Future Journal of Pharmaceutical Sciences

RESEARCH Open Access

Characterization of camptothecin by analytical methods and determination of anticancer potential against prostate cancer

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Abstract

Background: Objective of present research work is to develop and validate cost-effective analytical tool for determination of camptothecin (CPT) and determine its anticancer potential against prostate cancer LNCaP cell lines. Structural elucidation has been performed by mass spectrometry, Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy, and MTT assay utilized for in vitro cytotoxicity where spectrometric method was used for estimation of camptothecin.

Results: Mass spectra showed peak at 349.2 which matches to standard molecular weight of camptothecin. FTIR and NMR spectra conformed functional moieties and structure of isolated camptothecin which was nearly equal to values mentioned in standard structure of camptothecin. IC_{50} values of CPT against LNCaP cell lines was found to be 3.561 μg/ml. Lambda max of CPT was found to be at 225 nm and calibration curve found to be linear over the concentration range from 2 to 70 μg/ml of camptothecin. Developed method was found to be linear, accurate, and precise. LOD and LOQ were found to be 0.0524 μg/ml and 0.1614 μg/ml, respectively. Developed method has % relative standard deviation less than one which is reproducible hence % recovery was found to be 99.80%.

Conclusions: FTIR, NMR, and mass spectrometry results conforms isolated compound was camptothecin; cytotoxicity study proves it has strong potential in treatment of prostate carcinoma as competent alternative to chemotherapy in the form of herbal medicine and the developed UV method proves to be valid, sensitive, and applicable for rapid, accurate, precise, and economical determination of camptothecin.

Keywords: Camptothecin, Mass spectrometry, NMR, LNCaP, Anticancer, Accuracy, Precision

Background

Currently, the world is facing high threat of rapid rise of global cancer, and patients suffering from it badly needed complete cure from cancer [1]. According to globocan, there will be 9.6 million deaths and 18.1 million new cancer patients in 2018 worldwide. Among this, lung cancer is leading cause of mortality and diagnosed about 11.6% out of total cases. Lung cancer is highest in causing cancer deaths 18.4% of total carcinoma a death which is closely followed by breast cancer 11.6% and prostate cancer 7.1% for mortality. In men, lung cancer is leading cause of mortality which is subsequently followed by prostate, colorectal, liver, and stomach cancer [2]. Most frequently, surgery, radiation, and chemotherapy have been utilized to cure the carcinoma but it has ample of toxic and adverse effects. From ancient era, wide variety of drugs from natural herbal origin is available which prevent occurrence and cure of cancer [3]. Currently, the demand for herbal-based phytoconstituents is at peak due to its safety, efficacy, and limited side effects in treatment of cancer [4]. Phytoconstituents have unique multimolecular mode of action which potentially responsible to cure cancer

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along with its versatile pharmacological activities [5]. *Nothapodytes nimmoniana* is tropical and subtropical plant belongs to *Icacinaeae* predominantly found in western reason of Maharashtra India. Camptothecin (CPT) is key phytoconstituent of *Nothapodytes nimmoniana* which is reported to have broad spectrum of pharmacological activities such as antiviral [6], HIV [7], antibacterial, antifungal [8], colorectal cancer, malignancies, ovarian cancer [9–11], and breast cancer [12]. In 1966, Wall ME and Wani MC first identified camptothecin during screening of natural herbal products for anticancer potential. The broad cytotoxic potential of CPT mainly due to its quinoline alkaloid which inhibits DNA enzyme topoisomerase-I is mainly effective against hepatoma, leukemia, and gastric carcinoma along with tumor of head and neck. Chemically, CPT is (S)-4-ethyl-4-hydroxy-1H-pyrano[3',4':6,7] indolizino [1,2-b] quinoline-3,14-(4H, 12H)-dione. Applicability of CPT in treatment of cancer has ample of limitations such as poor water solubility, low bioavailability, inactivation at physiological pH by lactone ring hydrolysis and inadequate cellular uptake in acidic media through water soluble carboxylate [13]. Quantification of CPT content from *Nothapodytes nimmoniana* by different analytical methods like high-performance liquid chromatography (HPLC) [14] and high-performance thin layer chromatography (HPTLC) [15, 16]. Desorption electro spray ionization mass spectrometry (DESI-MS) [17, 18] and proton nuclear magnetic resonance spectroscopy (1H-NMR) [19, 20] methods have been reported. Normally, CPT was determined and validated by HPLC and RP-HPLC [21, 22] which was costly and consume ample of time; on the other hand, UV method is

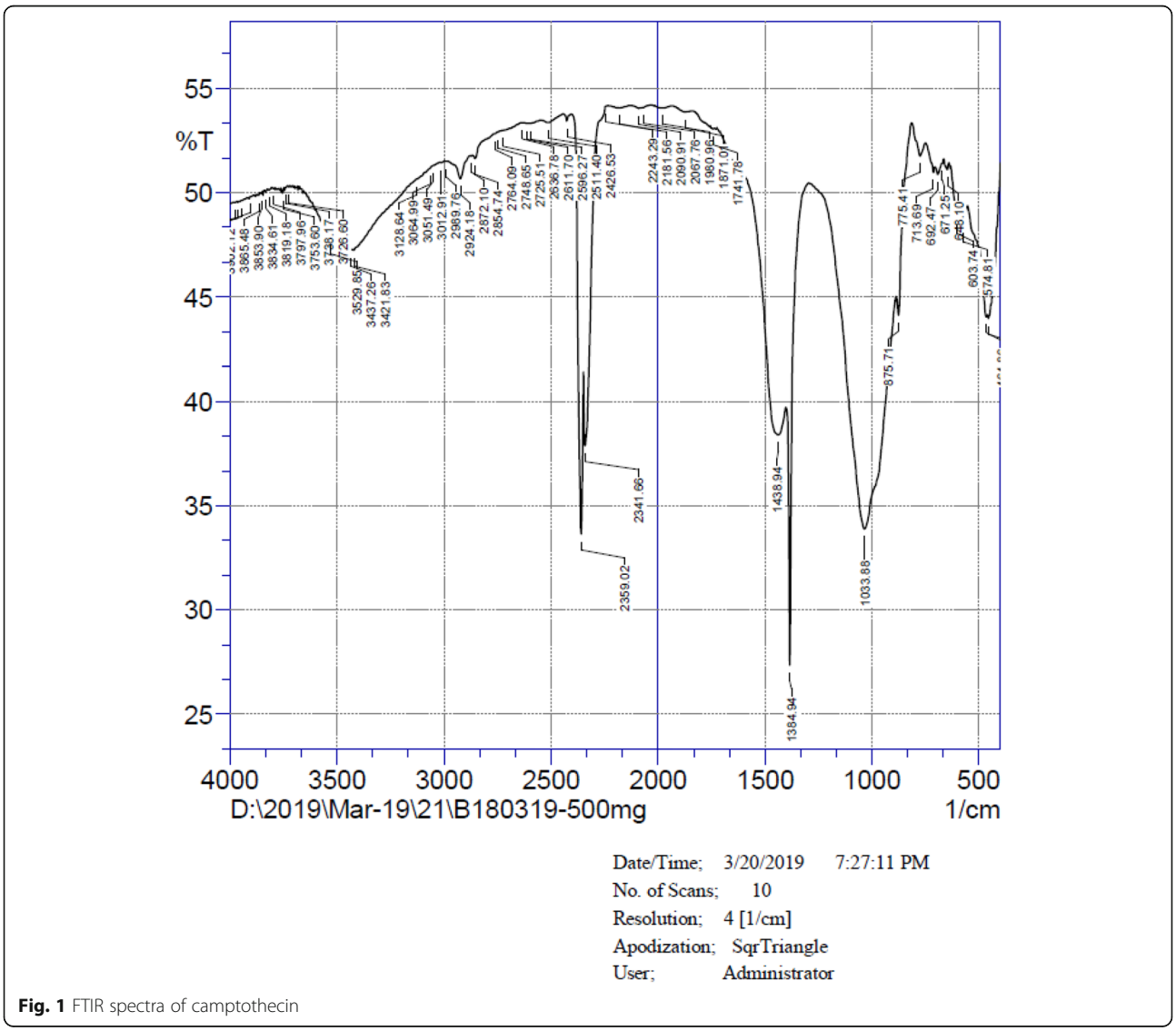

Fig. 1 FTIR spectra of camptothecin

rapid, reproducible, and cost-effective. In this present research work, first attempt has been made to treat prostate cancer with the help of CPT. Till date, there is no valid UV spectroscopic method for determination of camptothecin pure and bulk. The objective of current research work is to determine anticancer potential of CPT against prostate cancer LNCaP cell lines and to develop and validate cost-effective UV spectroscopic method for determination of camptothecin.

Methods

Chemicals and reagents

Standard camptothecin obtained from Aditya Imptex Pvt. Ltd. Mumbai, India. Methanol was supplied by OZONE International. Pvt. Ltd. Mumbai, Maharashtra. Chloroform, sodium carbonate, sulphuric acid, emulgen, and double distilled water were obtained from Unique Chemical Kolhapur. All chemicals and reagents used in the present research work were of analytical grade.

Preparation of extract

Microwave irradiation method was used for extraction of and isolation of CPT from *Nothapodytes nimmoniana* leaves using emulgen as surface active agent. Accurately weighted 5 g of leaf powder and placed in 250 ml conical flask along with 200 ml of emulgen solution having pH 8. Resulting solution was microwave irradiated for about 1 min for 350 V and cooled for 2 min. Resultant solution was maintained at pH 3–4 by using sulfuric acid and Mayer's reagent added to precipitate CPT [23, 24].

Fourier transform infrared spectroscopy (FTIR) study

FTIR (Perkin Elmer FTIR model-1615) is modern analytical tool which conforms characteristic peaks present in CPT which represents functional groups present and CPT sample was scanned 4000 to 400 cm^{-1} range at a resolution of 4 cm^{-1} [25].

Mass spectroscopy

In mass spectroscopy, LC ion trap method was utilized for determination of ions which are + ve and –ve in mass spectra. For both positive and negative ion mode, capillary voltage was set to – 3800 V and 4500 V, respectively and at the end ± 500 volts plate in +ve and –ve ion mode, respectively. With help of syringe, CPT sample was injected along with micro TOF-Q detector and Apollo ESI as ion source. Structural elucidation was confirmed by comparison of isotope and mass accuracy pattern [26].

Nuclear magnetic resonance (NMR) spectroscopy

Before analysis camptothecin samples were stored at – 80 °C. For dissolved sample, solvent was evaporated by stream of nitrogen then redissolved in $CDCl_3/CD_3OD$ (2:1). A high-resolution NMR spectrum of each single species was acquired on (Bruker DRX 600 MHz) NMR spectrometer equipped with TXI probe [26].

Cytotoxicity study

In a micro plate containing 96 wells, cells were seeded which are maintained overnight at 37 °C in 95% RH and CO_2 5%. Various concentrations ranging from 20 to 0.625 μg/mL of samples was treated. The

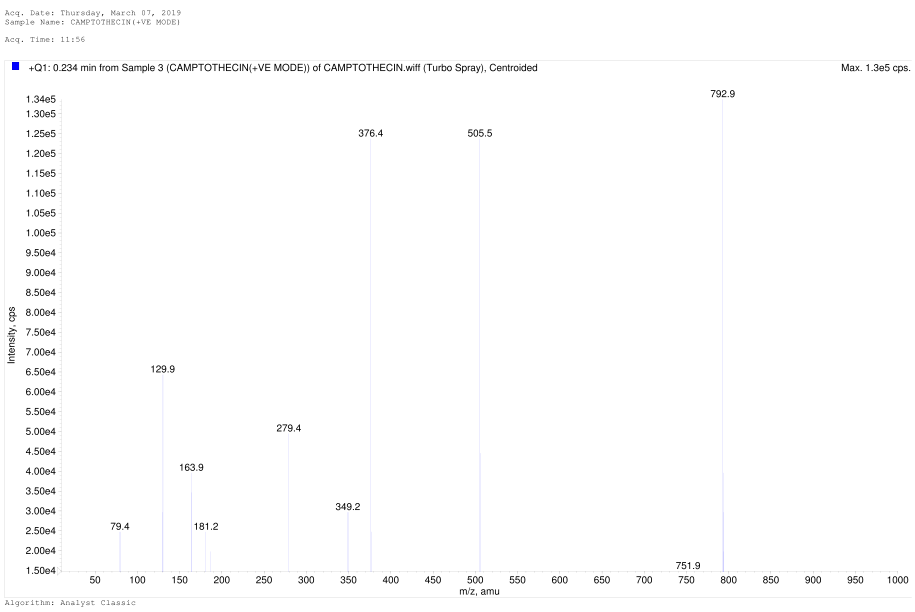

Fig. 2 Mass spectra of isolated camptothecin

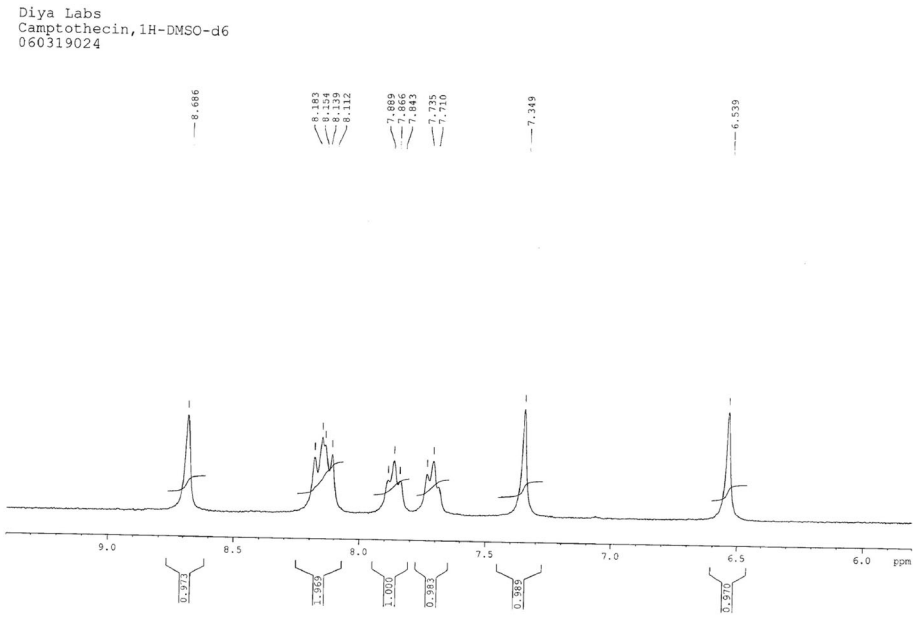

Fig. 3 NMR spectra **o**f camptothecin

cells were incubated for another 48 h. Phosphate buffer used for cleaning of wells and 20 μL of the MTT was used as staining solution was poured in every well and incubated at 37 °C. In every single well, DMSO was added after 4 h which dissolve formazan and with help of micro plate reader and absorbance was measured at 570 nm [27].

$$\%\text{Surviving cells} = \frac{\text{Mean OD of test compound}}{\text{Mean OD of Negative control}} \times 100$$

Standard stock solution

Precisely weighted camptothecin 10 mg and dissolved in 10 ml of organic solvent methanol and make up the volume up to 100 ml with same solvent. The solution obtained was standard stock solution having the strength of 1000 μg/ml. From standard stock solution, 10 ml was withdrawn and 100 μg/ml concentration solution was prepared by suitable dilution which was filtered before analyzing by Whatman filter paper [28].

Calibration curve

Working solution was prepared by suitably diluting primary stock solution at room temperature. Working solution was serially diluted at different concentrations and scanned in the range of 200–400 nm (Shimadzu-UV-1900). Linearity of calibration curve was measured diluting the working solution in the range of 1–100 μg/ml.

Accuracy

Accuracy of the developed analytical method has been determined by close comparison between actual observed and standard values. Recovery was performed by addition level of 80, 100, and 120% for test solution in to fixed standard solution [29].

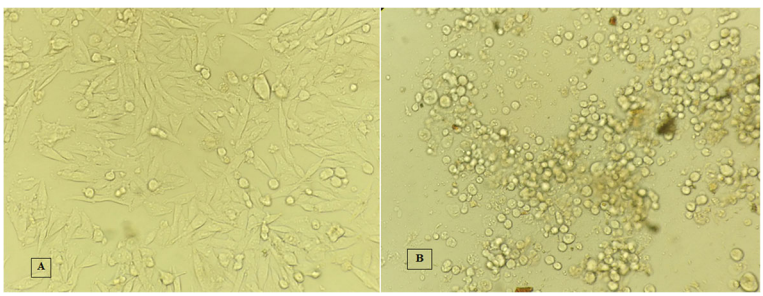

Fig. 4 LNCaP Cell lines. **a** Untreated. **b** Treated with CPT

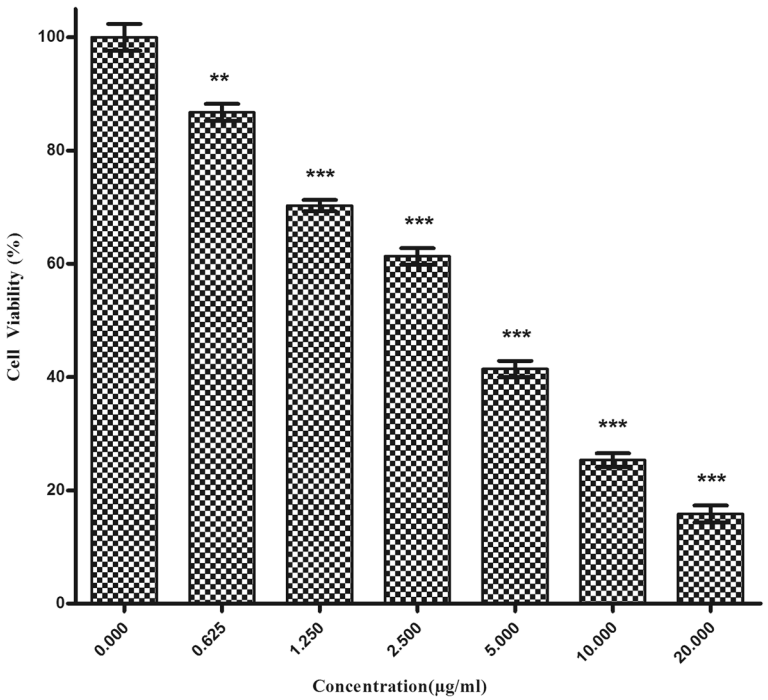

Fig. 5 Cell viability vs concentration on LNCaP cell lines

Precision

Both intra- and inter-day precision of developed analytical method was confirmed by observed values over a week from present day and on next 3 days at time intervals of 4 h. Observed results were calculated statistically and represented in the form of SD [30].

Repeatability

By analyzing 6 samples of CPT of 10 µg/ml the repeatability was determined and obtained results further utilized for statistical analysis [31].

Limit of detection and limit of quantification ((LOD and LOQ)

It is lowest detectible amount measured quantitatively by any analytical method. These variables were determined as per ICH guidelines by observed values along with its SD [32, 33].

LOD and LOQ were calculated using formula

$LOD = 3.3 * \sigma/s$ $LOQ = 10 * \sigma/s$

σ- Standard deviation and **S** -slope.

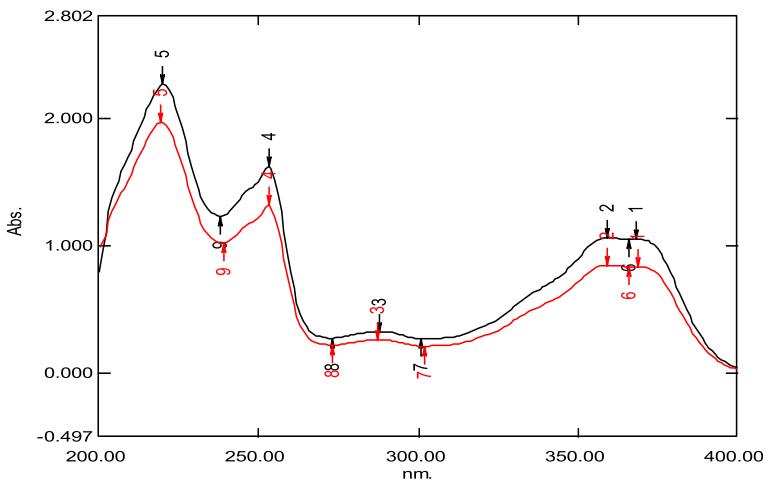

Fig. 6 λ max of camptothecin

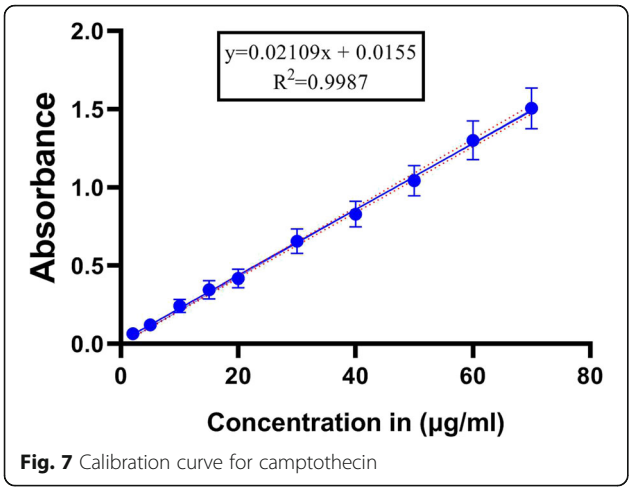

Fig. 7 Calibration curve for camptothecin

Results

Identification of camptothecin

FTIR spectroscopy

FTIR of CPT from *Nothapodytes Nimmoniana* extract showed functional peaks related to specific structural features as follows such as, OH stretching at 3437 cm^{-1}, Ester stretching at 1741 cm^{-1}, C=O stretching at 1642 cm^{-1}, C=C at 1621 cm^{-1}, C=N at 1438 cm^{-1}, C–O at 1033 cm^{-1} and peak at 775 cm^{-1} [34]. The values were near or equal to values mentioned in standard structure of camptothecin. Results were shown in Fig. 1.

Mass spectroscopy

Nothapodytes Nimmoniana is a rich source of the potent alkaloid camptothecin, 9-methoxy camptothecin, 9-Methoxy-mappacine-20-β-glucopyranoside and acetoxy-camptothecin-glycoside. It also contains palmitic acid, stearic acid, oleic acid, and linolenic acid. Mass spectroscopy principally determines mass and elemental structures of moieties. Mass spectra of CPT gives precursor m/z peak at 349.2 [M+H]+ which matches with standard molecular weight of CPT. Hence on the basis of concerned it was conformed that isolated compound was camptothecin [35]. On the other hand, mass spectra gives precursor m/z peak at 376.4 [M+H]+ and 505.5 [M+H]+ which more possibly matches with 9-Methoxy camptothecin and 9-Methoxy-mappacine-20-β-glucopyranoside or Acetoxy-camptothecin-glycoside respectively; precursor m/z peak at 792.9 [M+H]+ is an unidentified compounds [36] (Fig. 2)

NMR spectra of camptothecin

NMR spectra of camptothecin showed characteristic peaks at OH (1H)–8.686, N–H (1H)–8.183, aromatic protons–7.349 to 7.889, Ch (2H)–5.434 and 5.279, 1H (CH_2)–3.72 to 3.75 [35, 37]. From this, it was conformed that isolated compound was camptothecin. Results were shown in Fig. 3.

Cytotoxicity study

Cytotoxic potential of camptothecin on prostate cancer LNCaP cell lines was determined by MTT assay. On morphological evaluation of LNCaP cells in control group appear spindle shape; adhering to neighboring cells. On the other hand, cells treated with CPT lost their normal morphology. Morphological changes in LNCaP cell lines after treatment with CPT shown in Fig. 4. CPT showed concentration dependent % viability after 48 h having IC_{50} value 3.561 µg/ml against LNCaP prostate cancer cell lines shown in Fig. 5.

Cell viability

Absorption maxima (λ max)

Camptothecin showed strong and highest absorption of photon called as λ max at 225 nm as shown in Fig. 6.

Linearity

Calibration curve of CPT was evaluated by its correlation coefficient. Calibration curve of CPT was found to be linear in the range of 2–70 µg/ml. The regression line correlation coefficient (R^2) was calculated and found to be 0.9987 which is closest to 1 and indicative of good linearity of calibration curve with a *y*-intercept of 0.0155. Slope of the linearity was found to be 0.02109. Concerned results conformed linearity of calibration curve of CPT through the selected range. Results were shown in Fig. 7.

Precision

Inter- and intra-day precision study was performed for three continuous days. The % RSD was fond to be in the

Table 1 Results for intra-day and inter-day precision of camptothecin

Drug	Conc. (µg/ml)	Intra-day mean abs.	Absorbance ± S.D.	%RSD	Inter-day mean abs.	Absorbance ± S.D.	%R.S.D
CPT	15	0.3468	± 0.0016	0.503	0.3452	± 0.0015	0.336
	30	0.6238	± 0.0017	0.288	0.6368	± 0.0018	0.328
	60	1.3024	± 0.0024	0.272	1.3052	± 0.0023	0.313
Mean %RSD				**0.354**			**0.326**

*Each value represents mean ± S.D. of three observations

Table 2 Data showing repeatability of absorbances

Sr. no.	Concentration (μg/ml)	Absorbance	Mean ± S.D.	%R.S.D
1	10	0.2430	0.2421 ± 0.0037	0.521
2		0.2428		
3		0.2418		
4		0.2421		
5		0.2415		
6		0.2419		

S.D standard deviation, *R.S.D* relative standard deviation

range of 0.272–0.503 as shown in Table 1. This confirms the reproducibility of the developed method.

Repeatability

The repeatability of developed UV method was found to be significant for routine and frequent analysis of camptothecin in pure, bulk, and pharmaceuticals. A result of repeatability study confirms that absorbance remains unaffected on repetition of developed method (Table 2).

Accuracy

To ensure the accuracy of developed method, recovery study was performed by standard addition method at 80%, 100%, and 120% levels of CPT concentration. The results for the recovery study were found in the desired limits as shown in Table 3.

Discussion

Structural elucidation of camptothecin has been performed and conformed by different analytical techniques where peaks in FTIR spectra nearly equal to standard CPT [34]. Molecular mass of compound which is most crucial parameter in identification of isolated moiety was determined by mass spectroscopy. Molecular weight isolated CPT exactly matches with standard molecular weight of camptothecin, i.e., 349.2 [35, 36]. NMR spectroscopy mainly determines structure of specific compound. NMR spectra of CPT showed region of delta 8.686 to 5.279 the signals of H-7 related to structural features similar to standard camptothecin [35, 37]. It is of vital importance to evaluate the cytotoxicity of the CPT for determination of its broad spectrum anticancer potential. Cell viability of CPT on LNCaP prostate cancer exhibit concentration-dependent cytotoxicity having IC_{50} values 3.561 μg/ml. Drastic changes in morphology conformed that CPT has powerful cytotoxic activity against LNCaP cell lines which was proved by low cytotoxicity value. Normal cells showing higher confluency of monolayer and cells are adhering to the neighboring cells Fig. 4a. In contrast, CPT-treated LNCaP cells seems to be smaller in size, shrank, some cells became spherical, irregular in shape, and showed more significant reduction in the number along with detachment from the adherent site as shown in Fig. 4b [38]. The absorption maxima, i.e., lambda max of CPT, was found to be at 225 nm and calibration curve found to be linear over the range of 2–70 μg/ml. The correlation coefficient (R^2) was found to be 0.9987 which mainly indicative of functional relationship among variables. Results of precision indicate that developed analytical method is reliable, repeatable, and reproducible and can be applied for the determination of CPT in pure, bulk, and pharmaceuticals. Recovery results indicate the absence of interferences from the commonly encountered additives, excipients, and values of mean recovery were found to be 99.80%. High value of molar absorptivity by developed method $6.8252*10^4$ L/mol.cm which is indicative of CPT was strongly and potentially absorbed at specific wavelength, i.e., at lambda max. % RSD was found to be less than one and LOD and LOQ values were found to be 0.0524 μg/ml and 0.1614 μg/ml, respectively, which conforms CPT can be determined at lowest possible concentration in pure, bulk, and pharmaceuticals.

Table 3 Accuracy measurement by % recovery method

Standard CPT (μg/ml)	Level of addition (%)	CPT added (μg/ml)	Amount recovered (μg/ml)	% Recovery	Average% recovery
05	80	8	12.97	99.77	99.80
05	100	10	14.98	99.87	
05	120	12	16.96	99.76	

Conclusion

Structural determination by FTIR, NMR, and mass spectrometry confirms that isolated compound was camptothecin; cytotoxicity study proves it has great potential in treatment of prostate cancer as competent alternative to chemotherapy in the form of herbal medicine having IC_{50} values 3.561 μg/ml against LNCaP cell lines. As concern to results of accuracy, precision, repeatability, and recovery, it was concluded that developed method proved to be valid, sensitive, and applicable for rapid, accurate, precise, and economical determination of camptothecin in pure, bulk, and pharmaceuticals.

Abbreviations

UV: Ultraviolet; DMSO: Dimethyl-sulfoxide; nm: Nanometer; μg: Microgram; ICH: International Conference of Harmonization.; % RSD: Percent Relative Standard Deviation; λ max: Lambda maximum; S.D: Standard deviation; I.P: Indian Pharmacopeia; CPT: Camptothecin; FTIR: Fourier transform infrared spectroscopy; MS: Mass spectroscopy; O.D: Optical density; NMR: Nuclear magnetic resonance; RSD: Relative standard deviation; Surviving cells (%): Cell viability; Mean OD of test compound: Mean optical density of test compound; Mean OD of negative control: Mean optical density of negative control

Acknowledgements

The authors are thankful to Department of Pharmacy, Mandsaur University Mandsaur, Madhya Pradesh, Principal B.R. Nahata College of Pharmacy Mandsaur University Mandsaur for providing required guidance and support,

Diya Labs Mumbai for Characterization of camptothecin and Dr. Madhukar Bachulkar for authentification of *Nothapodytes nimmoniana*. Authors are also thankful to NCBI Pune for providing Prostate Cancer LNCaP Cell Lines and Maratha Mandal Dental College and Research Center Belagavi, for anticancer activity.

Plant authentication

The whole plant *Nothapodytes nimmoniana* specimen sample Family (Icacinaeae) was collected from Ajara Forest Resion and identified and authenticated by Dr. Madhukar Bachulkar, Taxonomist Shivaji University Kolhapur, Maharashtra, India, and the voucher specimen No.SGMCP/PH.COG/HERB/09-2019 herbarium was deposited in pharmacognosy department.

Authors' contributions

SG is a research scholar who contributed in concept, isolation, design of work, and determination of CPT and SG has major contributions in writing the manuscript. RT and DB were supervisors who contributed in research guidance and have major contribution in monitoring anticancer studies and discussion. All authors read and approved the final manuscript.

Funding

No funding was obtained for this study.

Availability of data and materials

The data sets used and/or analyzed during current research work are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they do not have any competing interests.

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Received: 12 August 2020 Accepted: 29 March 2021
Published online: 22 May 2021

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