per minute. 5 mL aliquot collected and replaced with fresh media to maintain sink condition and further processed to analyze UV spectrophotometrically at 270 nm.

2.11. Physical stability of nanoparticles [59]

To ensure stability of nanoparticles, accelerated stability was performed according to ICH guideline Q1A (R2). A dry powder of GA-loaded Chi nanoparticles was kept in vials sealed with paraffin film which were placed in stability chamber at temperature of 25 ± 2 °C and relative humidity 60 ± 5%

3. Result

3.1. Soxhlet extraction method [60]

Parameters for selecting an appropriate solvent for Soxhlet extraction as therapeutic value lies in non-polar constituents or bioactives, then a non-polar solvent is used. The high value of Total Extractable Content in polar solvent indicates the presence of more polar and water soluble components in bean seeds as compared to non polar ones. This applies the “like dissolves like” principle. Successful determinations of active phytochemicals from plant material is largely affected by the type of solvent used during extraction The extraction yield for chloroform, ethanol and ethyl acetate were found to be 27.08%, 35.45% and 42.51% respectively. After separation, ethyl acetate (high yield as compare to other solvents) part was concentrated and recrystallized with ethanol to get pure crystalline compound [27, 61].

3.2. Total phenolic content

The most abundant gallic acid in the extracts was anthecotuloide, novelty of our findings are the more phenolic content was found in of the ethyl acetate extract of amla fruit. Calibration curve from gallic acid showed linear equation at $y = 0.014x+0.395$, $R^2 = 0.996$). The content of phenolics in different solvents was as 25.73 ± 0.21, 42.09 ± 0.19 and 63.76 ± 0.29 mg GAE/g for chloroform, ethanol and ethyl acetate respectively [62].

3.3. Isolation and purification of bioactive molecule from amla fruit

3.3.1. Gas chromatography

The amounts of organic volatile impurities present in the residue powder after extraction were found to be 1305.3 ppm within the ICH limits. Hence the formulation was considered to be safe for the consumers and thus can be taken up for further *in vitro*, *in vivo* and clinical studies [63].

3.3.2. Thin layer chromatography

Mobile phase with ethyl acetate: methanol: toluene (8:2:1) screened for better separation. R_f value for sample was found to be 0.86 and compared with standard reference compound having 0.86 run in same respective solvent system under TLC densitometer [64].

3.3.3. Separation by using column chromatography

From glass column all fractions (A1 to A20) were concentrated in vacuo and bands observed were of solvent recovered by simple distillation of fractions [65]. The mobile phase, consisting of ethyl acetate: methanol: toluene (8:2:1), was the one that best separated compound for fraction no A16 again on TLC and further characterized by IR, ^{1}HNMR, and GCMS techniques.

3.3.4. Fractionation of bioactive compound by flash chromatographic technique

The UV spectra of fraction no FA4 phytoconstituent which gives absorbance at 270.5 nm also this absorbance confirmed with standard spectra (272 nm) and further characterization by HPLC and HPTLC. The percentage yield of gallic acid in fraction FA4 was found to be 33.4 mg/g.

3.4. Structural clarification of the bioactive molecules

The band of Fraction No.A16 from column chromatography and fraction no FA4 by flash chromatography were filtered, dried and stored at 4 °C for characterization of UV- Spectroscopy, FT-IR and ^{1}H-NMR, GC-MS, HPLC and HPTLC techniques [66].

3.4.1. UV-Visible Spectroscopy

Fraction A16 from column chromatography was detected by using UV/Vis spectrophotometer. The given sample solution scanned under UV and absorbance was recorded λ max at 270 nm [67].A good linearity was found from 2 to 18 μg/mL for gallic acid, and the linear regression equation was $y = 0.059x+0.018$ with correlation coefficient $r^2 = 0.995$.

3.4.2. FTIR spectroscopy of the isolated compound

The fraction A16 was scanned and peaks identified at 1697 cm^{-1} is considered the mainly significant as it is explicit to only for phenols. Peaks at 3268 cm^{-1} and 1697 cm^{-1} indicates the carbonyl absorption in carboxylic acid and at 1609 cm^{-1} with stretching of carbon-carbon in alkenes [67].

3.4.3. NMR spectroscopy of the isolated compound

^{1}H NMR for fraction A16 confirms the presence of gallic acid as it shows aromatic, acidic, and hydroxyl proton (Figure 1). 7 carbons in arrangement as d 9.136 (1H, H-7, s), 7.08 (1H, H-2, H-6, s) and 5.011 (1H, H-3, H-4, H-5, s) molecular formula as $C_7H_6O_5$. [68].

3.4.4. GC-MS spectrometry

The molecular weight (m/Z) gives major characteristic fragments of fraction A16 from column chromatography at 170.12 (M+, C6H6O+5), 155.1 (100 %, M + -1) and 125 (40 %, M + -COOH) (Figure 2). The molecular weight of isolated compound gives characteristic peak was confirmed by (m/e 170.12 g/mol) [69].

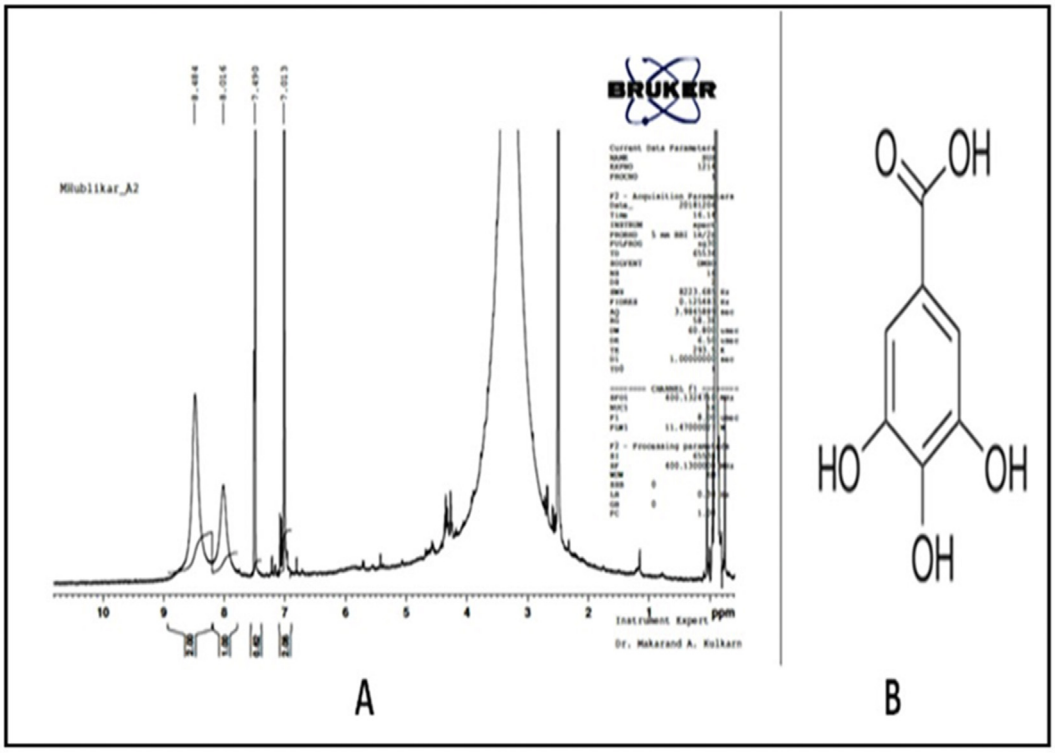

Figure 1. NMR Spectra of (A) Isolated compound, (B) Structure of compound (Gallic acid).

3.4.5. HPLC of isolated compound

The mobile phase selected as 40:60 (ACN: 2% Acetic Acid). A comparison between the spectra of standard gallic acid peak at 3.207 min (Figure 3A) and fraction no FA4 by flash chromatography peak at 3.165 min (Figure 3B) confirmed with that of at 270 nm. Separation carried at flow of 1.0 ml/min and injection loop as 20 μL A good linearity was found from 5 to 15 μg/mL gallic acid, and the linear regression equation was y = 8008x-397.0 (rc = 0.999). The gallic acid from amla fruit extract was fractionated by HPLC of which 27.15 ± 0.001 μg/mg GAE [70].

3.4.6. HPTLC of the isolated compound

Concentrations in the range of 40–240 ng/spot for plotting calibration curve of gallic acid (Standard) at 270 nm and R_f values shown in (Figure 4 A and B). The stock solution of the sample fraction no FA4 by

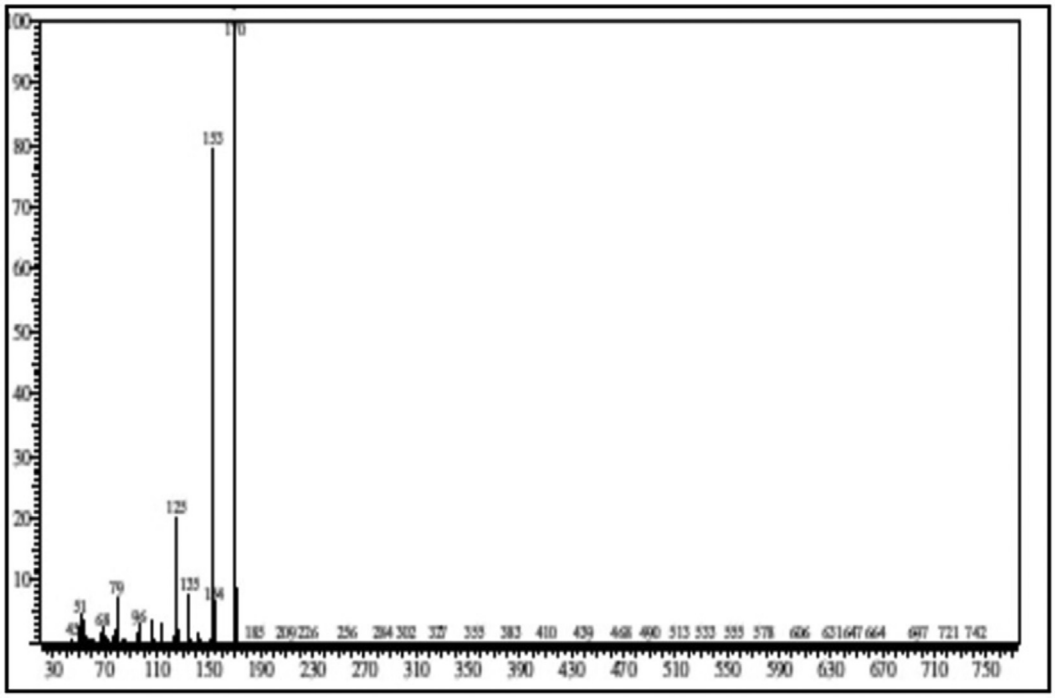

Figure 2. GC-MS Spectra of isolated compound.

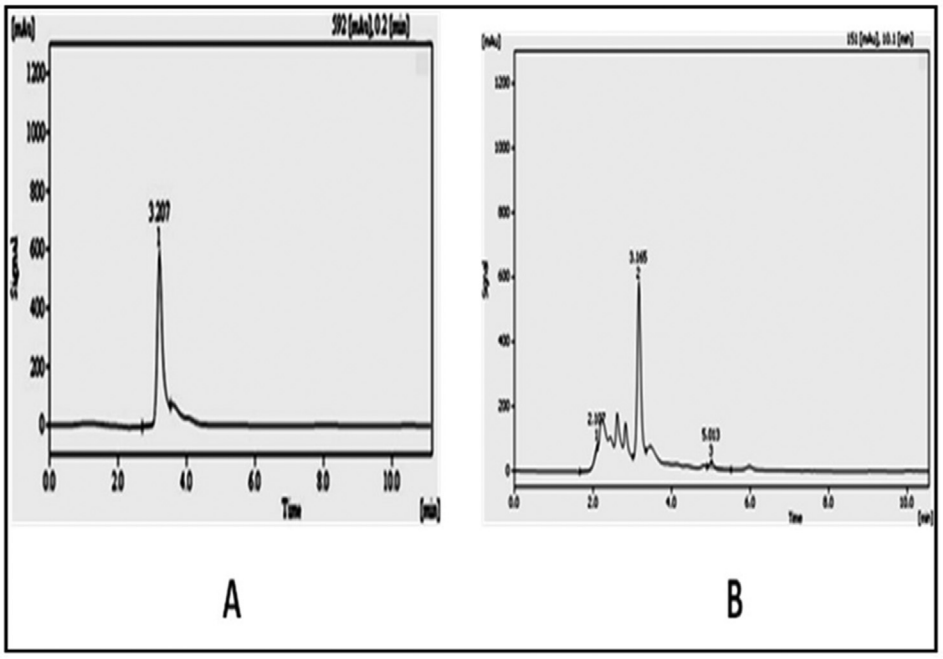

Figure 3. HPLC Profiles (A) Chromatogram of Standard gallic acid, (B) Chromatogram of amla extracts.

flash chromatography having concentration of 0.4 mg/mL is prepared. Track 1 (Figure 4C) showed (2 μL of standard) with their Rf value (0.84) is visible in test solution track at (0.83). Track 2 (Figure 4D) showed (5 μL of Sample) that total number of spot was three, respective Rf values: (0.33, 0.57 and 0.83) The regression analysis has shown good linear relationship with $y = 7.333x\text{-}39.73$ with $r^2 > 0.999$ for gallic acid at 270 nm [71].

3.5. Antioxidant activity by DPPH method [72]

Ascorbic acid used as standard and its IC_{50} was found to be 8.98 μg/ml. Effective concentrations at 50% (IC_{50}) were calculated from regression equations of calibration plots $y = 0.619x+0.528$; $r2 = 0.884$ with IC_{50} was found to be 25.74 μg/mL for amla extract and for fraction no A004 regression equation of calibration plot $y = 0.017x+0.528$; $r^2 = 0.904$ with IC_{50} was found to be 14.44 μg/mL [73, 74].

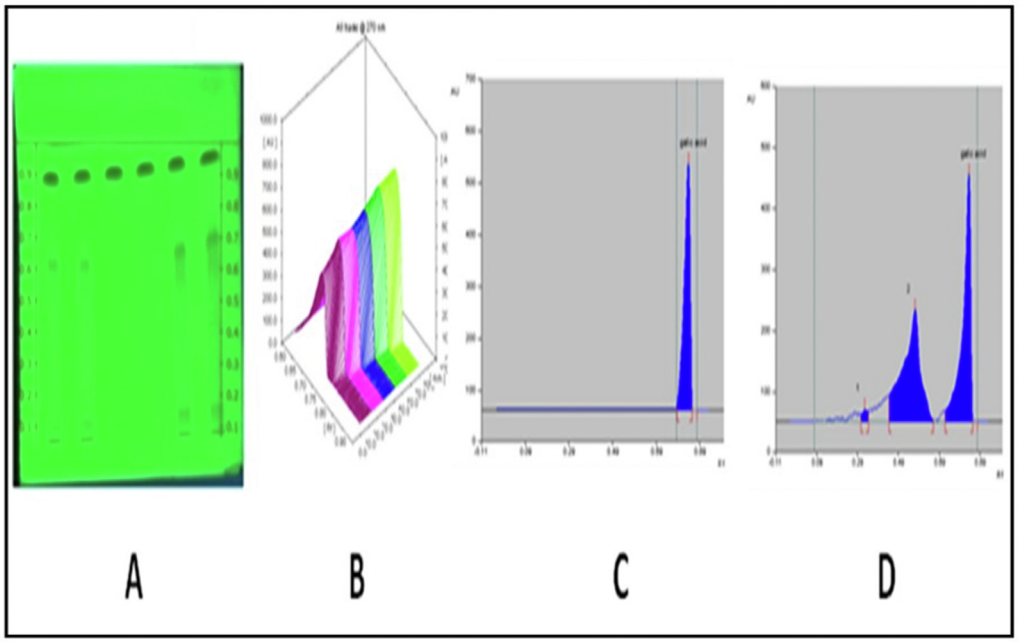

Figure 4. HPTLC Profile of amla fruit extracts (A) Fingerprinting profile, (B) 3 D display at 270 nm, (C) Chromatogram of standard gallic acid, (D) Chromatogram of extract.

3.6. Formulation of nanoparticles using central composite design

The goals for optimization in this study were to minimizing particle size and zeta potential. The optimum condition/central points of the best combination regions (optimum) corresponding to the following processing factors: Chitosan (gm) = 2.4 and Poloxamer 407 (gm) = 0.1. These conditions estimated the formulation of nanoparticles with (nm) = 218, and Zeta potential (mV) = 11.50. The values of central point given by overlay plot matched with batch F5, for that reasons batch F5 selected as optimized batch.

3.7. Characterization of GA-loaded chi nanoparticles

3.7.1. Analysis of particle size and zeta potential

0.1% poloxamer, 2.4% chitosan selected on basis of fact that higher the concentration of poloxamer, up to 0.15% and chitosan upto 3.6% were found to affect particle size in increasing order (Table 1). A mean diameter of gallic acid loaded chitosan particles was 180.8 ± 0.21 nm with zeta potential +24.2 mV.

3.7.2. FTIR of nanoparticles

In the spectrum of gallic acid (Figure 5A) there is a broad band at 3194.61 cm^{-1} related to OH stretching and hydrogen bonds between phenolic hydroxyl groups. The COOH stretch/bend is observed at 1255.93 cm^{-1} Aromatic ring stretching is observed at 1454.44 cm^{-1}. C–O stretching is at 1021.45 cm^{-1} [75] The characteristic groups for chitosan (Figure 5B) at 3285.15 cm^{-1} for OH stretching, 2875.66 cm^{-1} for C– H stretching and 1415.23 cm^{-1} for the amide CN stretching. The bands at 1150.54 cm−1 for asymmetric stretching of the bond C–O–C and 1062.04 and 1023.35 cm^{-1} for vibrations involving the C–O bonds of primary alcohols [76, 77].

The carbon chain of poloxamer 407 (Figure 5C) at 2881.11 cm^{-1} aliphatic C– H stretching, plane O–H bend at 1365.12 $cm^{-1}$and 1242.02 cm^{-1}, C–O stretch at 1096.99 cm-1, CH = CR_2 at 840.46 cm^{-1}.

The C=O functionality of GMO (Figure 5D) was seen with a strong peak at 1738 cm^{-1}. The spectra of the GA-loaded Chi nanoparticles showed that O–H stretch of gallic acid was disappeared (Figure 5E).

3.7.3. Differential scanning colorimetry

For gallic acid (Figure 6A), appearance of sharp endothermic peak at 259.68 °C was observed; which is corresponding to its melting point. Another peak was observed at 91.73 °C; which might be related to loss of water from it, indicating the crystalline nature of pure gallic acid. Poloxamer-407 (Figure 6B) showed the fundamental endothermic peak, pointed peak at 58.92 °C resultant of its melting. Whereas chitosan thermogram depicted a characteristic comparatively broad endothermic peak at about 84.37 °C (Figure 6C) [78]. The thermogram of the physical mixture showed no any changes, it represents just superimposition of the thermal profile of individual components indicating no interaction amongst them (Figure 6D). On contrary, the thermogram of GA-loaded Chi nanoparticles (Figure 6E) showed disappearance of the main peak of drug indicating complete encapsulation of gallic acid inside the polymeric nanoparticles.

3.7.4. X-ray diffracting studies (XRD)

Powder XRD studies showed peak of gallic acid, poloxamer 407 in a crystalline state but chitosan present in amorphous state. Powder XRD studies showed peak of gallic acid, poloxamer 407 in a crystalline state but chitosan present in amorphous state. The characteristic peaks of gallic acid at 8°, 16°, 19°, 33°, 42° and 43° (Figure 7A) Poloxamer 407 gives characteristic peaks at 2θ = 19° and 24° (Figure 7B) Normally, chitosan shows three XRD peaks, corresponding to two different crystalline structures. The hydrated crystalline structure shows a peak at 2θ = 10° (or two peaks at 2θ = 8 and 12°), whereas the anhydrous crystalline structure shows one peak at 2θ = 15°. Chitosan also shows a broad peak around 2θ = 20°, which is due to the existence of an amorphous structure. Here (Figure 7C) chitosan at 20° corresponding to the crystallographic planes [79, 80]. A GA-loaded Chi nanoparticle peaks at (Figure 7D) diffraction angle 2θ (10°, 18°, 21°, 25°, 36°, 41°) in amorphous form when compared with the pure forms it in crystalline state. It confirmed that gallic acid not present in crystalline nature because disappearance of large diffraction peaks and possible to distinguish the characteristic peaks of the gallic acid [81].

3.7.5. Scanning Electron Microscopy

Electron micrographs of the nanoparticulate system with chitosan and GMO were found to be spherical with small cracks with regular distribution depicted in (Figure 8A and B). GA loaded Chi nanoparticles with mannitol as a cryoprotectant in nanoformulation appears to be porous [82].

3.8. Entrapment efficiency and loading content

The effect of a change in concentration of chitosan and poloxamer 407 on the EE and LC of the system was investigated for graphical optimization of maximum Entrapment Efficiency and maximum Loading Capacity. On the basis of overlay plot generated by experiment work were analyzed statistically using Design-Expert 11 software (Stat-Ease Inc., USA). The statistical validity of the polynomials was established on the basis of ANOVA. Counter plot, 3D response surface graphs, normal probability and perturbation plot were generated to study interaction of independent variables with dependent variables. For the optimization of formulation goal is set as the in range maximum, minimum, target and none. Both numerical and graphical optimization techniques are used [83]. 3-Dimensional response surface plot as (Figure 9A) indicates regions were the chitosan and poloxamer 407 in nanoparticle formulation influences the EE of formulation and (Figure 9B) indicates influences the LC of formulation. It showed that EE and LC formulation was found to be

Table 1. Summary of experimental design.

Std., Run	Factor 1	Factor 2	Response 1	Response 2
	A: Chitosan (gm)	B: Poloxamer 407 (gm)	Particle Size (nm)	Zeta potential (mV)
1	1.2	0.05	150	8.34
2	1.2	0.10	155	9.27
3	1.2	0.15	165	10.02
4	2.4	0.05	180	10.22
5	2.4	0.10	218	11.50
6	2.4	0.15	235	12.10
7	3.6	0.05	275	12.41
8	3.6	0.10	290	14.70
9	3.6	0.15	310	15.20

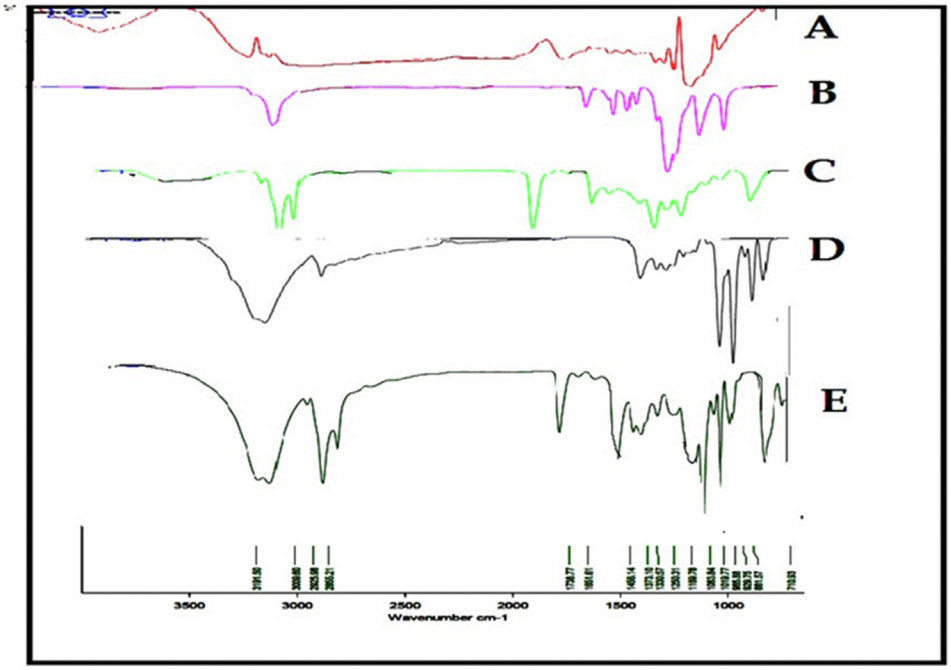

Figure 5. FTIR Spectra of GA-loaded Chi nanoparticles (A) Chitosan, (B) Poloxamer 407, (C) GMO, (D) Gallic acid, (E) Gallic acid NPs formulation.

increased with increasing concentration of chitosan and poloxamer 407 up to certain concentration after that further decrease in EE and LC take place. The over lay plot Criteria for optimization of nanoparticles shown in following Table 2 The values of central point given by overlay plot matched with batch F5, for that reasons batch F5 selected as optimized batch.

Figure 9C show two regions viz, yellow region describing an area of design space with feasible response values and grey region describing an area where response did not fit the quality product criteria. The optimum condition/central points of the best combination regions (optimum) corresponding to the above processing factors chitosan (mg) = 2.4% and poloxamer 407 (mg) = 0.1%. These conditions estimated the formulation of nanoparticles having Entrapment Efficiency (%) = 76.80%, and Loading Capacity (%) = 3.10% with desirability 1.000.

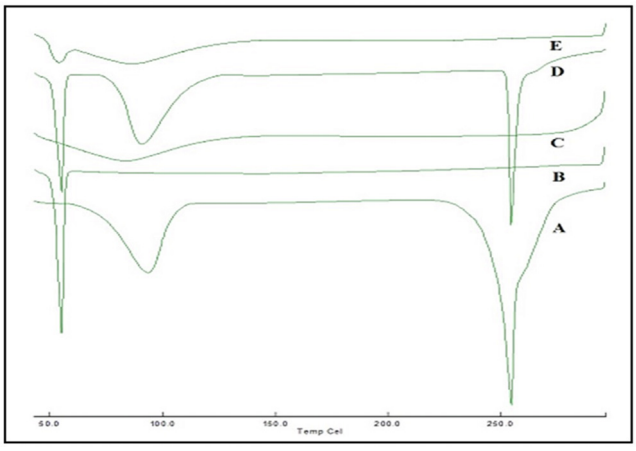

Figure 6. DSC thermogram of GA-loaded Chi nanoparticles (A) Gallic acid, (B) poloxamer-407, (C) Chitosan, (D) Physical mixture, (E) Gallic acid NPs formulation.

3.9. In vitro release studies

It shows better release of gallic acid after 8 h and for 24 h 77.56% in a sustained manner. To assess further drug release behavior, different kinetic models such as zero order, first order models as well as Higuchi's model, Hixson-Crowell cube root law and Peppas exponential model were used. If value of n lies in between 0.5 and 1.0; then drug release follows non-Fickian release, (anomalous) while in case of Fickian diffusion, $n = 0.5$ where as n values greater than 1 it indicate case II transport (Figure 10). At pH 7.4, gallic acid releases from nanoparticles were found to follow Peppas model and zero order release case II transport ($n = 2.09$) [84].

3.10. Physical stability of nanoparticles

Physical stability of formulation is fundamental for long duration storage of products. It is associated with performance of product over period of time. To assess the physical stability particles size and zeta potential is measured at predetermined intervals. GA-loaded Chi nanoparticles were found stable under stated conditions of temperature and relative humidity irrespective of stabilizing agent [85].

4. Discussion

The current study clarified that gallic acid extracted, isolated, characterized and formulated into chitosan nanoparticles. The results may provide significant data on the suitability of solvents for the extraction of gallic acid, various solvents used for extraction of gallic acid from amla fruit but as compare to other solvent ethyl acetate best solvent for extraction of amla fruit for determination of Total Extractable Components, Total Phenolic Content and for higher scavenging ability against DPPH hence this is suitable solvent for extraction of phenolics as gallic acid [86]. TLC method is the most basic method for confirming the presence of phenolic compound. For Thin layer chromatography different combination of mobile phases tried from that ethyl acetate: methanol: toluene (8:2:1) screened for better separation of gallic acid

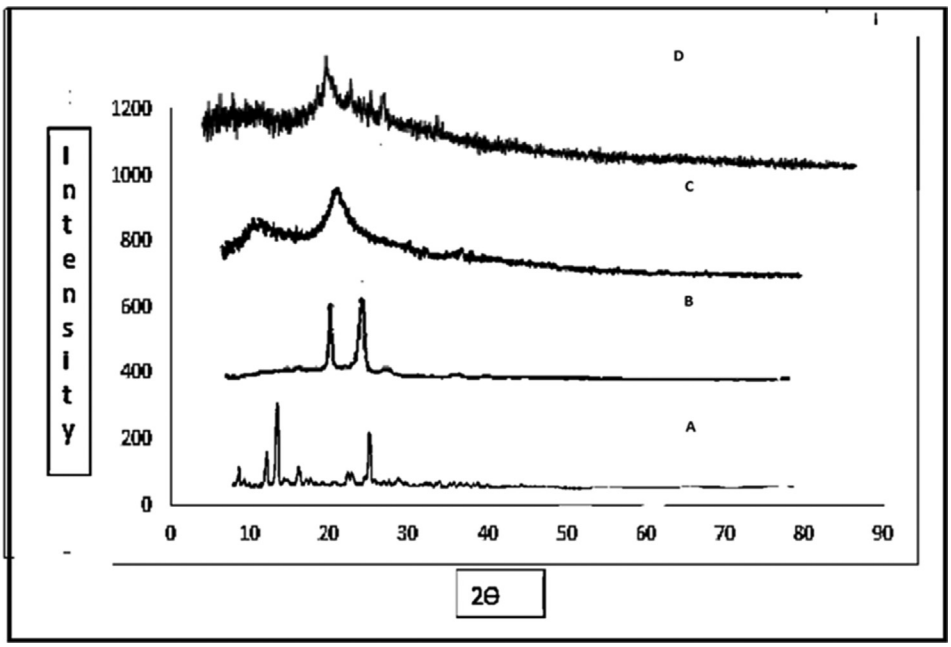

Figure 7. XRD Pattern of GA-loaded Chi nanoparticles (A) Gallic acid, (B) Poloxamer 407, (C) Chitosan, (D) Gallic acid NPs formulation.

from amla [87, 88]. Results of Rf value of TLC and HPTLC expressed the presence of gallic acid in the amla fruit extract [89]. For isolation of gallic acid from amla fruit extract column chromatography carried out by using same mobile phase to get a pure compound which is used for formulation of nanoparticles.

Moreover, the NMR spectroscopy results peak were observed at a slight different location but confirms the existence of interest compound [90]. The gallic acid was identified by comparing the observed GC-MS data and spectra in this work with those found in the literatures. The molecular weight (m/Z) gives major characteristic fragments of fraction A16 from column chromatography [91]. The observed data can correlate isolated composition fraction to phenolic acid [92]. Again for quantitative estimation of gallic acid from amla by HPLC method the mobile phase selected as 40:60 (ACN: 2% Acetic Acid) which gave better results [93].

In the present study, gallic acid estimated by HPTLC technique which results a quick, simple, reliable, reproducible and cheap method was developed for isolation of phenolic compound (Gallic acid) from amla extract this is in agreement with the previous studies [94]. Based on the report of TLC, UV, IR, HPTLC and HPLC amla fruit extracts contain

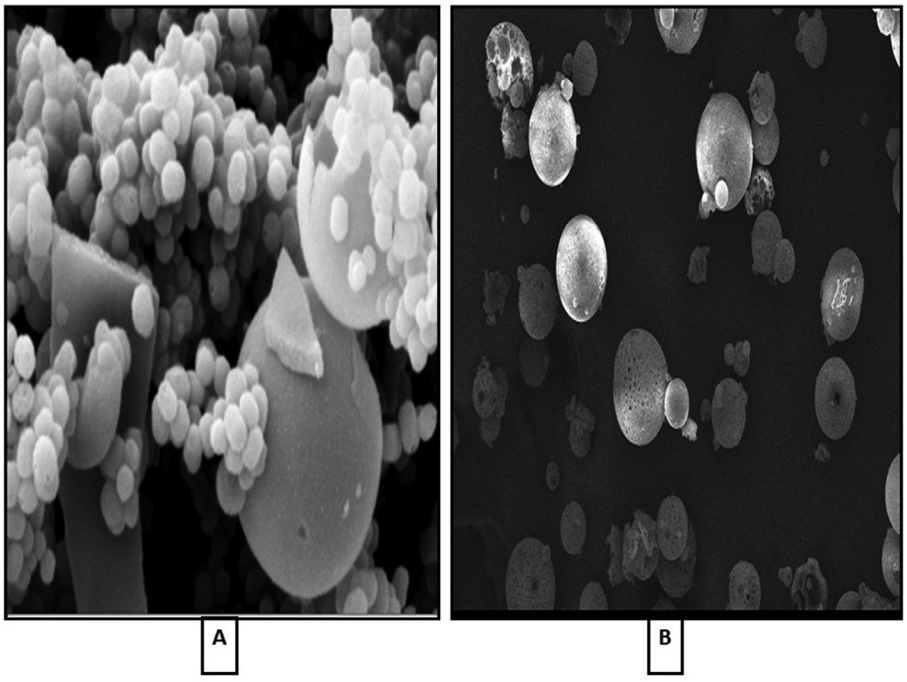

Figure 8. (A) and (B): SEM micrographs of GA-loaded Chi nanoparticles.

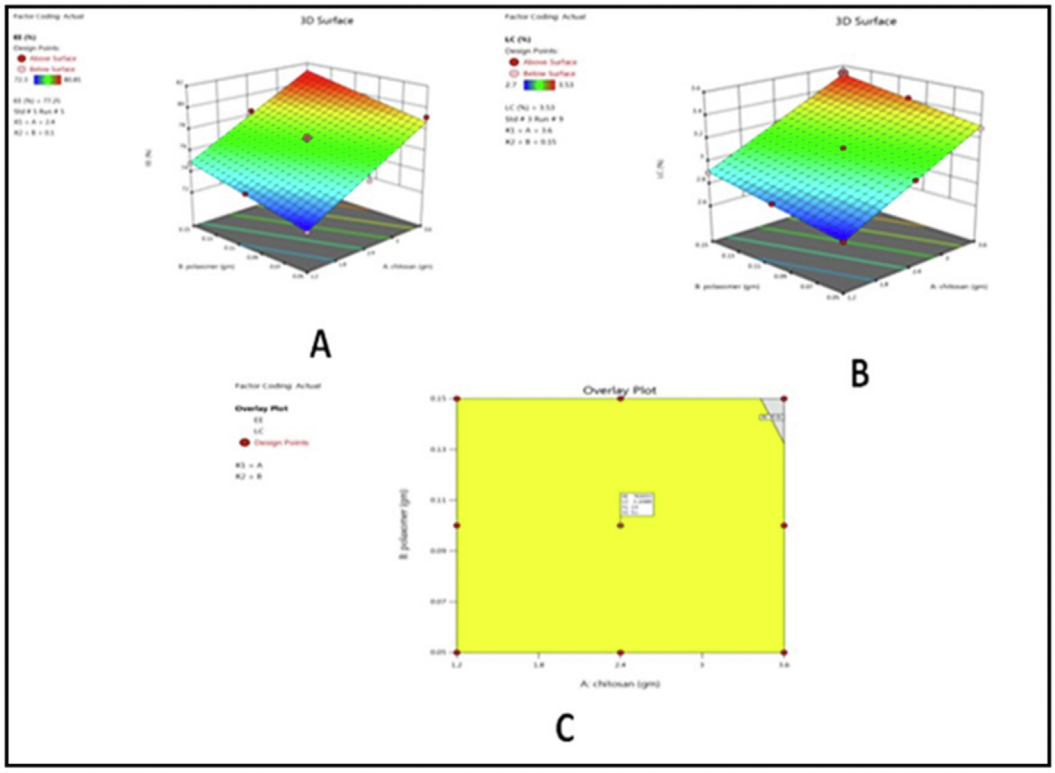

Figure 9. (A) 3-D plot of Encapsulation efficiency, (B) 3-D plot of loading Content, (C) Overlay plot showing optimized batch.

Table 2. Summary of experimental design.

Std., Run	Factor 1	Factor 2	Response 1	Response 2
	A: Chitosan (gm)	B: Poloxamer 407 (gm)	Encapsulation Efficiency (%)	Loading Content (%)
F1	1.2	0.05	72.30	2.70
F2	1.2	0.10	73.70	2.81
F3	1.2	0.15	74.80	2.90
F4	2.4	0.05	74.89	3.01
F5	2.4	0.10	76.80	3.10
F6	2.4	0.15	78.43	3.20
F7	3.6	0.05	79.15	3.29
F8	3.6	0.10	79.90	3.42
F9	3.6	0.15	80.81	3.53

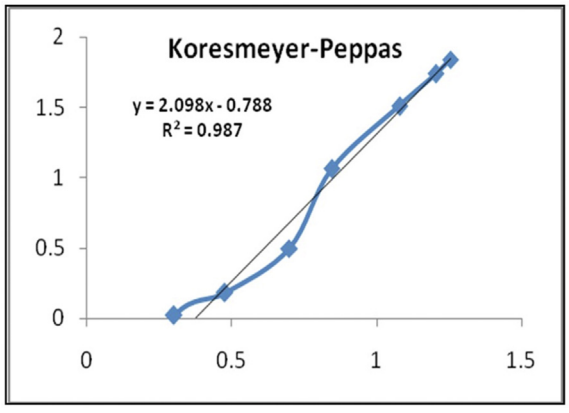

Figure 10. *In-vitro* drug release plot.

remarkable levels of gallic acid [95]. Moreover, the fraction A4 have maximum DPPH* scavenging activity as compare to extract hence this fraction was selected for further biological activity because of their molecular structures, which include an aromatic ring with hydroxyl groups containing mobile hydrogen [28]. Additionally, it has been revealed that the zeta potential of GA-loaded Chi nanoparticles is positive due to the coating of chitosan.

Here all data mentioned for FTIR results conclude that encapsulation of gallic acid into chitosan nanoparticles with intermolecular hydrogen bonding occurred in the nanoformulation which correlated with the less crystalline compared to gallic acid [96]. There are several studies that have demonstrated that hydrogen bonding can affect the transformation of drug crystal to amorphous state [97]. Thus, XRD data supports the DSC studies which indicated the reduced crystallinity of gallic acid in the prepared GA-loaded Chi nanoparticles with lower values of enthalpy and melting points [98]. Furthermore, Scanning Electron Microscopy result were found to be rough surface and agglomeration which seem to be due

to chitosan as a natural polymer with less elasticity compared to synthetic polymer [99].

It is expected that studies with natural products of *in vitro* release of gallic acid from GA-loaded Chi nanoparticles showed negligible exploded release, due to inexplicable surface binding or strongly bound chitosan coating [100]. It has been shown that the stabilized particle sizes and surface charges can benefit the drug by ameliorating their biodistribution with enhancing passively targeted delivery [101]. To date, chemotherapies in many post-clinical studies with natural compounds directed against colorectal cancerous cells are unfortunately very limited. Further research is warranted to identify the specificity and target ability, as well as precise molecular mechanisms of this natural compound against colorectal cancer, while sparing non-cancerous/normal cells healthy.

5. Conclusion

From all our findings, it can be concluded that work will facilitate extraction, design and fabrication of nanoparticles for protection and sustained release of gallic acid biomolecule particularly to colonic region. FT-IR, DSC, XRD and SEM studies suggested that there was no chemical interaction between gallic acid and chitosan polymer. The results suggest the suitability of polar solvents for the extraction of antioxidant compound from amla fruit, with high values of Total Phenolic Content in non-polar solvents indicate that most of the phenolic compounds present in amla fruit are non-polar in nature. The study also suggests that antioxidant effectiveness of isolated fragment of amla, as biomolecule gallic acid is more effective than entire plant matrix.

The nanoparticle system comprised of polymer along with stabilizer to construct Glyceryl monooleate/chitosan nanostructure has been found altered physicochemical properties of gallic acid. Model hydrophobic drug with nano particle size range, positive charge on particle with good value and sustained *in-vitro* gallic acid release especially in wide pH range of entire gastrointestinal tract from nanoparticles were special findings associated with studies. Therefore, discovery and development of new nanoformulation based on natural products have been the focus of much research and proposed carrier system can be beneficial to target the gallic acid to colorectal region.

Declarations

Author contribution statement

Poournima Patil: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Performed the experiments; Analyzed and Wrote the paper.

Suresh Killedar: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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