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Chapter

Colon Available Bioactive Compounds Exhibits Anticancer Effect on *In-Vitro* Model of Colorectal Cancer

Poounima Patil and Suresh Killedar

Abstract

The current work was addressed to characterize gallic acid from amla fruit and quercetin from peels of pomegranate fruit and formulated into Chitosan (CS) nanoparticles and to evaluate their cytotoxicity towards human colorectal cancer (HCT 116) cell lines. Identification of the biomolecules was performed by chromatographic and spectroscopic techniques and characterization of gallic acid and quercetin loaded chitosan nanoparticles carried out by using FT-IR, X- ray diffraction, entrapment efficiency and loading content confirmed successful encapsulation of biomolecules into nanoparticles. *In vitro* drug release studies done by using simulated fluids at various pH (1.2, 4.5, 7.5, and 7.0) to mimic the GIT tract and achieved drug releases 77.56% for gallic acid 79.06% for quercetin at 24 hr. in a sustained manner. The human HCT116 cell line by MTT assay results inferred that the synthesized CS nanoparticles demonstrated shows more effective antiproliferative potential with IC_{50} value of 36.17 ug/ml than polyherbal extract 60.32ug/ml.

Keywords: gallic acid, quercetin, chitosan nanoparticles

1. Introduction

The cancer is one of the most dreaded and threatening diseases in the world, causing more than 6 million deaths a year [1]. Colon cancer is recognized as the third most common cancer worldwide with high morbidity and mortality, and the fourth common cause of death [2]. Various cytotoxic drugs are used for the treatment of colorectal cancer like 5-Fluorouracil, Oxaliplatin and Cisplatin drugs are their hydrophobic nature and their susceptibility to develop drug resistance [3, 4].

In these current work great efforts for the discovery and development of nanoformulation based on natural products on *in vitro* HCT 116 cell lines study. Here gallic acid isolated from amla fruit (*Emblica officinalis*) and quercetin isolated from peels of pomegranate fruit (*Punica granatum*). Gallic acid 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 [5, 6]. Gallic acid exerts an anticarcinogenic activity, antiproliferative and antiapoptotic activities against pancreatic cancer cells [7–9]. Flavonoids are a group of naturally occurring class of plant secondary metabolites having polyphenolic

structure in addition quercetin has anti-tumor properties, anti-inflammatory, anti-proliferative, anti-angiogenic [10–15].

There have been particular efforts to evaluate the therapeutic role of these active constituents present in plants rather than using whole extracts. The fundamental method of reasoning behind these systems contributes greatly to enhance the targeting delivery and bioavailability of phenolic and flavonoid remarkable formulation development can be made by preparation of nanotechnology products. The eco-friendly synthesis of gallic acid and quercetin loaded chitosan nanoparticles (CS nanoparticles) through green route from plant extracts have renowned a wide range of application in the field of modern science, due to increased drug efficacy and less toxicity in the nanosized mediated drug delivery model. At the same time, the use of gallic acid and quercetin in pharmaceutical formulations is limited due to its poor water solubility, poor bioavailability and instability in physiological medium [16, 17].

Lack of site specificity is one of the major reasons for the drug in reaching the target site in therapeutic concentrations in colorectal cancer [18]. Chitosan (CS), as the only naturally occurring positive charge polysaccharide, has remarkable properties including high bioavailability, super biodegradability, high biocompatibility, non-toxicity etc., On the other hand it causes sustained release of the drug from the particle in the tumor environment [19]. The main rationale behind using these types of polymers is their ability to prevent drug degradation in the gastric environment in the stomach and their ability to release the drug after entering the distal ileum [20]. Poloxamer 407 is a hydrophilic nontoxic copolymer used for its stabilizing properties and incorporation of hydrophobic drugs capability to increase the solubility of biomolecules [21]. Here combined biomolecules synergistic activity of nanotechnology approach has been developed to improve the bioavailability as to entrap these natural biomolecules into biodegradable polymeric CS nanoparticles [22].

The system of glyceryl monooleate (GMO)/chitosan is a surface-modified nanoparticulate system consisting of GMO as a lipid portion and chitosan as a coating polymer to target colonic area with poloxamer 407 as a stabilizer. Therefore, the purpose of this study was to formulate CS nanoparticles of where quercetin isolated from peels of pomegranate fruit and gallic acid isolated from amla fruit as a model hydrophobic biomolecules followed by lyophilization using probe sonicator and High Pressure Homogenization (HPH) method. CS nanoparticles prepared and optimized using Quality by design approach by using central composite factorial design. Optimized formulation further characterized for different parameters as particle size, zeta potential, FT-IR and. Release kinetic studies performed using method for conventional nanoparticle release behavior assessment.

In this study, we systematically analyzed the *In vitro* anti-cancer potential of the gallic acid and quercetin loaded chitosan nanoparticles synergy approach for combined active biomolecules and compared to their activity to combined extracts on HCT 116 human colon cancer cell lines and the mechanism of action of CS nanoparticles in regulating the growth of CRC cells. The human HCT116 cell line by MTT (3-(4,5-dimethyl-2-tiazolyl)-2,5-diphenyl-2-tetrazolium bromide) assay was exposed to cytotoxicity of polyherbal extracts, chitosan nanoparticles and cisplatin (Standard) and activity is dependent up to the concentration of 6.25-100ug/mL for 24 h followed by MTT cellular assays [23]. To determine the potential anti-cancer effect of, we synthesized CS nanoparticles using gallic acid and quercetin biomolecules, which is a phenolic and flavonoid predominantly found in amla fruit and peels of pomegranate fruit. HCT116 cells exposed to gallic acid and quercetin for 24 h exhibited significant loss of cell viability and proliferation in a dose-dependent manner.

The IC50 of polyherbal extract, chitosan nanoparticles and standard cisplatin after 48 h treatment it was found to be 60.32 and 36.17 and 8.915 ug/ml respectively. The obtained result inferred that the synthesized CS nanoparticles demonstrated shows more effective antiproliferative potential on HCT-116cell lines with IC_{50} value of 36.17 ug/ml than polyherbal extract 60.32 ug/ml were discussed briefly in this manuscript.

2. Materials and methods

2.1 Materials

Poloxamer 407 from BASF, Chitosan 90% dda obtained from CIFT Cochin, GMO from Mohini organics, standard gallic acid and quercetin purchased from Loba Chemie., 10% fetal bovine serum (Invitrogen Life Technologies USA). RPMI 1640 and McCoy's 5A medium (Fisher Scientific, Waltham, USA). All the solvents and chemicals used were procured from Himedia Laboratories, Research Lab. Mumbai.

2.2 Plant material

The sample of different parts of plant of amla and pomegranate was collected from Kolhapur district and was authenticated by Dr. Madhukar Bachulkar, Principal, Arts and Science College Peth Vadagaon Kolhapur. The voucher herbarium (PSP-1 and 2) has been deposited in the department of Pharmacognosy Bharati Vidyapeeth College of Pharmacy, Kolhapur. Amla fruit and peels of pomegranate fruit were collected in season (Feb-March), were dried under shade for 10–15 days in air.

2.3 Soxhlet extraction method

In order to extract Flavonoid and phenolics from plants with a high degree of accuracy, various solvents of differing polarities were tried as chloroform, ethanol and ethyl acetate. The dried powder of amla fruit and peels of pomegranate powder extracted with 800 ml in various solvents for 6 hours separately. All extracts were filtered and evaporated to dryness under reduced pressure at 60 °C by a rotary evaporator and to determine percentage yield for all three different solvents [24].

2.4 Phytochemical screening

2.4.1 Qualitative test

Phytochemical analysis was carried out to detect the presence of primary and secondary metabolites were used to identify the biomolecules present in the plant extract [25]. The phytochemical tests carried out for amla and peels of pomegranate extract include alkaloids, glycosides, saponins, tannins, triterpenoids, steroid, flavonoids and carbohydrate [26].

2.4.2 Quantitative tests

2.4.2.1 Determination of total phenolic content for amla extract

Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity. Folin–Ciocalteu's method (FC) is a colorimetric

method based on transfer of electrons between reagents and polyphenols. Different solvent extract chloroform, ethanol and ethyl acetate of amla fruit used for determination of phenolic content. The reaction mixture was prepared by mixing 1 ml of methanolic solution of all extracts, 2.5 ml of 10% Folin–Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% NaHCO3. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin–Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of NaHCO3. The samples were thereafter incubated in a thermostat at 45° C for 45 min. The same procedure was repeated for the standard solution of gallic acid (Standard) in methanol (10to100μg/ml) and for blank then calibration line was construed and absorbance measured at λmax 765 nm [27]. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained.

2.4.2.2 Determination of total flavonoid content for pomegranate peel extract

Flavonoids are group of polyphenolic compound used for different activities and their potency depends on the number and position of free hydroxy groups. As a basis quantitative determination, flavonoid contents in pomegranate peel extract were determined using aluminum chloride colorimetric method with sufficient modification. In this process, flavonoid content was determined using quercetin standard (5 to 320 μg/mL) to make the calibration curve. Different solvent extracts chloroform, ethanol and ethyl acetate of pomegranate peel used for determination of flavonoid content. All procedure followed for preparation of different extracts of sample solution, blank and for standard and their corresponding absorbances were measured at 415 nm with a UV-1800 spectrophotometer [28].

3. Techniques of isolation and purification of bioactive molecules from extracts

3.1 Fractionation of bioactive compound by flash chromatographic technique

Flash chromatography instrument consisting of (Analytical technologies limited, Shanghai china) consisting of TBP2H02pump along with TBD2000 UV detector and automatic fraction collector was used for analysis. System equipped with Chromo station software was used for data monitoring during the analysis. The separation was carried out on OROCHEM OROFLO-4SiHPS column made up of silica particles [29, 30].

3.2 Gas chromatography

The gas chromatography used for estimation of residual class 3 ethyl acetate solvent in both crude extracts was performed using a Gas Chromatography system 7890 B with Agilent DB 624 column with helium gas at 1 ml/min flow mode reference solution tetrahydrofuran. GC temperature was set at 50 °C (hold for couple of min) to 250 °C at 20 °C/min. (hold up to 5 min) [31, 32].

4. Structural clarification of the bioactive molecules

The flash chromatographic fractions of amla fraction no FA004 and pomegranate fraction no FP004 were filtered, dried and kept at 4 ° C for characterization of FT-IR and ^{1}H-NMR techniques and quantitavely estimated by HPLC technique [33, 34].

4.1 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.

4.2 NMR spectroscopy of the isolated compound

Only fraction A16 and Fraction P4 was additionally elucidated by ^{1}HNMR by using solvent D_6 + $CDCL_3$ MIX. The analysis was done at the BRUKER instrument of 400 MHz [35].

4.3 HPLC of isolated compounds

HPLC PU-2080 Plus (Systronics) with UV-2075 plus intelligent detector and HPLC C18 column (250 × 4.6 mm, 5 μm) was set at 270 nm for estimation of gallic acid and 259 nm for estimation of quercetin [36, 37]. HPLC PU-2080 Plus (Systronics) with UV-2075 plus intelligent detector and HPLC C18 column (250 × 4.6 mm, 5 μm) was set at 270 nm for estimation of gallic acid and 259 nm for estimation of quercetin. The mobile phase Acetonitrile and 2% Acetic Acid with ratio 40:60 used for elution of both compounds. Flow of mobile phase and injection loop was set at 1.0 ml/min and 20 μL respectively. Quantitative determination of gallic acid and quercetin content in fraction concentrations (FA004 by flash chromatography of amla extract) in the range 0.01 to 0.5 mg/ml and (Fraction FP004 by flash chromatography of pomegranate extract) in the concentration range of 0.01 to 0.5 μg/ml used.

5. Determination of solubility of isolated compound

The two isolated compounds were analyzed for their solubility in different solvents as in DMSO, ethanol, methanol and acetone.

6. Melting point determination

Melting points of two isolated biomolecule compounds was done in thermonic apparatus to determine its identity and purity. The observed melting point of isolated compound was compared with the standard melting point of respective gallic acid and quercetin.

7. Antioxidant activity by DPPH method

2, 2-diphenyl-1-picryl-hydrazyl (DPPH) is a stable organic free radical used to estimate the antioxidant activity of various compounds. The scavenging action on DPPH radical from amla fruit and isolated fraction (FA004 by flash chromatography) and peels of pomegranate extract and isolated fraction (FP004 by flash chromatography of peels of pomegranate extract) determined by following method [38, 39]. As of different concentrations was mixed with an aliquot of DPPH (1 ml, 0.004% w/v) and analyzed at 517 nm. Then the scavenging capacity was calculated using equation number (1).

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

8. Formulation of nanoparticles

An o/w nanoemulsion of gallic acid and quercetin was prepared by using a GMO/chitosan framework as reported by with slight modifications. Briefly, isolated gallic acid (100 mg) and quercetin (100 mg) were dissolved in molten GMO (2 g), then add 12.5 ml of 0.1% poloxamer 407 sonicated at 18 W for 3 min in probe sonicator. To this emulsion, dropwise 12.5 ml of 2.4% chitosan solution was added again using probe sonicator at 16 W for 4 min [40, 41]. Finally this phase was subjected to twelve cycles of HPH at 15,000 psi to give the nanoemulsion. Then, lyophilized with 2% mannitol as a cryoprotectant for 48 hr. Central composite design [42] was applied to examine the combined effect two variables, each at 2 levels and the possible 9 combinations of CS nanoparticles.

9. Characterization of polyherbal nanoparticles

9.1 Particle size and zeta potential

Average particle size and zeta potential of the CS nanoparticles were determined by Particle Size Analyzer (Zetasizer Ver System; Malvern Instruments Ltd., Malvern, UK). To analyze particle size, nanosuspension was diluted with filtered (0.22 lm) ultra pure water [43–45].

9.2 By FTIR spectroscopy

FTIR has proven to be a valuable tool for identification of functional groups present in compound from 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).

9.3 *In vitro* release studies

CS nanoparticles were tested in various simulated fluids at different pH to evaluate the release of nanoparticles at particular pH and also to determine the drug release [46]. Four milligrams of CS nanoparticles were dispersed in a freshly prepared phosphate-buffered saline (PBS; pH = 2.0, 4.5, 6.8, 7.4) as a release medium in a dialysis membrane sac (mw cut-off 12 kDa; Sigma Aldrich) to simulate ileo-colon conditions for 24 hr [47]. The enclosed dialysis sac was immersed in a beaker containing 50 mL of the release medium. The beaker was placed in a shaking incubator at 37 °C under mild agitation (90–100 rpm) PBS; pH = 2.0 for first four hour, pH = 4.5 for next five to nine hour, pH = 6.8 for next ten to thirteen hour and finally pH = 7.4 for fourteen to twenty-four hour. The supernatant 5 ml withdrawn at specified time intervals and assayed for drug release in UV spectrophotometrically gallic acid at 270 nm and quercetin at 259 nm.

10. *In vitro* anticancer activity (cytotoxicity) by MTT assay

10.1 Cell culture

A human colorectal adenocarcinoma cell line (HCT116) were cultured with RPMI 1640 and McCoy's 5A medium (Fisher Scientific, Waltham, MA, USA), respectively [48]. All cell culture mediums contained 10% fetal bovine serum. Cells were incubated

in a CO2 incubator at 37 °C with 5% CO2. After reaching confluency, cells were isolated from the dish with Trypsin–EDTA. The cell suspension was centrifuged at 1000 r/min for 5 min and then re-suspended in growth medium for further experiments.

10.2 Cell viability assay

Cell viability was studied using an MTT assay. Cells were grown in a medium containing 10% FBS, seeded in 96-well plates at a density of $2x10^5$ cells/well, and incubated at 37 °C in CO2 incubator with 5% CO2 for 24 h [49]. Then, polyherbal extract, CS nanoparticles and standard cisplatin were added (final concentrations of 6.25, 12.5, 25, 50, and 100 ug/ml) to the mono-layers of cells, which were subsequently incubated for at 24 and 48 h, media were aspirated and MTT solution at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) buffer was added 20 ml/well. After further incubation (3 h), the media was removed and replaced with 100 ml of DMSO. Plates were washed with 1% acetic acid, air-dried, and then 10 mM Tris base pH 7.4 (150 μl) was added to the wells to solubilize the dye. The plates were shaken vigorously for 5 min and color absorbance was measured at 540 nm using an ELISA microplate reader. (ELISA reader Denver Jasco Model 7800 UV/VIS Spectrophotometer Jasco Tokyo, Japan) Untreated cells were used as positive controls with 100% viability and cells without assay reagents were used as a blank**.**

11. Result and discussion

11.1 Soxhlet extraction method

Soxhlet extraction method carried out for extraction of amla fruit and peels of pomegranate fruit by using three solvents as chloroform, ethanol and ethyl acetate separately. Ethyl acetate solvent gives highest yield 42.51% (Amla fruit) and 42.89% (Pomegranate peels) hence used for extraction of phenolics and flavonoids [50].

11.2 Phytochemical screening

11.2.1 Qualitative tests

Qualitative tests of Phytochemical screening of amla fruit and pomegranate peel extract gives positive test for presence of flavonoids, alkaloids, tannins and carbohydrate.

11.2.2 Quantitative test

11.2.2.1 Total phenolic content

Calibration curve of standard gallic acid showed linear equation at y = 0.014x + 0.395, R2 = 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. As compare to other solvent ethyl acetate gave more yields hence this is suitable solvent for extraction of phenolics.

11.2.2.2 Total flavonoid content

The concentration of flavonoid standard quercetin on the calibration line was based on the calculated absorbance at y = 0.017 × +0.412, R2 = 0.990 (**Figure 1**)

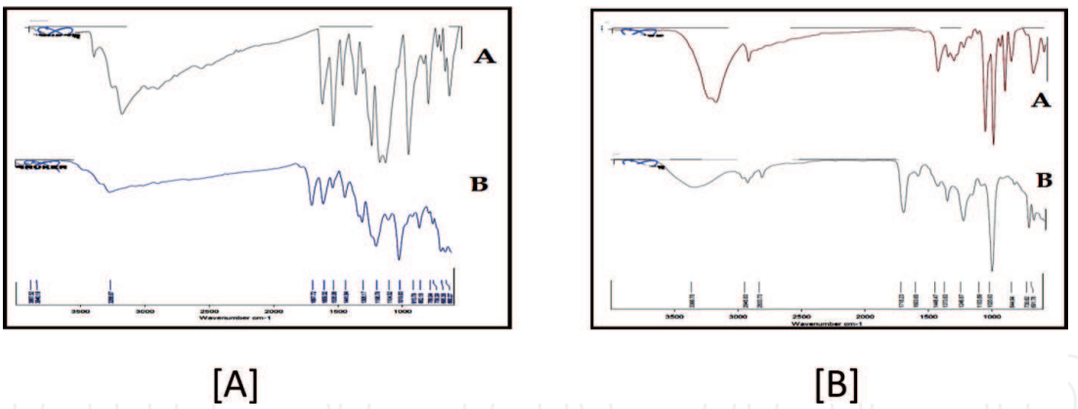


Figure 1.
IR spectra of isolate of [A] Amla fruit [B] pomegranate peel extract.

then, the content of flavonoids in pomegranate peels of different solvent extract was expressed in terms as mg QE/g. The content of flavonoid in different solvents was as 32.88 ± 0.26, 42.11 ± 0.29 and 70.8 ± 0.1732 mg QE/g for chloroform, ethanol and ethyl acetate respectively. As compare to other solvents ethyl acetate gave more yields.

11.3 Techniques of isolation and purification of bioactive molecule from amla fruit and pomegranate peel extract

11.3.1 Fractionation of bioactive compound by flash chromatographic technique

The mobile phase used as ethyl acetate: methanol 100:0 to 0:100 with flow rates were kept at 4 ml/min with wavelength for amla fruit at 270 nm and pomegranate peel extract at 263.5 nm. Column was loaded with 8.0 gm slurry (3 g extract +5 g silica gel) in 25 gm of silica gel (200–400 mesh size).

1 gm of amla extract and peels of pomegranate powder extract separately mixed with 3 gm of silica gel and triturated properly in mortar and pestle. Then, properly mixed extract samples were loaded in sample holder. The separation was completed in 15 minutes only. The Five fractions were isolated by linear gradient with peak tube volume was 14 ml and run time was 15 min. Different fractions no. FA001 to FA005 from amla extract and FP001 to FP005 from peels of pomegranate extract were isolated and dried on buchi roto evaporator (R-210 water bath B-491) for dryness.

Among all five fractions of amla extract fraction number the UV spectra of fraction no FA004 phytoconstituent which gives absorbance at 270.5 nm and this absorbance confirmed with standard gallic acid solution spectra at 272 nm. (**Figure 2A**) The percentage yield of fraction FA004 was found to be 33.4 mg/gm. The five fractions of peels of pomegranate extract fraction no FB004 gives maximum absorbance at 263.5 nm and also this absorbance confirmed by standard quercetin sample absorbance at 345 nm (**Figure 2B**) scanning with UV Spectrophotometry summarized in **Tables 1** and **2** . The percentage yield of fraction FP004 was found to be 42.6 mg/gm. Further these two isolated fractions no FA004 and FP004 characterized for IR, H1NMR, HPLC and HPTLC techniques for better results.

11.3.2 Gas chromatography

The study represented a simple gas chromatographic method for estimation of ethyl acetate contents in both amla and pomegranate extract. The GC analysis of the crude

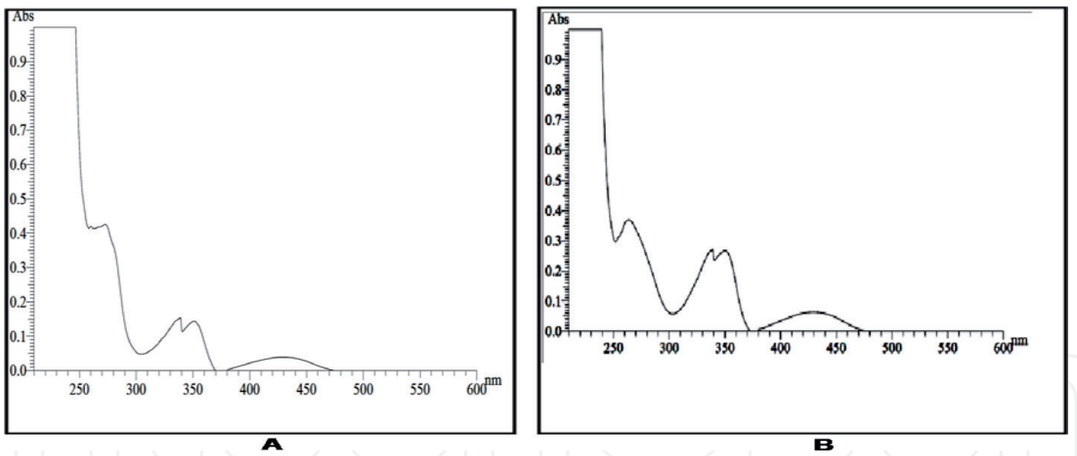


Figure 2.
Flash chromatogram of [A] Amla fruit [B] pomegranate peel extract.

Peak	Start (nm)	Apex (nm)	End (nm)	Height (Abs)	Area
FA001	600.0	430.5	379.0	0.039	1.257
FA002	379.0	350.5	340.5	0.144	2.708
FA003	340.5	339.5	304.0	0.154	3.471
FA004	304.0	270.5	262.0	0.426	11.114
FA005	262.0	239.0	210.0	1.971	70.223

Table 1.
Flash chromatography of amla extract.

Peak	Start (nm)	Apex (nm)	End (nm)	Height (Abs)	Area
FP001	600.0	429.5	379.0	0.064	2.745
FP002	379.0	350.0	340.5	0.266	5.440
FP003	340.5	335.0	302.5	0.272	5.920
FP004	302.0	263.5	252.0	0.369	12.189
FP005	252.0	230.5	210.0	1.893	53.258

Table 2.
Flash chromatography of peels of pomegranate extract.

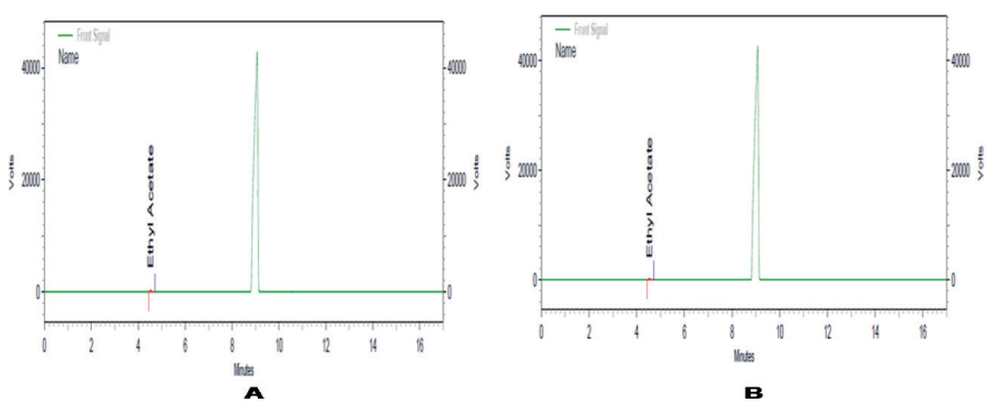


Figure 3.
Gas chromatogram of [A] Amla fruit [B] pomegranate peel extract.

ethyl acetate extracts of amla gives retention time at 4.526 min (**Figure 3A**) and for pomegranate fruit at 4.528 min. (**Figure 3B**) Ethyl acetate concentration in amla fruit was found to be 1305.376 ppm and in pomegranate fruit was found to be 1538.440 ppm. Excellent results were obtained within the worldwide accepted validation reference values and particularly taking into account the low concentration levels investigated [51].

11.4 Structural clarification of the bioactive molecules

The isolated compounds (Fraction No. FA004 from amla extract and FP004 from peels of pomegranate extract by flash chromatography) was characterized by using FT-IR, ^{1}H-NMR and quantitatively estimated by using HPLC technique [52].

11.4.1 FTIR spectroscopy of the isolated compound

FT-IR spectra of isolate of amla fruit extract resulted in presence of functional groups hydroxyl (-OH) stretch, C-H stretch of alkenes, C=O stretch for acid and aromatic benzonoid ring (**Figure 1A**) and FT-IR spectra of isolate of Pomegranate peel extract resulted in presence of functional groups hydroxyl (-OH) stretch at 3366 cm^{-1}, C-H stretch of alkenes at 2945 cm^{-1}, C=O stretch for lactone and aromatic benzonoid ring 1020 cm^{-1} (**Figure 3**).

11.4.2 NMR spectroscopy of the isolated compound

The analysis was done at the BRUKER instrument of 400 MHz 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). ^{1}H NMR of isolate of amla fruit showed the aromatic proton, acidic proton and hydroxyl proton and presence of 7 carbons in structure (**Figure 4A**) given molecular formula as $C_7H_6O_5$ [53] ^{1}H-NMR signals of isolate of Pomegranate peel extract shows signals at 12 (S 1H OH Pyran), 6.2 (S 2H Aromatic OH), 6.9 (S1H Aromatic OH), 7.1 (S1H Aromatic OH), 7–8 (S Aromatic proton) ^{1}H NMR showed the aromatic proton and hydroxyl proton and presence of 15 carbons (**Figure 4B**) in structure given molecular formula as $C_{15}H_{10}O_7$.

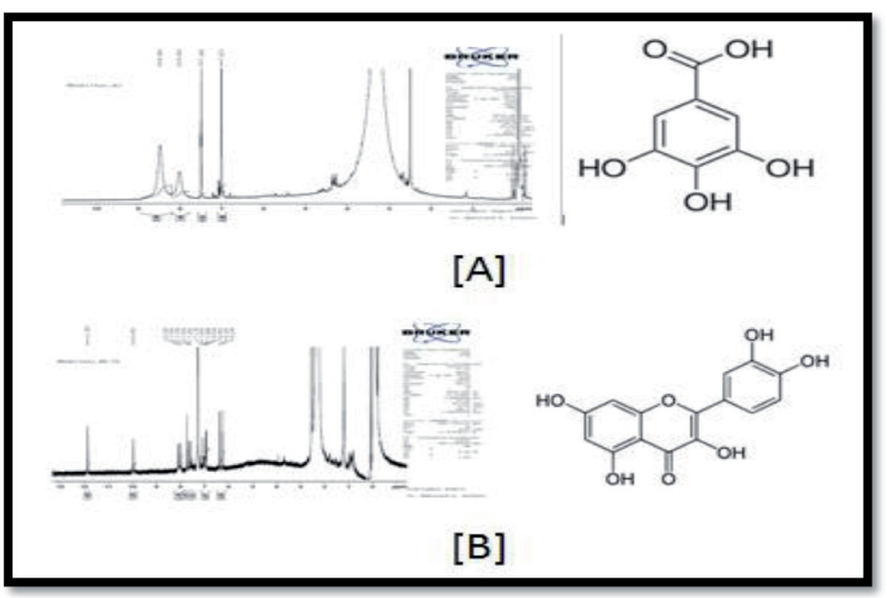


Figure 4.
NMR spectra of isolated compound and structure of compounds [A] Amla fruit (Gallic acid) [B] pomegranate peel extract (quercetin).

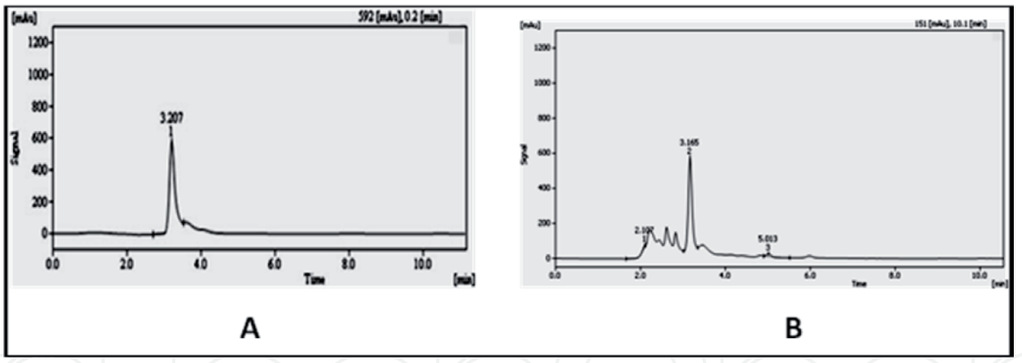


Figure 5.
Chromatogram of [A] standard Gallic acid [B] isolated fraction of amla extract.

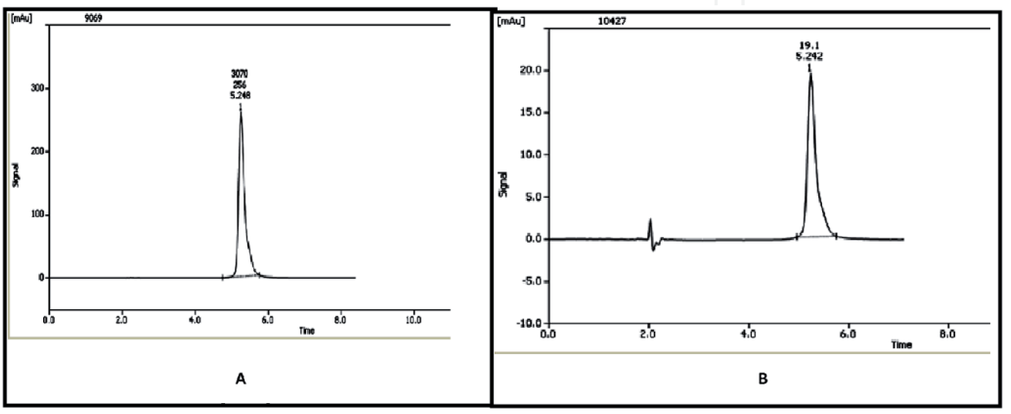


Figure 6.
Chromatogram of [A] standard quercetin [B] isolated fraction of pomegranate peel extract.

11.4.3 HPLC analysis of isolated compounds

A comparison between the spectra of fruits of amla extract (Fraction no A004 by flash chromatography) peak at 3.165 min confirmed with that of standard gallic acid peak at 3.207 min respectively (**Figure 5A** and **B**). A good linearity was found from 5–15 μg/mL gallic acid, and the linear regression equation was y = 8008x-397.0 (rc = 0.999) where y is the peak height. The gallic acid from amla fruit extract was fractionated by HPLC of which 27.15 ± 0.001 μg/mg GAE equivalent by HPLC method were characterized.

Same comparison between the spectra of peels of pomegranate (Fraction no B004 by flash chromatography) peak at 5.242 min with that of standard quercetin confirmed that the retention time of the analyte was 5.248 min respectively (**Figure 6A** and **B**). Linearity for the developed method was found over the concentration range 3–18 μg/ml with a linear regression equation was y = 16.01x + 25628 where y is the peak height correlation coefficient of 0.999.

11.5. Antioxidant activity

11.5.1 Antioxidant activity by DPPH method

The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical-scavenging activities of antioxidants. The scavenging activity on 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical of both the fruits extract and isolated fractions was determined by following method. The extracts of different concentrations were mixed with an aliquot of DPPH (1 ml, 0.004% w/v) [54].

The mixtures were vigorously shaken and left to stand for 30 min in the dark at room temperature. For this method the absorbance were recorded at 517 nm. The percentages of remaining DPPH in the presence of the amla and pomegranate peel extract (**Figures 7** and **8**) and its fractions at different concentrations are shown in **Table 3**.

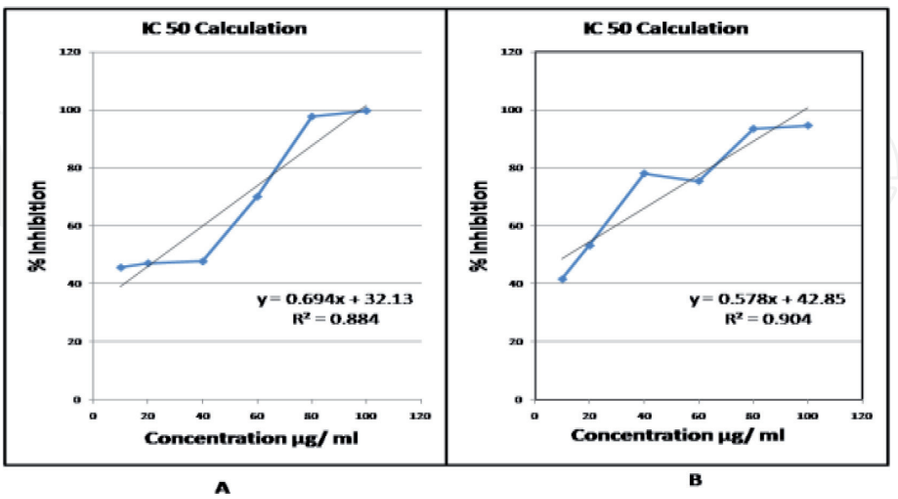


Figure 7.
DPPH radial scavenging activity (A) Amla extract (B) isolated fraction of FA004 by flash chromatography.

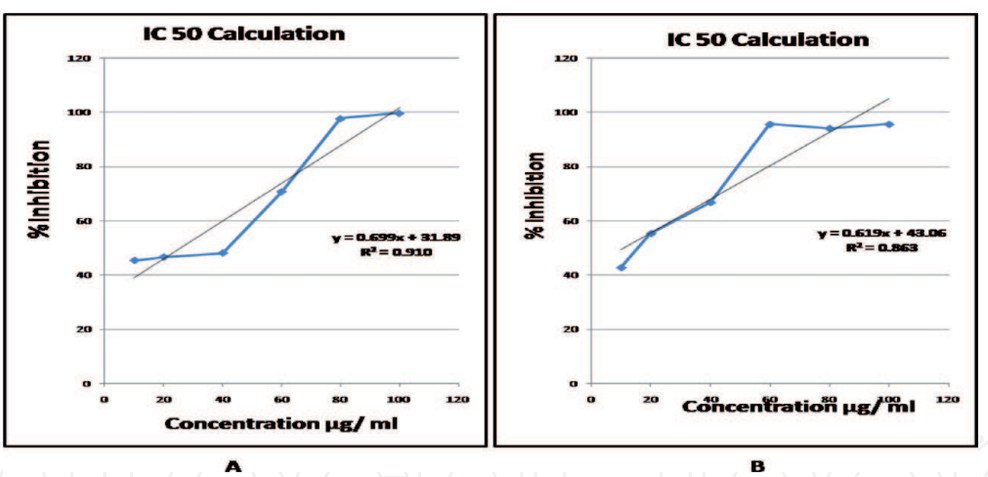


Figure 8.
DPPH radial scavenging activity (A) peels of pomegranate extract (B) isolated fraction no FP004 by flash chromatography.

Sample	R^2	IC_{50}
Ascorbic acid (Standard)	0.996	8.98 µg/ml
Amla extract	0.884	25.74 µg/ml
Isolated Fraction [FA004]	0.904	14.44 µg/ml
Pomegranate peel extract	0.863	29.89 µg/ml
Isolated Fraction [FP004]	0.910	11.21 µg/ml

Table 3.
Antioxidant activity of amla and pomegranate extract by DPPH.

11.6 Determination of solubility of isolated compound

The isolated compound was analyzed for their solubility in different solvents. White colored powder of amla extract (FA004 Flash chromatography) which is soluble in ether, ethanol, methanol, glycerol and acetone. Yellow colored crystalline powder of pomegranate extract (FP004 Flash chromatography) practically insoluble in water and soluble in DMSO, ethanol, methanol and acetone.

11.7 Melting point determination

Melting point of compound was done in thermonic apparatus to determine its identity and purity. The observed melting point of isolated compound of amla extract (FA004 Flash chromatography) was 255–257 ° C compared with the standard melting point (260 °C) of respective isolated gallic acid. The observed melting point of isolated compound of pomegranate extract (FP004 Flash chromatography) was 313–316 °C compared with the standard melting point (316 °C) of respective isolated quercetin.

11.8 Formulation of CS nanoparticles

In this study the goals for optimization were to minimizing particle size and maximum Zeta potential. Desirability ramp showing optimum conditions to formulate CS nanoparticles as chitosan 2.4%, and Poloxamer (407) 0.1% to achieve particle size 218.33 nm and zeta potential11.50 mV with desirability 1.000.

11.9 Characterization of CS nanoparticles

11.9.1 Analysis of particle size and zeta potential

A mean diameter of particle size of CS nanoparticles was found to be 214.2 ± 1.28 nm with +14.7 mV zeta potential [14, 55, 56]. Chitosan on the other hand has a positive charge in acidic solutions due to the presence of protonated amino groups which was appropriate adhere negatively charged intestinal mucus layer. This explains that outer coating of nanoparticles was CS only.

11.9.2 FTIR of CS nanoparticles

The characteristic groups of chitosan at (**Figure 9A**) 3285.15 cm^{-1} for O-H stretching 2875.66 cm^{-1} for C-H stretching and 1415.23 cm^{-1} for amide C-N 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 [57]. The carbon chain of poloxamer 407 (**Figure 9B**) 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 9C**) was seen with a strong peak at 1738 cm^{-1}. In the spectrum of gallic acid (**Figure 9D**) 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} [58]. C- O stretching is at 1021.45 cm^{-1} In the spectrum of quercetin (**Figure 9E**) there is a broad band at 3194.61 cm^{-1} related to OH stretching and hydrogen bonds between phenolic hydroxyl groups. O-H stretch at 3190.38 cm^{-1}, =C-H stretch at 2935.23 cm^{-1}, aromatic C=C stretch at 1454.09 cm^{-1} and aromatic C-O stretch at

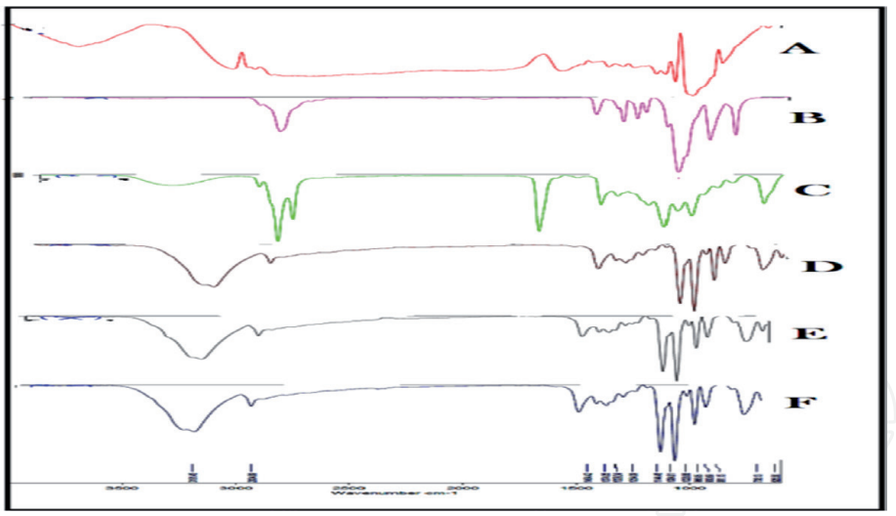


Figure 9.
IR spectra of CS nanoparticles (a) chitosan (B) Poloxamer 407 (C) GMO (D) quercetin (E) Gallic acid (E) CS nanoparticles.

1145.06 cm^{-1}. The COOH stretch/bend is observed at 1255.93 cm^{-1} The spectra of the gallic acid and quercetin loaded CS nanoparticles showed that O-H stretch of gallic acid and quercetin was disappeared (**Figure 9F**). Here all data of FTIR results conclude that encapsulation of gallic acid and quercetin into CS nanoparticles with intermolecular hydrogen bonding occurred in the nanoformulation which correlated with the less crystalline compared to both pure biomolecules.

11.9.3 In vitro release studies

As a result CS nanoparticles have indicated improved drug releases 77.56% for gallic acid 79.06% for quercetin at 24 hr. respectively. So the CS nanoparticles can be considered as a potential barrier, which can release the biomolecules at colonic pH [59]. By engineering chitosan approach gallic acid and quercetin biomolecules achieved sustained and controlled release and also benefitted by its targeting property to colonic region. To describe the mechanism of gallic acid and quercetin release from the CS nanoparticles, [60] the data was plotted into a few kinetic models and best fitted information into the Korsmeyer–Peppas power law model.

11.10 Methods of anticancer activity determination

11.10.1 In vitro cytotoxicity by MTT assay

After 24 hours of incubation, cell viability was determined by the MTT assay. The nanoparticles induced cell cytotoxicity in a concentration dependent manner, as illustrated. Cytotoxicity of polyherbal extracts, CS nanoparticles and cisplatin (Standard) was dose on HCT 116 cell lines and activity is dependent up to the concentration of 6.25–100 ug/mL. The IC50 of polyherbal extract, chitosan nanoparticles and standard after 48 h treatment it was found to be 60.32 and 36.17 and 8.915 ug/ml respectively summarized in **Table 4**.

The antiproliferative potential of all samples shown as cytotoxicity of standard cisplatin (**Figure 10A**) CS nanoparticles (**Figure 10B**) polyherbal extract (**Figure 10C**) was done on HCT 116 cell lines and activity is dependent up to the concentration of 6.25–100 ug/mL. MTT assay determined the cytotoxic effect of all samples by decreasing the cell viability of HCT116 colon cancer cells with different serial dilutions. The half maximal inhibitory concentration (IC50) was evaluated to determine the effectiveness of CS nanoparticles in inhibiting

Name of Samples	Concentrations ug/ml					
	Untreated	**6.25**	**12.5**	**25**	**50**	**100**
Standard (A)	1.157	0.680	0.582	0.19	0.0885	0.06
	IC_{50} = 10.55 ug/ml					
Polyherbal extract (B)	1.157	1.677	1.301	0.794	0.478	0.182
	IC_{50} = 60.32 ug/ml					
CS nanoparticles (C)	1.157	0.906	0.892	0.79	0.141	0.028
	IC_{50} = 36.173 ug/ml					

Table 4.
MTT data analysis of HCT-116 cell lines.

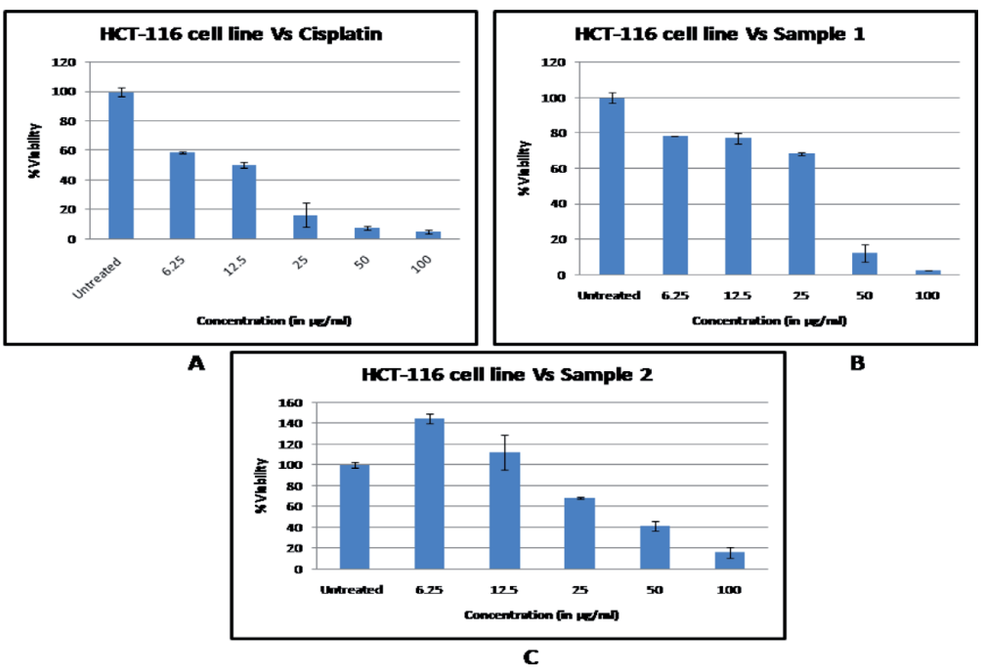


Figure 10.
Concentrations used for MTT assay (A) standard (cisplatin) (B) Polyherbal extract (C) CS nanoparticles.

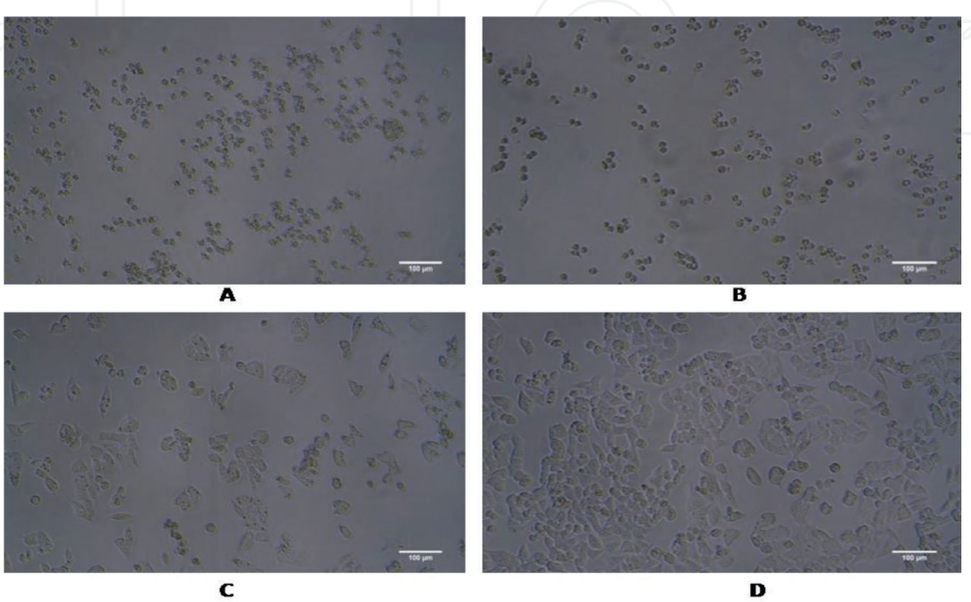


Figure 11.
Microscopy imaging of cellular uptakes (A) standard (B) CS nanoparticles (C) Polyherbal extract (D) untreated HCT116 cell lines.

biological or biochemical functions. CS nanoparticles shows a higher cytotoxic effect on HCT116 cells with low concentrations (IC50 = 36.173 μg/ml) than polyherbal extract (IC50 = 60.32 μg/ml) that might be due to the active biomolecules capped to the nanoparticles.

HCT 116 cell lines considered to have more prominent take-up for CS nanoparticles and more stable even at low concentrations and longer interval than polyherbal extract. Microscopy imaging of cellular uptakes shows as standard cisplatin (**Figure 11A**) CS nanoparticles (**Figure 11B**) polyherbal extract (**Figure 11C**) and untreated HCT116 cell lines (**Figure 11D**) HCT 116 cell lines subjectively were deemed to have had greater uptake for CS nanoparticles and more stable even at low concentrations than polyherbal extract expected to be longer interval than polyherbal extract.

12. Conclusion

In conclusion, the presence of phenolic compound (gallic acid) and flavonoid (quercetin) could be one of the contributing factors for mechanism of *in vitro* studies on HCT 116 cell lines. Model hydrophobic biomolecules with nanoparticle size range, positive charge on particle with good value and sustained in-vitro releases of gallic acid and quercetin especially in wide pH range of entire gastrointestinal tract from nanoparticles were special findings associated with colonic site. Therefore, discovery and development of new nanoformulation based on natural products have been the reported to have a controlled effect on cancer cell lines; therefore, they have the potential to be used as an important therapeutic anticancer biomolecules. Further studies are warranted to decipher the probable mechanism by which gallic acid and quercetin nanoparticles exert anticancer effect.

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