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Original Article

A Stability-Indicating Green Chromatographic Method for Simultaneous Estimation of Gefitinib and Ellagic Acid: Saturated Solubility, Assay, and In Silico Profiling

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ABSTRACT

Background: The present study focused on the development and validation of a cost-effective green analytical high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of gefitinib and ellagic acid. The study was also extended to the in vitro stability profiling of both molecules. **Methods:** The chromatographic analysis was performed using a mobile phase composed of 40 mM ammonium acetate buffer (pH 2.5 ± 0.05), methanol, and acetonitrile in a ratio of 68:30:02 %v/v. The mobile phase was isocratically maintained at a flow rate of 1 mL. min^{-1}. The samples (10 µL) were injected with an autoinjector and analysed at 252 nm. Forced degradation studies were conducted in conjunction with solubility assessments of individual molecules and their assays in a blended powder mixture. **Results**: The developed method demonstrated linearity for ellagic acid and gefitinib in the range of 1 to 11 µg.mL^{-1} for both molecules. The DL and QL were 0.13 and 0.39 µg.mL^{-1} for ellagic acid, and 0.105 and 0.31 µg.mL^{-1} for gefitinib, respectively. The method exhibited high sensitivity and specificity with good accuracy (>95%) and precision (RSD <2%). Forced degradation studies revealed that ellagic acid is sensitive to acidic, alkaline, and oxidative conditions, whereas gefitinib showed sensitivity to oxidative stress, especially at higher temperatures. **Conclusion:** The validated RP-HPLC method was recognised as a "green" analytical approach, serving as an eco-friendly, cost-effective, and reliable tool for pharmaceutical research, solubility assessment, and assay determinations in complex formulations.

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INTRODUCTION

Lung cancer is a major global health crisis, responsible for around 18,00,000 deaths and affecting 22,00,000 new cases in 2020.[1] However, in 2022 alone, around 25,00,000 new cases were recorded.[2,3] This significant increase in this number is a challenge for the healthcare sector. In India, lung cancer alone accounts for around 6%, whereas it is the main cause of around 8% of cancer-related deaths.[2] The occurrence of lung cancer and related deaths is almost twice as high in males compared to females.[4] In India, this is also an alarming indication that immediate action is needed to control this number.

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The maximum recorded cases (85%) are non-small-cell lung cancer (NSCLC), with its highest existing subtypes, adenocarcinoma (40%) and squamous cell carcinoma (25%).[5] AstraZeneca Pharmaceuticals in the United Kingdom developed a tyrosine kinase inhibitor, Gefitinib (GIFT, Iressa®) (Figure. 1A), for treating metastatic NSCLC by blocking the epidermal growth factor receptor (EGFR).[6] Inhibiting EGFR tyrosine kinase (EGFR-TK) effectively impedes tumour growth, metastasis, and angiogenesis while considerably increasing tumour cell apoptosis. Importantly, EGFR signalling is a key contributor to various cancers, such as lung, colon, breast, and ovarian cancers.[7] Its absolute bioavailability in cancer patients is very low due to its low aqueous solubility. Because it is basic, it is practically insoluble in the basic and near-neutral regions of the distal small intestine, especially in the colonic area. Although most patients initially responsive to gefitinib develop acquired resistance (AR) within 0.5 to 12 months, leading to treatment failure. Factors such as EGFR overexpression, increased Akt and mTOR activity, MET amplification, phenotypic transformation, and other genetic changes are known mechanisms for gefitinib resistance.[8–10] GIFT has synergistic anticancer activity in vitro

and in vivo studies when combined with other cytotoxic agents.[11] In recent years, researchers have found that functional natural phytoconstituents, particularly polyphenolic compounds and alkaloids, are promising compounds used as potential anticancer agents.[12,13] Due to therapeutic limitations, complexity and diversity of resistance, multidrug treatment is necessary, using both molecularly targeted anticancer drugs like GIFT and natural phytoconstituents.[14]

Ellagic acid (EA) (Figure. 1B), a polyphenolic di-lactone compound derived from natural sources, especially natural gallic acid dimers, has gained attention for its potential to combat oxidative stress, which may help mitigate conditions such as autoimmune disorders, cardiovascular diseases, diabetes, neurodegenerative diseases, obesity, and even cancer.[15] It has limited aqueous solubility and permeability, which classifies the molecule as BCS class IV.[16] It also has extensive first-pass metabolism, gastrointestinal degradation, and interactions with proteins and cellular DNA, all of which compromise its oral bioavailability and therapeutic efficacy.[17] Due to its antioxidant and antiproliferative properties, ellagic acid is considered a promising candidate for anti-cancer therapies. EA exerts anti-cancer effects by arresting cells in the G_0/G_1 phase of the cell cycle and inhibiting metastasis in breast cancer (BC). EA induces apoptosis by downregulating the AKT/mTOR pathway, thereby disrupting PI3K/AKT and MAPK activity. Additionally, EA inhibits HIF-1α-induced VEGF/VEGFR-2 signalling.[18,19]

Multidrug treatment has been used by researchers as an effective tool for the treatment of complex diseases, including HIV and cancer, due to the combination or synergistic effect of the drugs, to mitigate the limitations of pure drugs. In addition to the synergistic effect, it produces fewer adverse events, reduces the development of drug resistance, enhances patient compliance, and lowers management and manufacturing costs compared with monotherapy.[20,21] Therefore, the combination of gefitinib and ellagic acid could be a promising approach to treating lung cancer by achieving synergistic effects of the two drugs in a single formulation. Therefore, an analytical method for the simultaneous quantification of these two molecules is urgently needed. Also, for the development of a new formulation, a stability profiling of both molecules is essential.

Several methods have been reported using RP-HPLC for the quantification of gefitinib and ellagic acid individually, as well as for their combination with other drugs. Sathishbabu et al. developed a simultaneous RP-HPLC method for the estimation of gefitinib and resveratrol.[22] Faivre and colleagues developed a simultaneous method for gefitinib and erlotinib.[23] In contrast to ellagic acid, no HPLC-UV method has been reported for the simultaneous detection of gefitinib and ellagic acid, nor has one been reported in combination with other molecules. Similarly, very few studies have been conducted for stability-indicating forced degradation testing using the HPLC method. Furthermore, the majority of reported analytical methods used harmful, environmentally destructive solvents at higher concentrations, which could be detrimental to the environment, human health, and the analyst. Hence, an eco-friendly validated HPLC method is highly preferable in the current scenario.

For a multi-drug formulation, the storage and stability of the combined drugs are the primary criteria for pharmaceutical product development, manufacturing, and warehousing. Potential chemical interactions and incompatibilities between two molecules can lead to instability and alter the therapeutic safety and efficacy of the active pharmaceutical ingredients. Thus, forced degradation studies are the primary and essential way to assess these interactions using HPLC under distinct environmental and stressed conditions, such as humidity, temperature, and light. The study is not helpful only for the control of manufacturing and processing parameters in the development of pharmaceutical formulations of a combination of two molecules, but also for deciding the storage conditions for raw materials and finished products and expiry date & retest periods of finished products.[24,25] Several studies have reported the stability parameters of gefitinib and ellagic acid, in their pure form or in a nanoformulation, along with their process impurities.[26–28] However, none of the studies reported the forced-degradation stability-indicating assay (SIA) for a combination of GIFT and EA.

The present study aims to develop and validate a cost-effective green analytical RP-HPLC method for the simultaneous quantification of GIFT and EA. Using an isocratic elution method with methanol, acetonitrile, and ammonium acetate buffer, this method achieves high sensitivity, accuracy, and precision, in accordance with ICH guidelines. It provides an SIA for the effective identification of both molecules in combination during stress testing under hydrolytic (acidic and alkaline), oxidative, thermal, and photodegradation conditions. Furthermore, using green analytical metrics confirms its eco-friendliness, making it an efficient, environmentally friendly, and cost-effective tool for both individual and simultaneous estimation of GIFT and EA in pharmaceutical research.

MATERIALS AND METHODS

Materials

GIFT was supplied by Neon Laboratories Ltd., Mumbai, India, as a gift sample. EA was purchased from TCI, Hyderabad. Ethanol was purchased from G Biosciences, Noida, UP. The organic solvent used in mobile phase, i.e., acetonitrile (HPLC gradient grade, 99.8%) and methanol (HPLC grade, 99.8%) were procured from Qualigens, Thermo Fisher Scientific India Pvt. Ltd. Analytical grade ammonium acetate and Triethylamine (extra pure AR, 99% purity) was used in buffer preparation; glacial acetic acid (extra pure AR, 98% purity) for the pH adjustment;

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Sodium hydroxide pellets (extra pure AR, 98%) and potassium dihydrogen phosphate (PDP, 98% purity) were procured from SRL (Sisco Research Laboratories) Pvt. Ltd., Mumbai, India. Apart from that, Loba Chemie Pvt. Ltd. (Mumbai, India) provided hydrochloric acid (extra pure, 35%) for hydrolytic degradation and hydrogen peroxide (30% w/v H_2O_2, 100 volumes) for oxidative degradation, both used in stress degradation testing.

Instrumentation

Basic instrumentation included a UV Spectrophotometer (UV-1900i, Shimadzu Co., Kyoto, Japan) for scanning the spectra of both molecules, and an Aczet digital weighing balance (CM-6A) from Aczet Pvt. Ltd., Maharashtra, for robust, effective weighing of analytical samples. For accurate pH adjustment and buffer preparation, an Eutech digital pH meter was used. A Lab Man digital ultrasonicator and a vacuum filtration assembly were used for buffer and mobile-phase preparation (Lab Man Scientific Instruments, Chennai). High-purity Millipore water was acquired from the integrated assembly of distilled water Synergy (Kit IN (SYNSOHFIN)) and the Merck Millipore Synergy system. Moxcare 0.22 µm PTFE/nylon syringe filters (NXSFN2522-1) from Moxcare Products Inc., Haryana, were used to filter all samples for effective analysis, while maintaining analytical precision.

Chromatographic conditions

Chromatographic evaluation was performed using a Shimadzu N-series HPLC with an SPD-40 UV-Vis detector, a DGU-405 degasser, a CBM-40LITE controller, LC-40D XR pumps, and an SIL-40C XR autosampler. Separation was attained with an Intersil ODS-3V C18 (250 × 4.6 mm, 5 µm particle size, 100Å) column, protected by a Shim-pack GWS C18 guard column. All the data were processed using LabSolution software. An isocratic elution method was employed for effective analysis, with a constant flow rate of 1.0 mL.min^{-1}. A ternary solvent system was used, with 40 millimolar ammonium acetate buffer (AB, pH 2.5 ± 0.05) as eluent A, methanol as eluent B, and acetonitrile as eluent C. The mobile phase ratio remained constant at 68:30:2 (A:B:C) throughout the 15 min run time. Analytes were detected at 252 nm using the selected wavelength, with a 10 µL injection volume. The column was held at 25 ± 1 °C, and the sample tray at 15 ± 2 °C during sample analysis.

Selection of wavelength

Stock solutions of 10 µg.mL^{-1} of ellagic acid and gefitinib were prepared in ethanol separately, and aliquots were placed in quartz cell cuvettes, using ethanol as a blank, and scanned over wavelengths with absorption maxima (λ_{max}) from 200 to 400 nm using a UV Spectrophotometer. To simultaneously quantify and streamline chromatographic analysis, a common wavelength was chosen.

Methods

Buffers

Ammonium acetate pH 2.5

To prepare the 40 mM ammonium acetate buffer (AB), a measured amount of ammonium acetate was dissolved in a 500 mL volumetric flask containing deionised water. 0.1% triethylamine was added, and the pH was adjusted to 2.5 with glacial acetic acid[29]

Phosphate buffer, pH 7.4

To prepare a 200 mM potassium dihydrogen phosphate buffer (PB), pH 7.4, equimolar (200 mM) concentrations of PDP and sodium hydroxide (NaOH) were prepared individually. To make the desired buffer (1000 mL), PDP solution (250 mL) was combined with NaOH (195.5 mL) in a volumetric flask, then shaken, volume adjusted with deionised water, and finally shaken again with careful pH adjustment.[29]

Preparation of calibration standards and controls for gefitinib and ellagic acid

First, standard stock solutions of both molecules (GIFT and EA) were prepared in high-purity ethanol (GC grade 99.99%, G Biosciences) at 500 µg.mL^{-1} for each. From these stock solutions, a primary working standard solution (50 µg.mL^{-1}) was prepared again in the same high-purity ethanol for both molecules by diluting the stock solution to 10 mL. Then, the distinct concentration solutions for the linearity and quality control (QCs) samples were prepared by mixing the solutions of both molecules in the required amounts and diluting with the mobile phase. The calibration ranged from 1 to 11 µg.mL^{-1} with the distinct concentrations of 1, 3, 5, 7, 9, and 11 µg.mL^{-1}, whereas the QC samples had three levels, i.e., low (LQCC, 1.2 µg.mL^{-1}), medium (MQCC, 6.0 µg.mL^{-1}), and high (HQCC, 10.8 µg.mL^{-1}).

Method validation

To verify that the newly developed simultaneous isocratic analytical method for quantifying both molecules met all performance criteria, a comprehensive validation was conducted using rigorous laboratory assessments. For method validation, the ICH Q2(R2) guidelines apply to critical characterisation, including system suitability, linearity, sensitivity (through DL & QL), robustness, precision (repeatability and intermediate), and recovery.[30,31]

System readiness

System readiness, also known as system suitability, which was carried out to evaluate the resolution and reproducibility of the chromatographic conditions. To evaluate system readiness, the average peak area, retention time (R_T), number of theoretical plates (NTP), and tailing factor were calculated, followed by the

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calculation of the percentage relative standard deviation (%RSD) from six replicate injections containing both molecules.[32]

Specificity

Specificity is measured by identifying each analyte without interference from other analytes, matrix components, or degradation products. GIFT and EA, individually and in a combined solution, were analysed using the developed analytical method, and chromatograms were recorded to assess the absence of other molecules or excipients that might interfere.

Furthermore, specificity was also estimated by stress-degradation studies of a combined solution of GIFT and EA under oxidative, hydrolytic, thermal, and photodegradation conditions, and by evaluating the interference of degradation products at the retention times of GIFT and EA.

Matrix interference

Potential matrix interference was confirmed by comparing blank injections with a combined GIFT and EA solution at a medium-quality control (MQCC) standard of 6 $\mu g.mL^{-1}$. Each analyte was analysed thrice to confirm that neither the molecules nor the mobile-phase peaks/retention times overlapped or interfered with the retention times of the other molecules.

Stability-indicating assay

Stress testing was performed to assess the specificity and to accurately quantify the molecules using the developed analytical method, in accordance with ICH Q1 stability guidelines.[33] It covers the simultaneous estimation of GIFT and EA, along with their stress-activated degradants, and assesses the assay's specificity (i.e., the absence of interference from degradants with both active pharmaceutical constituents). Hydrolytic (acidic and alkaline), oxidative, solvent and buffer stability, as well as photostability, were assessed to identify degradants and verify the reliability (specificity) of the developed method. Here, 0.1N and 1N HCl were utilised to assess the acidic degradation of the molecules. The alkaline stability was performed in 0.1N and 1N NaOH. Similarly, 1% H_2O_2 and 6% H_2O_2 were used for oxidative degradation. Further analysis was performed in potassium dihydrogen phosphate buffer (pH 7.4), ethanol, and the mobile phase. All the above-mentioned samples were then exposed to different thermal and storage conditions, including 4 °C, room temperature (RT, 25 °C), and 80 °C for 48 h, in amber-coloured vials.

Initially, a stock solution was prepared by dissolving both molecules in high-purity ethanol to 500 $\mu g.mL^{-1}$; a specific amount of the stock was then added to the respective solvents and buffers (those used for the stress-indicating study) to produce 50 $\mu g.mL^{-1}$, which was then exposed to the different stress conditions as mentioned above. Sample collection was performed at 0, 24, and 48 h. The samples were brought to a working concentration of 6 $\mu g.mL^{-1}$ after appropriate dilutions with the mobile phase. Each sample was analysed three times using the developed analytical method after filtering (0.22 µm).

Hydrolytic degradation

Hydrochloric acid (0.1N and 1N) for acidic hydrolysis and sodium hydroxide (0.1N and 1N) for alkaline hydrolysis were used. The other solvents, including phosphate buffer (pH 7.4), ammonium acetate (pH 2.5), ethanol, and the mobile phase, were also used in this study. All the samples were stored under the previously mentioned distinct conditions in amber-coloured vials in the mentioned solvents. At predefined time points, analysis was carried out after appropriate dilution and filtration.

Oxidative stress degradation

Oxidative stress degradation for both molecules was carried out with hydrogen peroxide (H_2O_2) solution (1% and 6%). The samples were subjected to media containing 1% and 6% H_2O_2. At predetermined time points, analysis was carried out after appropriate dilution and filtration.

Thermal stress degradation

The impact of temperature on the simultaneous estimation of both molecules was also studied. Samples were prepared in HCl (0.1N and 1N), NaOH (0.1N and 1N), H_2O_2 (1% and 6%), phosphate buffer (pH 7.4), mobile phase, and ethanol, then exposed to the three specified temperature conditions for 24 and 48 h. After that, the collected samples were analysed after appropriate dilution and filtration.

Photolytic degradation

For photolytic degradation, samples were prepared in distinct media, including ammonium acetate (pH 2.5) and phosphate buffer (pH 7.4). The prepared samples were grouped into two sets. One set of samples was exposed to UV light at 395 nm, 100 W, 220 V, AC power, with a 12-h on and 12-h off cycle for 48 h in transparent vials. Another set of samples was stored completely in the dark in amber-coloured vials to prevent light-induced or other degradation factors. After storage for the specified time periods (0, 24 and 48 h), aliquots were collected, diluted, filtered, and analysed by the developed method.

Linearity

Six distinct concentrations ranging from 1 to 11 $\mu g.mL^{-1}$ were analysed. Each sample was analysed in six replicates; serial dilutions were prepared in the mobile phase and analysed using the developed HPLC method after filtration through a 0.22 µm syringe filter. The calibration curve was generated by plotting peak area against concentration.

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Precision

The concentration levels LQCC, MQCC, and HQCC were assessed six times to evaluate the intra-day precision (repeatability) and inter-day precision (intermediate) of the newly developed analytical method. Repeatability samples were analysed in the morning and evening, with a 12 h gap, whereas intermediate samples were analysed the next day, with a 24 h gap. The relative standard deviation was calculated for the peak area.

Recovery

The closeness to truth was evaluated in this study, in which the known concentrations of standards and test samples were assessed at three distinct levels: 75%, 100%, and 125%. The standard samples were prepared using the working standard, whereas the test samples were prepared using the allegation method.[33] The following relation gives the recovery for both molecules by integrating the respective values. Further, the relative standard deviation was calculated for the recovery data.

$$\text{Percent recovery}\ (\%) = \frac{\text{Recovery from test}}{\text{Recovery from standard}} \times 100) \quad (1)$$

Robustness

A robustness study of the simultaneous isocratic method was performed to assess its reliability under purposefully varied chromatographic conditions. It was evaluated by modifying several parameters, including the key robustness parameters: injection volume (10 ± 2 µL), detector wavelength (252 ± 2 nm), pH of the mobile phase (2.5 ± 0.2), column oven temperature (25 ± 2 °C), and flow rate (1.0 ± 0.2 mL.min^{-1}). A sample of 6 µg.mL^{-1} was studied in this study.

Detection limit (DL) and quantification limit (QL)

DL and QL were calculated to identify the lowest concentration thresholds for both molecules that could be reliably detected and quantified with this method. The DL and QL were calculated using the "Standard Deviation and Slope Method," with the slope (Sl) obtained from the standard calibration curve and the response standard deviation (SD).

$$\text{DL} = 3.3 \times \frac{\text{SD}}{\text{Sl}} \quad (2)$$

$$\text{QL} = 10 \times \frac{\text{SD}}{\text{Sl}} \quad (3)$$

Greenness assessment

Method greenness is another crucial approach for assessing environmental safety, human health impacts, and energy efficiency when organic solvents are used. It is a formal evaluation that measures how well your analytical method aligns with the principles of Green Analytical Chemistry (GAC). This emphasises the development of a streamlined green analytical protocol by selecting less toxic reagents, minimising power consumption, and limiting waste generation. By prioritising waste generation, the number of distinct steps used, the samples analysed by this method, and the volumes of chemical (organic) and operational energy consumed, the greenness of the developed method was estimated using greenness assessment metrics, including AGREE, MoGAPI, ComplexMoGAPI, and BAGI.[34]

Analytical greenness (AGREE) matrix calculator v.0.5 beta accessed from https://mostwiedzy.pl/en/wojciech-wojnowski,174235-1/AGREE [35]. For this score calculation. Twelve distinct characteristic parameters were analysed for greenness assessment by AGREE. The pictograms were characterised by distinct colours, with "green" signifying eco-friendliness or the least impact from hazardous substances. The colour "yellow" depicts moderate or acceptable levels of hazardous substances. The colour "red" signifies high impact on environmental conditions.

The Modified Green Analytical Procedure Index (MoGAPI) is an online, software-supported metric for evaluating greenness, available at https://fotouhmansour.github.io/MoGAPI/. It has primarily analysed three parameters, including sample preparation procedure, solvents and reagents used in the analytical method, and instrumentation used to determine the method's greenness. Similarly, complexMoGAPI addresses several other conditions, such as yield and conditions, relation to the green economy, workup and purification, etc., which makes it more precise and accurate than MoGAPI. Like AGREE, the analytical method requires entering inputs into the software and produces a pictogram with a cumulative score ranging from 0 to 100. Values above 80 are considered "fully acceptable and environmentally optimal", scores between 60 and 79 are "good", whereas 40-59 indicate "moderately green".

The Blue Applicability Grade Index (BAGI) is a metric used to evaluate the eco-friendliness of an analytical method, reflecting its practical applicability, throughput, and routine feasibility. The tool was accessed on the website https://bagi-index.anvil.app/. It mainly focuses on the analytical procedures and highlights the major strengths and analytical constraints of the developed analytical method. BAGI uses 10 characteristic parameters that need to be entered in the software, such as AGREE and MoGAPI, and then calculates scores. The scores range from 25 to 100, and the higher the BAGI score, the more eco-friendly the developed analytical method.

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Method applicability

The final validated HPLC method was used for individual and simultaneous quantification of GIFT and EA. The saturation solubility studies (each molecule) and assay (blended powder mixture) were evaluated by the validated HPLC method.

Saturation solubility studies

The solubility study was conducted using the modified shake-flask method. Excessive amounts of GIFT and EA were added to the microcentrifuge tubes containing 2 mL of phosphate buffer (pH 7.4). The final suspension was shaken for 48 h at 37 °C and 100 rpm on a shaker. The samples were collected at 48 h, filtered through a 0.22 µm syringe filter, and analysed using the developed HPLC method.

Assay

A blended powder mixture of molecules was prepared in a 1:1 ratio. A final blended powder (1 mg) was collected, solubilised in 2 mL of ethanol, diluted with mobile phase, filtered, and analysed by the validated HPLC method. Analyses were performed in triplicate, and the mean assay was calculated using Equation 4.

$$\text{DC}\,(\%) = \frac{\text{Obtained drug content}}{\text{Total drug content}} \times 100 \qquad (4)$$

In silico toxicity prediction of gefitinib and ellagic acid

The potential toxicity of GIFT and EA was evaluated using the in silico toxicity prediction tool ProTox 3.0 (https://tox.charite.de/protox3/index.php?site=home). ProTox 3.0 utilises various machine-learning algorithms and molecular similarity models to predict multiple toxicity endpoints. By inputting the chemical structures and their canonical SMILES, probability scores ranging from 0 to 1 were generated for each toxicity endpoint. Thus, the toxicity of both molecules was assessed based on the software-suggested probability score. A probability score ≤ 0.7 indicates a lower probability of toxicity, whereas a score ≥ 0.7 indicates a higher probability of toxicity. This study provided insights into oral and organ toxicity, toxicological pathways, and potential toxicity targets, aiding understanding of the molecular mechanisms underlying these toxic responses. Furthermore, the predicted toxicity class and median lethal dose (LD50, expressed in mg/kg body weight) were determined for both compounds. A toxicity evaluation of GIFT and EA was conducted to assess their relative toxicological risks.[36]

RESULTS AND DISCUSSION

Method development

All analytical operations and sample preparation for both molecules were performed in well-closed, amber-coloured glassware and microcentrifuge tubes. Both molecules have two distinct wavelengths, as mentioned earlier in the methodology. Although different absorption maxima were observed for ellagic acid and gefitinib, i.e., 255.0 nm and 250.0 nm, respectively (Figure. 1C), the selected wavelength of 252 nm falls within the hyperchromic absorption bands of both molecules. Therefore, at this wavelength, higher sensitivity, robustness, and acceptable molar absorptivity are anticipated for both molecules. Therefore, a common wavelength of 252 nm was chosen to streamline chromatographic analysis and simultaneously quantify both molecules.

A newly developed RP-HPLC method for the simultaneous estimation of GIFT and EA was developed using AB (pH 2.5) as the aqueous phase, and methanol and acetonitrile as the organic phase. Preliminary trials were conducted with different pH levels of the acetate buffer. Finally, the effective separation of GIFT and EA was achieved simultaneously in AB (pH 2.5), using methanol and ACN in a 68:30:02 ratio as a mobile phase and Intersil ODS-3V C18 (250 × 4.6 mm, 5 µm particle size, 100Å) as a stationary phase in an isocratic elution mode at a constant flow of 1.0 $mL.min^{-1}$. GIFT and EA were eluted at 8.076 and 5.726 min, respectively (Figure. 1D).

Method validation

The primary focus of validating the newly developed isocratic RP-HPLC method for the simultaneous estimation of GIFT and EA was to demonstrate that the method is highly robust, reliable, and suitable for all analyses. Compliance with the ICH (International Council for Harmonisation) guideline was confirmed through the simultaneous detection of both molecules and all essential parameters, as discussed in the following sections.

System suitability

Using the simultaneous, optimised isocratic analytical technique, a series of six replicate injections of a mixture of GIFT and EA at 6 $\mu g.mL^{-1}$ (molecular concentration) was evaluated. The results showed high resolution, with well-accepted observed retention times, peak areas, tailing factors, and theoretical plate counts.

No significant differences were observed in these responses after repetition. Also, the relative standard deviation for these parameters was below 2%, confirming the suitability of the developed method. All the findings are tabulated in Table 1 below.

Table 1. System suitability data for simultaneous estimation of gefitinib and ellagic acid

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Table 1

Critical Quality Attributes (CQAs)	Gefitinib*		Ellagic acid*	
	Mean ± SD	% RSD	Mean ± SD	% RSD
Peak area (uV)	228291.00 ± 2717.00	1.19	780911.00 ± 12771.48	1.64
R_t (min)	8.12 ± 0.01	0.15	5.51 ± 0.01	0.13
Column efficiency (NTP)	5668.17 ± 15.86	0.27	4373.83 ± 9.47	0.22
Tailing factor	1.15 ± 0.002	0.207	1.16 ± 0.002	0.16

- Study repeated 6 times.

Selectivity and specificity

Matrix interference

To verify the specificity or selectivity of the newly developed method, the mobile phase composition was tested as a blank, and a matrix containing gefitinib and ellagic acid at 6 $\mu g.mL^{-1}$ was analysed for potential matrix interference. The results showed that no extraneous peaks were observed at the RT of either molecule. The peak resolution and symmetry are very high for both GIFT and EA, with no significant tailing or fronting. As shown in Figure. 1D, the blank solvent does not interfere with either molecule, and both elute separately, confirming the selectivity and specificity of the developed method.

Fig. 1. Molecular structure of Gefitinib **(A)**; molecular structure of Ellagic Acid **(B)**; Schematic representation of wavelength spectra of gefitinib (red line) and ellagic acid (blue line) **(C)**; RP-HPLC chromatogram: black line represents gefitinib (GIFT, retention time-8.076 min) and ellagic acid (EA, retention time-5.726 min), whereas a pink line represents blank (mobile phase) **(D)**; and acidic stress degradation of gefitinib and ellagic acid in 0.1 N and 1 N HCl at different thermal conditions for 24 and 48 h **(E)**.

Stress degradation

In accordance with the ICH guideline, different stress conditions were chosen, including 0.1N HCl and 1N HCl for acidic hydrolysis, and 0.1N NaOH and 1N NaOH for alkaline hydrolysis. 1% and 6% H_2O_2 for oxidative stress, photolytic degradation, other buffer systems, mobile phase and environmental conditions. This study was critical for determining possible degradation products of both molecules and assessing their interference with the parent compounds.

This investigation serves as an important tool for developing novel formulations and enhancing pharmaceutical solubility, enabling the simultaneous assessment of multidrug therapies. It provides the necessary data for selecting specific solvents or buffers for formulation and development. Also, the stability of the individual molecules provides information that may be useful during processing, storage, and analysis. The comprehensive findings are depicted in Table 2.

Hydrolytic degradation

Under acidic stress, EA was prone to degradation across almost all temperature conditions, whereas GIFT was moderately stable. As shown in Table 2, in a 0.1N HCl solution, EA after 24 h showed degradation of up to 84.09 ± 9.33%, 71.76 ± 2.23%, and 69.82 ± 3.17% at 4 ℃, RT, and 80 ℃, respectively. This further degraded to 91.09 ± 11.53%, 85.05 ± 0.66%, and 79.22 ± 2.83%, respectively, after 48 h. Similar trends were observed in more concentrated solutions, such as 1N HCl, where EA was degraded by 73.24 ± 6.62 %, 59.67 ± 13.15%, and 48.74 ± 6.63% at 4 ℃, RT, and 80 ℃, respectively, after 24 h, with further degradation up to 85.59 ± 3.14%, 76.57 ± 2.84%, and 69.47 ± 8.48%, respectively, after 48 h. Stability in acidic conditions is low at 4 ℃ and RT, compared to 80 ℃, which is unusual. Similar findings were also reported by Kumar et al., who stated that EA stability is not affected by temperature.[16] Hasegawa and colleagues reported that lower recovery in 0.1N HCl is due to its conversion to a mono-deprotonated form, as EA is mono-deprotonated below pH 5.6.[37] Further, with increasing temperature, recovery in acidic media increases due to greater solubility and more ionisations.

For GIFT, degradation was low in 0.1N HCl, as shown in Table 2. After 24 h, the degradation of GIFT was 11.87 ± 1.93%, -11.50 ± 3.00%, and -23.92 ± 5.77% at 4 ℃, RT, and 80 ℃, respectively. This further increased to 20.17 ± 3.76%, -14.67 ± 1.48%, and -31.71 ± 4.18%, respectively, after 48 h. This indicates its high stability in 0.1N HCl under all temperature conditions. As GIFT has higher solubility in 0.1N HCl, increasing the temperature further enhances its solubility, which affects the degradation. For this reason, minimum degradation was noticed at 80 ℃. Whereas more concentrated 1N HCl played a destructive role in the stability of GIFT, as it degraded around 10.84 ± 0.39%, -9.92 ± 3.10%, and 23.23 ± 1.54% at 4 ℃, RT, and 80 ℃, respectively, over 24 h, and after 48 h, degraded to 14.33 ± 2.94%, -6.88 ± 1.20%, and 40.75 ± 1.31%, respectively. This confirmed the temperature-dependent degradation of the GIFT in 1N HCl. It is moderately stable at 4 ℃ and RT, but at 80 ℃, it showed significant degradation over time. This indicated that, though GIFT is stable in 0.1N HCl, it becomes unstable in 1N HCl,

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Table 2

Stress Mediums	Time (h)	Temperature Condition	Degradation (%) Mean ± SD	
			Ellagic acid*	Gefitinib*
0.1N HCl	24	4 °C	84.09 ± 9.33	11.87 ± 1.93
		RT	71.76 ± 2.23	-11.50 ± 3.00
		80 °C	69.82 ± 3.17	-23.92 ± 5.77
	48	4 °C	91.09 ± 11.53	20.17 ± 3.76
		RT	85.05 ± 0.66	-14.67 ± 5.33
		80 °C	79.22 ± 2.83	-31.71 ± 4.18
1N HCl	24	4 °C	73.24 ± 6.62	10.84 ± 0.39
		RT	59.67 ± 13.15	-9.92 ± 3.10
		80 °C	48.74 ± 6.63	23.23 ± 1.54
	48	4 °C	85.59 ± 3.14	14.33 ± 2.94
		RT	76.57 ± 2.84	-6.88 ± 1.20
		80 °C	69.47 ± 8.48	40.75 ± 1.31
0.1N NaOH	24	4 °C	70.49 ± 0.69	26.28 ± 0.06
		RT	94.31 ± 1.23	-23.64 ± 0.36
		80 °C	91.15 ± 0.12	-14.88 ± 0.19
	48	4 °C	91.16 ± 0.95	8.84 ± 0.38
		RT	98.12 ± 0.59	-28.29 ± 6.94
		80 °C	95.19 ± 1.15	-31.29 ± 3.34
1N NaOH	24	4 °C	84.53 ± 1.04	15.74 ± 1.35
		RT	97.72 ± 0.62	11.27 ± 1.79
		80 °C	97.03 ± 0.32	23.91 ± 7.88
	48	4 °C	92.81 ± 0.65	19.85 ± 1.75
		RT	98.49 ± 0.22	17.92 ± 5.63
		80 °C	97.63 ± 0.17	27.51 ± 2.82
1% H_2O_2	24	4 °C	85.49 ± 1.72	14.95 ± 0.36
		RT	73.64 ± 8.11	16.10 ± 2.23
		80 °C	93.86 ± 0.72	80.58 ± 2.50
	48	4 °C	91.54 ± 0.68	22.24 ± 3.76
		RT	87.38 ± 3.18	26.43 ± 5.70
		80 °C	95.90 ± 0.67	95.81 ± 2.20
6% H_2O_2	24	4 °C	92.11 ± 3.61	23.09 ± 2.19
		RT	85.29 ± 1.77	20.84 ± 1.80
		80 °C	96.05 ± 0.06	98.16 ± 3.65
	48	4 °C	95.52 ± 3.38	28.82 ± 0.84
		RT	97.39 ± 0.30	18.55 ± 1.68
		80 °C	96.18 ± 0.17	98.67 ± 4.43

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Table 2: (Continued)

Stress Mediums	Time (h)	Temperature Condition	Degradation (%) Mean ± SD	
			Ellagic acid*	Gefitinib*
MP	24	4 ℃	26.42 ± 9.10	-4.90 ± 0.01
		RT	26.44 ± 0.72	-3.16 ± 0.21
		80 ℃	5.27 ± 0.48	-14.19 ± 8.65
	48	4 ℃	37.21 ± 0.95	-3.86 ± 1.06
		RT	48.90 ± 11.78	-6.82 ± 0.72
		80 ℃	32.32 ± 2.50	-17.78 ± 5.90
PB (pH 7.4)	24	4 ℃	12.05 ± 0.36	18.23 ± 2.52
		RT	6.80 ± 1.68	11.94 ± 1.28
		80 ℃	14.53 ± 2.26	11.48 ± 0.44
	48	4 ℃	11.50 ± 0.79	4.68 ± 1.12
		RT	14.32 ± 0.92	14.57 ± 0.58
		80 ℃	21.90 ± 3.18	19.51 ± 3.16
Ethanol	24	4 ℃	11.72 ± 2.42	4.38 ± 1.40
		RT	3.77 ± 0.65	8.46 ± 0.33
		80 ℃	9.78 ± 1.91	9.39 ± 1.53
	48	4 ℃	15.39 ± 0.97	8.46 ± 1.98
		RT	-0.81 ± 0.89	1.75 ± 1.24
		80 ℃	0.83 ± 0.25	6.12 ± 1.34
Photolytic degradation of ellagic acid and gefitinib in different buffers for 24 & 48 h				
Medium	**Condition**	**Time (h)**	**Degradation (%) Mean ± SD**	
			Ellagic Acid*	**Gefitinib***
AB (pH 2.5)	Light	24	81.22 ± 1.03	-4.05 ± 0.22
		48	78.32 ± 4.62	-2.52 ± 0.45
	Dark	24	81.33 ± 0.39	-2.06 ± 0.30
		48	85.27 ± 0.38	1.97 ± 0.30
PB (pH 7.4)	Light	24	6.69 ± 2.48	9.89 ± 1.55
		48	26.10 ± 4.15	13.18 ± 2.25
	Dark	24	-1.48 ± 0.29	-1.72 ± 0.33
		48	12.51 ± 0.94	13.00 ± 0.28

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whereas EA is unstable in both media. This is shown in Figure. 1E, where the peak intensity decreased for EA, and under higher acidic conditions, the degradation peak occurs at 4.7 min. The major acidic degradation of GIFT happened due to hydrolysis. As inferred from Patra et al., under several stress conditions, GIFT produces around seven degradants, among which three major degradants (I-III) were produced in acidic conditions due to cleavage of the quinazoline system and the removal of HCHO, methylmorpholine, and the hydroxyl group.[38]

Under alkaline stress (Table 2), EA was highly degraded with increasing temperature and time. Degradation of 70.49 ± 0.69 %, 94.31 ± 1.23%, and 91.15 ± 0.12 % was observed at 4 ℃, RT, and 80 ℃ after 24 h in 0.1N NaOH; after 48 h, it reached 91.16 ± 0.95%, 98.12 ± 0.59%, and 95.19 ± 1.15%, respectively, which indicates significant degradation in 0.1N NaOH. In 1N NaOH, EA showed a more destructive effect, with 84.53 ± 1.04% degraded at 4 ℃ within 24 h, and 97.72 ± 0.62% and 97.03 ± 0.32% degraded at RT and 80 ℃, respectively. After 48 h, it reached 92.81 ± 0.65% at 4 ℃, 98.49 ± 0.22% at RT, and 97.63 ± 0.17% at 80 ℃. This suggests that the molecule degrades under almost all distinct thermal conditions in alkaline media. EA is susceptible to alkaline conditions; as shown in Figure. 2A, an EA-degradant peak was observed at 12.5 min in the chromatogram after 24 h, which disappeared after 48 h, suggesting complete EA degradation. Though the EA is freely soluble in an alkaline medium, its stability decreases significantly with increasing basicity. The decline in stability is attributed to the breakdown of the polyphenolic lactone ring in an alkaline medium. In addition, interactions between phenolic groups form less stable dimers that readily degrade in an alkaline medium.[39,40]

In the case of GIFT, alkaline degradation was very low, with 26.28 ± 0.06%, -23.64 ± 0.36%, and -14.88 ± 0.19% degraded after 24 h at 4 ℃, RT, and 80 ℃, respectively, whereas 8.84 ± 0.38%, -28.29 ± 6.94%, and -31.29 ± 3.34% were degraded after 48 h. On the other hand, at higher alkaline concentrations (i.e., 1N NaOH), degradation of GIFT was observed at 15.74 ± 1.35%, 11.27 ± 1.79%, and 23.91 ± 7.88% after 24 h at 4 ℃, RT, and 80 ℃, respectively. The GIFT degradation was similar, with values of 19.85 ± 1.75%, 17.92 ± 5.63%, and 27.51 ± 2.82% after 48 h at 1 N NaOH. Compared with acidic hydrolysis, in an alkaline medium, with increasing temperature and time, the solubility of gefitinib increases, resulting in higher recovery at 80 ℃. Solvent evaporation can also be another reason for higher recovery at higher temperatures. Alkaline degradation chromatograms of GIFT and EA are shown in Figure. 2A. Alkaline degradation occurred due to the opening of the morpholine moiety, O-dealkylation of the benzene ring and N-dealkylation of the aminium moiety.[38]

In compliance with ICH guidelines, hydrolytic stress degradation was also performed in PB (pH 7.4), ethanol and in the mobile phase (MP 68:30:02). As shown in Figs 2B and 2C, both molecules have better stability in the PB (pH 7.4) and ethanol, whereas stability was decreased in the mobile phase composition after 48 h and at higher temperatures, as shown in Figure. 2B. Though EA showed greater stability in PB (pH 7.4), GIFT showed slight degradation across all temperature conditions and time points. As detailed in Table 2, degradation of 12.05 ± 0.36%, 6.80 ± 1.68%, and 14.53 ± 2.26% was observed in EA under PB (pH 7.4) after 24 h of storage at 4 °C, RT, and 80 °C, respectively. This further degraded to 11.50 ± 0.79%, 14.32 ± 0.92%, and 21.90 ± 3.18% after 48 h under the same storage conditions. Similarly, GIFT exhibited 18.23 ± 2.52%, 11.94 ± 1.28 %, and 11.48 ± 0.44% degradation in PB (pH 7.4) after 24 h, and 4.68 ± 1.12%, 14.57 ± 0.58%, and 19.51 ± 3.16% degradation after 48 h at 4 °C, RT, and 80 °C, respectively. The higher recovery and lower degradation at higher temperatures could be due to increased solubility.

In ethanol, EA degradation was 11.72 ± 2.42%, 3.77 ± 0.65%, and 9.78 ± 1.91% after 24 h at 4 °C, RT, and 80 °C, respectively, whereas it was 15.39 ± 0.97%, -0.81 ± 0.89%, and 0.83 ± 0.25% after 48 h at the same temperatures. On the other hand, GIFT showed a degradation of 4.38 ± 1.40% at 4 °C after 24 h, increasing to 8.46 ± 0.33% at RT and 9.39 ± 1.53% at 80 °C. After 48 h in ethanol, degradation was 8.46 ± 1.98% at 4 °C and 1.75 ± 1.24% and 6.12 ± 1.34% at RT and 80 °C, respectively. These irregularities in degradation can be anticipated with an increase in temperature and the volatile nature of ethanol. After 48 h, degradation of both molecules decreased at higher temperatures, which could be due to increased solubility and solvent evaporation of the volatile ethanol. The chromatograms of GIFT and EA under stress-degradation conditions are shown in Figure. 2C.

For the mobile phase composition (68:30:02), after 48 h of storage, moderate degradation was observed. As detailed in Table 2. For EA, degradations of 26.42 ± 9.10%, 26.44 ± 0.72%, and 5.27 ± 0.48% were observed after 24 h at 4 °C, RT, and 80 °C, respectively. This further degraded, and after 48 h, the final degradations were 37.21 ± 0.95%, 48.90 ± 11.78%, and 32.32 ± 2.50% at the above-mentioned temperatures. For GIFT, -4.90 ± 0.01% degradation was found at 4 °C, and at RT and 80 °C, the degradations were -3.16 ± 0.21% and -14.19 ± 8.65%, respectively, after 24 h. After 48 h, the values became -3.86 ± 1.06%, -6.82 ± 0.72%, and -17.78 ± 5.90% at 4 °C, RT, and 80 °C, respectively. This shows that at lower temperatures, almost negligible GIFT degradation was observed; as the temperature increased, recovery decreased, and degradation increased. This suggests that samples should be stored at lower temperatures, e.g., 4 °C, during handling and analysis. Second, the study suggests that, for the analysis, a fresh mobile phase should be preferred.

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Oxidative stress degradation

Oxidation plays a major role in the degradation of GIFT and EA. Between them, EA shows the greatest degradation under oxidative stress. Table 2 shows that EA degraded by 85.49 ± 1.72%, 73.64 ± 8.11%, and 93.86 ± 0.72% at 4 °C, RT, and 80 °C, respectively, over 24 h. Degradation of 91.54 ± 0.68%, 87.38 ± 3.18%, and 95.90 ± 0.67% occurred after 48 h in the presence of 1% H_2O_2 media. Similar levels of oxidation were observed with 6% H_2O_2, with 92.11 ± 3.61%, 85.29 ± 1.77%, and 96.05 ± 0.67% degradation of EA at 4 °C, RT, and 80 °C, respectively, after 24 h. After 48 h, almost complete degradation of EA occurred, with degradation values of 95.52 ± 3.38%, 97.39 ± 0.30%, and 96.18 ± 0.17%, respectively.

GIFT also showed a slight decrease in oxidative stress, but this effect is temperature- and time-dependent. Major degradation occurred at 80 °C and after 48 h. In 1% H_2O_2 media, GIFT degraded to 14.95 ± 0.36% at 4 °C, 16.10 ± 2.23% at RT, and 80.58 ± 2.50% at 80 °C after 24 h, whereas after 48 h, degradation increased to 22.24 ± 3.76%, 26.43 ± 5.70%, and 95.81 ± 2.20%, respectively, at the distinct temperatures. On the other hand, at higher oxidative concentrations, i.e., 6% H_2O_2, degradation of GIFT was observed at 23.09 ± 2.19%, 20.84 ± 1.80%, and 98.16 ± 3.65% after 24 h at 4 °C, RT, and 80 °C, respectively. The GIFT degradation increased, with values of 28.82 ± 0.84%, 18.55 ± 1.68%, and 98.67 ± 4.43% after 48 h. This indicates severe GIFT degradation at 80 °C, with a degradation peak near 12 min, as shown in Figure. 2D. Oxidation of GIFT mainly leads to the formation of GIFT N-oxide.[41] This oxidative degradation mainly happened due to several reasons, such as the removal of the methanol group, N-dealkylation of the aniline moiety, or dealkylation or deformylation of the formamide moiety.[38]

Fig 2. Alkaline stress degradation of gefitinib and ellagic acid in 0.1 N and 1 N NaOH at different thermal conditions for 24 and 48 h **(A)**; Hydrolytic stress degradation of gefitinib and ellagic acid in the mobile phase (MP 68:30:02) and PB pH 7.4 at different thermal conditions for 24 and 48 h **(B);** Hydrolytic stress degradation of gefitinib and ellagic acid in ethanol at different thermal conditions for 24 and 48 h **(C)**; and Oxidative degradation in the presence of 1% and 6% H_2O_2 of gefitinib and ellagic acid at different thermal conditions for 24 and 48 h **(D)**.

Thermal stress degradation

Thermal degradation is a major concern not only in manufacturing and analysis, but also in transportation, storage, and handling. For the thermal degradation study, all samples are prepared in different media, including 0.1N and 1N HCl, 0.1N and 1N NaOH, 1% and 6% H_2O_2, mobile phase, ethanol, and PB and kept at 4 °C, RT (25 °C), and 80 °C. The thermal behaviour of EA shows an anomaly in acidic conditions; at lower temperatures (e.g., 4 °C), more degradation was observed, whereas at higher temperatures, degradation was less, as detailed in previous sections. Apart from that, in almost all other conditions, such as alkaline, oxidative, and hydrolytic conditions, with increasing temperature, degradation increased. This depicts the impact of temperature on the EA's stability. EA showed almost equal recovery in ethanol across all temperature conditions, indicating higher thermal stability in ethanol.

On the other hand, GIFT's degradation was lower at RT (25 °C) and 80 °C than at 4 °C in acidic and alkaline media. Due to the higher ionisation and increased solubility at higher temperatures, as the pKa values of GIFT are 5.28 and 7.17, it can be easily ionised at lower pH, and temperature influences its solubility. Similar results were also found under hydrolytic stress. Under oxidative conditions, only at 4 °C did GIFT remain relatively more stable than at RT and 80 °C. Higher temperatures can lead to the oxidation of the morpholine ring and dechlorination under photolytic and oxidative conditions. It is important to note that O-demethylation and morpholine ring oxidation are reported to dominate in the metabolism of the GIFT.[42] The results are reported in Table 2.

Table 2. Forced degradation and photolytic degradation study of ellagic acid and gefitinib under different stress conditions for 24 and 48 h

Photolytic degradation

Photolytic degradation was performed in AB (pH 2.5) and PB (pH 7.4) to verify the photolytic stability of both molecules, as detailed in Table 3. EA showed almost identical stability in both light and dark conditions when stored in AB (pH 2.5). In the presence of light and AB (pH 2.5), degradation was 81.22 ± 1.03% and 78.32 ± 4.62% after 24 and 48 h, respectively, and in the dark, it was 81.33 ± 0.39% and 85.27 ± 0.38% after 24 h and 48 h, respectively. This shows at least a non-significant effect of light on EA in this buffer. This study revealed that the major degradation occurred in this medium (AB, pH 2.5), which was not significantly affected by light.

Fig 3. Photolytic degradation in different buffers of gefitinib and ellagic acid for 24 and 48 h.

But light played a significant role in the PB (pH 7.4), with 6.69 ± 2.48% and 26.10 ± 4.15% degradation after 24 h and 48 h of storage, respectively. In the absence of light, almost -1.48 ± 0.29% of EA was degraded after 24 h, and 12.51 ± 0.94% degradation was found after 48 h, as shown in Figure. 3. This shows that EA stability was slightly affected by light. This study recommends protecting this molecule and its formulation from light during handling, formulation, storage, and analysis. The study showed slightly better stability in light after 24 h than reported in the literature.[16] in PB (pH 7.0). The present study showed slightly better recovery of EA, especially after 24 h, which may be due

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Table 3

Repeatability and intermediate precision data of ellagic acid and gefitinib												
Peak area (uV) of Ellagic Acid												
Levels	**Repeatability**						**Intermediate**					
	LQCC		**MQCC**		**HQCC**		**LQCC**		**MQCC**		**HQCC**	
Mean ± SD*	1,43,866 ± 1,279		7,79,620 ± 1,119		15,39,530 ± 6,909		1,47,447 ± 2,812		7,26,457 ± 10,149		15,44,691 ± 1,880	
RSD (%)	0.8893		0.1435		0.4488		1.9071		1.3970		0.1217	
Peak area (uV) of Gefitinib												
Levels	**Repeatability**						**Intermediate**					
	LQCC		**MQCC**		**HQCC**		**LQCC**		**MQCC**		**HQCC**	
Mean ± SD*	46,574 ± 223		2,27,315 ± 522		3,92,576 ± 3,588		43,191 ± 220		2,25,944 ± 257		3,98,189 ± 5,282	
RSD (%)	0.479		0.230		0.914		0.510		0.114		1.327	
Recovery data for ellagic acid and gefitinib												
Level (%)	**Spiked Conc. (µg/mL)**	**Ellagic Acid***					**Gefitinib***					
		Reference		**Test**		**Recovery (%)**	**Reference**		**Test**		**Recovery (%)**	
		Mean ± SD	**RSD (%)**	**Mean ± SD**	**RSD (%)**	**Mean ± SD**	**Mean ± SD**	**RSD (%)**	**Mean ± SD**	**RSD (%)**	**Mean ± SD**	
75	4.5	105.70 ± 1.60	1.54	99.85 ± 3.60	3.60	96.68 ± 2.97	101.55 ± 5.22	5.14	106.43 ± 0.10	0.10	104.76 ± 0.10	
100	6.0	97.26 ± 3.82	3.90	101.63 ± 4.34	4.27	103.95 ± 2.95	101.31 ± 1.72	1.70	99.89 ± 0.01	0.01	98.43 ± 2.02	
125	7.5	102.70 ± 6.69	6.50	102.48 ± 0.52	0.51	100.10 ± 6.10	101.94 ± 0.003	0.003	105.87 ± 5.17	4.88	103.85 ± 6.14	

to the slightly higher pH of the buffer used in the study, which is responsible for the better ionisation of EA at an alkaline pH, contributing to improved solubility. However, a reduction in recovery after 48 h (in the presence or absence of light) indicates its time-dependent recovery/sensitivity in this buffer. The findings of enhanced solubility at an alkaline pH are consistent with previous studies.[43]

As detailed in Table 2. GIFT is highly stable in AB (pH 2.5) under both light and dark conditions. As depicted in AB (pH 2.5), degradations of -4.05 ± 0.22% and -2.52 ± 0.45% were obtained after 24 and 48 h in light, and similar results were observed in dark conditions as well. In the dark, -2.06 ± 0.30% and 1.97 ± 0.30% degradation were achieved after 24 and 48 h, respectively. In PB (pH 7.4), there was no degradation in the dark for up to 24 h, and after 48 h, only 13.00 ± 1.44% had degraded. In the light, only 9.89 ± 1.55% and 13.18 ± 2.25% degradation were observed after 24 and 48 h, respectively. The chromatogram of photolytic degradation is shown in Figure. 3.

Linearity

For EA and GIFT, standard plots were generated in the range of 1 to 11 µg/mL of both molecules to assess linearity. The analysis yielded high correlation coefficients (r^2) for EA (0.999) and GIFT (0.9953). The calibration curve equations for EA and GIFT were y = 127146x - 3924.8 and y = 35719x + 6450.6, respectively, based on mean peak area and concentration. Figure. 4 shows the calibration curves of EA (A) and GIFT (B).

Precision

Repeatability and intermediate precision were assessed to evaluate the analytical method's precision by following the ICH guidelines. The relative standard deviation for repeatability precision was ≤ 1%, whereas for intermediate precision it was ≤ 2%, demonstrating that the developed analytical method for the

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simultaneous estimation of EA and GIFT is precise. The precision data were presented in Table 3.

Table 3. Repeatability, intermediate precision and recovery data of ellagic acid and gefitinib

Recovery

The recovery of EA and GIFT with the developed method was assessed by the close agreement between the derived and true values, as expressed as percentage (%) recovery. The overall % recovery of the 75%, 100%, and 125% samples was 96% to 105% for both ellagic acid and gefitinib. Results for the analytical method's accuracy are shown in Table 3. The recovery (%) for EA and GIFT was within the acceptance range, indicating that the proposed method is accurate for the simultaneous estimation of both drugs.

Fig 4. Calibration curve of **A**. Ellagic Acid, **B.** Gefitinib and Schematic representation of the results of the Greenness assessment tools: **C.** AGREE, **D.** BAGI, **E.** MoGAPI, **F.** Complex MoGAPI.

Robustness

The results of the consequences of the purposeful changes of the operating conditions, such as injection volume (10 ± 2 µL), detector wavelength (252 ± 2 nm), pH of the mobile phase (2.5 ± 0.2), column oven temperature (25 ± 2 °C), and flow rate (1 ± 0.2 mL/min), are tabulated in Table 4. The observed relative standard deviation for each condition was less than 2, within the permissible limits. This suggests that the developed isocratic analytical method for the simultaneous estimation of EA and GIFT is robust and reliable for the routine estimation of both molecules, individually and together.

Detection limit (DL) and quantification limit (QL)

To assess reliable detection (DL) and quantification (QL), thresholds for both molecules were determined. EA exhibited a DL of 0.13 µg.mL^{-1} while GIFT's DL was 0.105 µg.mL^{-1}. The QL for EA and GIFT were 0.39 and 0.31 µg.mL^{-1}, respectively. These findings revealed that this method can reliably detect and quantify both EA and GIFT with adequate sensitivity.

Greenness assessment

The environmental sustainability of the developed and validated analytical method was assessed using AGREE, MoGAPI, ComplexMoGAPI, and BAGI software. The data obtained revealed that this analytical method is eco-friendly and poses minimal environmental risks. As shown in Figure. 4C, the green AGREE pictogram value is 0.75, which is very close to 1, indicating the analytical method was green with negligible environmental impact.[44] The BAGI score of 85.0 (Figure. 4D) indicates the eco-friendliness of the developed method. On the other hand, the MoGAPI score (Figure. 4E) obtained was 76, and 5 out of 16 factors were completely green, indicating that this analytical method is environmentally compatible and highly green. A more complex MoGAPI score (Figure. 4F) was also calculated to provide a more detailed evaluation of the greenness of this analytical method, yielding a score of 80, indicating that the method passed all greenness assessment tools and was eco-friendly. With a very low ACN percentage (only 2%), the developed analytical method met all metrics, validating its "green" and sustainable performance under all conditions.

Method applicability

Saturated solubility studies

A saturation solubility study was conducted separately for standard GIFT and EA in a phosphate buffer (pH 7.4). The solubility of GIFT and EA after 48 h was 0.0038 ± 0.0009 µg.mL^{-1} and 1.2700 ± 0.0500 µg.mL^{-1} , respectively. It has also been reported that GIFT shows limited solubility in neutral and alkaline buffers, as well as in a few solvent-buffer combinations.[45,46] Similarly, for EA, limited solubility is reported in phosphate buffer (pH 7.4).[47]

Assay

The assay of both molecules in the blended mixture of GIFT and EA were 105.97 ± 4.37% and 100.09 ± 8.46%, respectively.

Table 4. Robustness data of the developed simultaneous method for ellagic acid and gefitinib

In silico toxicity prediction of gefitinib and ellagic acid

The in silico toxicity of GIFT and EA was assessed using ProTox 3.0.GIFT is classified as toxicity class 5, with a predicted LD_{50} value of 2935 mg.kg^{-1}. However, it demonstrates a high likelihood of causing organ toxicities, particularly in the lungs (0.98), neurons (0.83), and liver (0.73). Notable toxicological endpoints include immunotoxicity (0.99), clinical toxicity (0.87), and blood-brain barrier (BBB) toxicity (0.78), indicating potential risks associated with prolonged use. These effects are attributed to specific structural characteristics of GIFT, such as its electrophilic quinazoline core, which can lead to off-target activity, and its high lipophilicity, which results in accumulation in lipid-rich organs like the liver and lungs.[48,49]

EA is classified as a toxicity class 4 compound with a predicted LD_{50} of 2991 mg.kg^{-1}. This suggests that it has lower toxicity than GIFT. It particularly demonstrates its two main toxicities, i.e., respiratory toxicity (0.84) and nutritional toxicity (0.71). The toxicity of EA primarily arises from its structural features, which include four phenolic hydroxyl groups attached to a fused aromatic ring system. These groups increase acidity, leading to deprotonation of moist mucous membranes in the respiratory

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Table 4

Sr. No.	Parameter	Level	Ellagic acid*								Gefitinib*							
			R_t (min)		Area (uV)		NTP		TF		R_t (min)		Area (uV)		NTP		TF	
			Mean ± SD	RSD %	Mean ± SD	RSD %	Mean ± SD	RSD %	Mean ± SD	RSD %	Mean ± SD	RSD %	Mean ± SD	RSD %	Mean ± SD	RSD %	Mean ± SD	RSD %
1.	Injection volume (µL)	8	5.43 ± 0.002	0.04	565252 ± 4906.32	0.86	3915 ± 2.94	0.07	1.18 ± 0.002	0.20	7.99 ± 0.007	0.09	182213 ± 1702.90	0.93	5650.67 ± 5.73	0.1	1.14 ± 0.001	0.10
		12	5.45 ± 0.008	0.15	856234.7 ± 345.73	0.85	3894.33 ± 16.63	0.42	1.17 ± 0.002	0.20	8.08 ± 0.01	0.21	272062 ± 2458.76	1.08	5651.67 ± 12.49	0.22	1.146 ± 0.001	0.07
2.	pH	2.3	4.45 ± 0.01	0.2	727570 ± 6408.30	0.88	4137.30 ± 39.16	0.94	1.22 ± 0.001	0.11	5.85 ± 0.003	0.06	228933 ± 2963.50	1.29	5006.60 ± 18.37	0.36	1.13 ± 0.001	0.15
		2.7	5.46 ± 0.01	0.18	717960.70 ± 10044	1.39	4549.30 ± 47.44	1.04	1.19 ± 0.002	0.2	9.33 ± 0.01	0.18	228716 ± 2495.60	1.09	6058 ± 26.87	0.44	1.16 ± 0.002	0.24
3	λ max (nm)	250	5.45 ± 0.004	0.09	697224 ± 6288.04	0.90	3896 ± 6.48	0.16	1.17 ± 0.001	0.10	8.01 ± 0.009	0.11	223332 ± 3027.20	1.35	5608.60 ± 6.79	0.12	1.14 ± 0.002	0.18
		254	5.44 ± 0.004	0.07	705378 ± 5132.30	0.70	3904.60 ± 7.76	0.19	1.17 ± 0.0004	0.03	8.00 ± 0.006	0.08	224289 ± 2736.59	1.20	5556.30 ± 6.84	0.12	1.15 ± 0.002	0.24
4	Flow rate (mL/min)	0.8	5.97 ± 0.02	0.46	784240 ± 6551.30	0.80	4221 ± 55.25	1.31	1.20 ± 0.003	0.31	8.76 ± 0.04	0.55	251260 ± 3722.80	1.40	5886.30 ± 28.11	0.47	1.15 ± 0.004	0.37
		1.2	4.92 ± 0.005	0.11	643582 ± 5800.20	0.90	3778.60 ± 4.64	0.12	1.16 ± 0.001	0.14	7.21 ± 0.009	0.13	207035 ± 3128.20	1.51	5191.67 ± 11.44	0.22	1.14 ± 0.003	0.29
5	Column temperature (°C)	23	5.41 ± 0.007	0.13	710562 ± 6979.40	0.98	3967.67 ± 29.48	0.74	1.18 ± 0.002	0.17	7.94 ± 0.01	0.16	227219 ± 2458.70	1.08	5651.60 ± 12.49	0.22	1.14 ± 0.0008	0.07
		27	5.43 ± 0.004	0.07	733575 ± 8690.40	1.18	4754.30 ± 11.08	0.23	1.19 ± 0.003	0.31	7.98 ± 0.009	0.11	227538 ± 2195.70	0.96	5813.30 ± 14.88	0.25	1.15 ± 0.002	0.25

tract. As a result, this can cause mucosal irritation, inflammation, coughing, and difficulty in breathing. Nutritional toxicity is attributed to its hydrophobic core and strong hydrogen-bonding affinity for digestive enzymes, such as trypsin and amylase, as well as dietary proteins in the gut. These interactions can lead to protein cross-linking, reducing enzyme activity and making it harder for proteins to be digested and absorbed. Additionally, the free -OH groups of EA can act as chelators, binding to essential multivalent metal ions such as Fe^{2+} and Zn^{2+}. This interaction may hinder absorption of minerals across the intestinal lining, potentially leading to nutritional deficiencies over time.[50] However, this toxicity is mild, as the score is 0.71. But it doesn't have high hepatotoxicity, neurotoxicity, respiratory toxicity, immunotoxicity, BBB barrier, or clinical toxicity, like GIFT.

Therefore, this combination can balance each other's toxicity and may be the best for cancer treatment. However, it should be noted that these toxicity predictions are based on computational models and require further validation through in vitro and in vivo studies to accurately reflect real-world conditions.

CONCLUSION

The present work represents an analytical method for the simultaneous estimation of EA and GIFT, along with an SIA. The developed method can accurately, precisely, and effectively

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detect and quantify both molecules simultaneously in a single run, facilitating in vitro analysis in multi-drug treatment. The developed method also demonstrates excellent linearity, high sensitivity, and robustness, indicating that it meets the ICH validation guidelines and is suitable for complex pharmaceutical research and large-scale quality-control sample analysis.

Apart from validation and primary quantification, this research also focused on stress degradation studies of both molecules and on the identification of their degradation products under various hydrolytic, oxidative, thermal, and photolytic conditions. The degradation analysis suggested that protecting EA from acidic, alkaline, and oxidative conditions, and protecting GIFT from oxidative stress, particularly at higher temperatures, would improve the shelf-life of molecules and formulations. Under other stress conditions, GIFT showed moderate to high stability. In the case of photolytic degradation, EA degraded more in AB (pH 2.5), regardless of light or dark conditions, whereas in PB (pH 7.4), it showed greater stability. The results showed higher stability of GIFT across media and time. Indicating a need for proper storage conditions and handling. The higher stability in ethanol over 48 h, as well as at high temperatures, indicates its use as a solvent in formulation and development.

Furthermore, the environmental impact of this method was estimated using multiple metrics, including AGREE, MoGAPI, ComplexMoGAPI, and BAGI, which assess its eco-friendliness. Developing an environmentally friendly, sustainable analytical method for the simultaneous estimation of two anticancer molecules not only enables the quantification of samples from in vitro and in vivo studies but also significantly promotes cost-effectiveness in Green Analytical Chemistry (GAC) in modern laboratory environments.

The developed analytical method was applied for the assay of GIFT and EA in the blended powder mixture as well as for the saturated solubility assessment of both GIFT and EA individually in phosphate buffer (pH 7.4). The study confirmed the suitability and reproducibility of the developed method. The solubility of both molecules is limited at the specified pH. Still, the assay for both molecules was above 100%, confirming that the molecules can enhance each other's solubility in a blended powder mixture. This may be more interesting to further explore.

The toxicity data obtained from in silico studies provide valuable insights into improving the safety, quality, and efficacy of a formulation containing GIFT and EA. The study suggested that the combination of these two molecules may be a promising approach for new formulation development, as it may balance their respective toxicities.

FUTURE SCOPE

Combining both molecules into a single formulation could be a promising approach for treating cancers. Therefore, this environmentally friendly analytical method for the simultaneous quantification of GIFT and EA would be useful for aspects of their new formulation development, including pre-formulation, prototype, scale-up, and commercialisation studies. The stability-indicating assay method for this multi-drug combination under various stress conditions guides the manufacturing of multi-drug formulations and their storage conditions. Furthermore, identification of degradants is recommended using suitable analytical techniques to distinguish them from the parent compound and other degradants, as well as to protect them from further degradation. The combination of both molecules may exhibit synergistic activity with reduced toxicity; however, further in vivo studies in suitable animal models are recommended.

AUTHOR CONTRIBUTION STATEMENT

Pritam Jana: Data curation; formal analysis; investigation; methodology; validation; writing-original draft. **Ganesh Pandav**: Data curation; formal analysis; investigation; methodology. **Arka Karmakar**: Data curation; formal analysis; investigation; methodology. **Lalit Kumar:** Conceptualization; investigation; supervision; writing-review & editing.

DATA AVAILABILITY STATEMENT

The data will be made available upon appropriate request.

DECLARATION OF COMPETING INTERESTS

The authors declare that they have no conflicts of interest related to this research.

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ABBREVIATIONS

AB: Ammonium acetate buffer; AGREE: Analytical Greenness metric; AR: Acquired resistance; BAGI: Blue Applicability Grade Index; BBB: Blood-brain barrier; BC: Breast cancer; DL: Detection limit; EA: Ellagic acid; EGFR: Epidermal growth factor receptor; EGFR-TK: EGFR tyrosine kinase; GAC: Green Analytical

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Chemistry; GIFT: Gefitinib; ICH: International Council for Harmonisation; LD50: Median lethal dose; LQCC/MQCC/HQCC: Low/medium/high-quality control concentration; MoGAPI: Modified Green Analytical Procedure Index; NSCLC: Non-small-cell lung cancer; NTP: Number of theoretical plates; PB: Phosphate buffer; PDP: Potassium dihydrogen phosphate; QC: Quality control; QL: Quantification limit; RP-HPLC: Reverse-phase high-performance liquid chromatography; RSD: Relative standard deviation; RT: Retention time/room temperature; SIA: Stability-indicating assay.

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