UV-Vis spectroscopy combined with chemometrics for discriminating gamma-irradiated Javanese long pepper (*Piper retrofractum* Vahl) extracts

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Abstract. Javanese long pepper (*Piper retrofractum* Vahl) is an Indonesian spice known for its main bioactive compound, piperine, which exhibits various pharmacological properties. This study aimed to develop a rapid, non-destructive analytical approach to classify Javanese long pepper extracts exposed to different doses of gamma irradiation using UV-Vis spectroscopy combined with chemometrics. The absorption spectra of 25 extract samples treated with irradiation doses of 0, 250, 500, 750, and 1000 kGy were recorded in the wavelength range of 200–700 nm. Partial Least Squares–Discriminant Analysis (PLS-DA) and Hierarchical Cluster Analysis (HCA) were applied to identify spectral variations and construct classification models. The chemometric models successfully differentiated between non-irradiated and irradiated extracts, indicating that gamma irradiation significantly influenced the UV-Vis spectral characteristics of the samples. These findings demonstrate that combining UV-Vis spectroscopy and multivariate analysis provides a reliable and efficient method to monitor irradiation-induced changes in *P. retrofractum* extracts. This approach offers potential applications for quality control and authentication of irradiated herbal materials.

1 Introduction

Javanese long pepper (*Piper retrofractum* Vahl) is a spice native to Indonesia valued for its economic importance and medicinal properties. The primary bioactive constituent in this plant is the alkaloid piperine, comprising approximately 2–7% of the pepper fruit (1,2). Piperine is mainly responsible for the pungency of pepper and has been reported to exhibit diverse pharmacological activities, including antioxidant and anti-inflammatory effects (3). In addition to piperine, Javanese long pepper contains chavicine (a geometric isomer of piperine) and various phenolic compounds that contribute to its antioxidant activity, anti-inflammatory properties, and digestive stimulant effects. However, post-harvest conditions and processing methods highly influence the levels and efficacy of these bioactives.

Gamma irradiation has been explored as a post-harvest treatment to improve microbiological safety and preserve bioactive compounds in herbal materials. Gamma rays from Cobalt-60 (Co-60) offer a sterilization method that can decontaminate spices and medicinal plants without leaving harmful chemical residues (4). Applying gamma irradiation to spices and medicinal herbs has effectively reduced microbial contamination and can induce modifications in the chemical structure of bioactive compounds. High-energy ionizing radiation generates free radicals that react with chemical constituents, leading to oxidation or degradation of target molecules (1). Consequently, the phytochemical profile of the material may change significantly depending on the irradiation dose. While low to moderate doses of gamma rays (e.g., ≤10 kGy) generally do not cause substantial quality changes in spices (1,4). Higher doses have the potential to induce more pronounced chemical alterations.

Gamma irradiation can also alter the physicochemical characteristics of biological materials. Previous studies have shown that gamma rays can induce conformational changes in biomolecules (e.g., proteins) and even disrupt complex biomass structures, which in turn may facilitate the release of bound bioactive compounds (5). Moreover, numerous studies report that appropriately dosed gamma irradiation can maintain or even enhance the antioxidant activity and phenolic compound content of herbal materials (5). For instance, total phenolics and antioxidant capacity have been found to increase significantly after gamma treatment in various herbal extracts (6). In other words, gamma irradiation is regarded as a promising pre-treatment strategy to obtain herbal extracts with high yields and potent bioactivity (5). The mechanisms of dose-dependent gamma-induced chemical changes in P.

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retrofractum extracts have not been well-elucidated, underscoring the need for a comprehensive molecular-level investigation.

Ultraviolet-visible (UV-Vis) spectroscopy is a rapid and non-destructive analytical technique that provides an overall profile of compounds with chromophore groups (such as alkaloids and phenolics) in an extract. This technique has been employed, for instance, to quantify piperine content in pepper samples rapidly (2) and to assess the quality of various herbal products. However, interpreting UV-Vis spectra can be challenging because they represent the superimposed absorption of multiple components. Therefore, multivariate analysis (chemometrics) is required to extract meaningful information from complex spectral data. Partial Least Squares–Discriminant Analysis (PLS-DA) is one of the chemometric methods widely used for classifying samples based on their spectral fingerprints (7,8). The combination of UV-Vis spectroscopy with multivariate analyses (such as PLS-DA and clustering) has been successfully applied in the authentication and classification of various herbal and food products (7,8).

In this study, we aim to evaluate the capability of UV-Vis spectroscopy combined with chemometric analysis to discriminate *P. retrofractum* extracts subjected to different gamma irradiation doses (0, 250, 500, 750, and 1000 kGy). This analysis aims to identify patterns of spectral changes resulting from varying irradiation doses. Furthermore, this approach is expected to provide a scientific basis for developing radiation-based authentication and quality control methods for herbal materials.

2 Materials and methods

2.1 Materials and gamma irradiation treatment

The plant material used in this study was dried fruit of Javanese long pepper (*Piper retrofractum* Vahl) sourced from Madura, East Java, Indonesia. The fruits were sun-dried to a moisture content of about 10% and ground into a fine powder. The pepper powder was subjected to gamma irradiation using a Co-60 source at the National Nuclear Energy Agency (BATAN) irradiation facility. Five levels of gamma dose were applied: 0 kGy (control), 250 kGy, 500 kGy, 750 kGy, and 1000 kGy. Irradiation was performed at room temperature with a constant dose rate. Samples were sealed in containers during irradiation to prevent contamination and oxidative degradation. Five replicate samples (5 g powder each) were prepared for each dose level to ensure data reproducibility. After irradiation, samples were stored in a dry, calm environment prior to extraction.

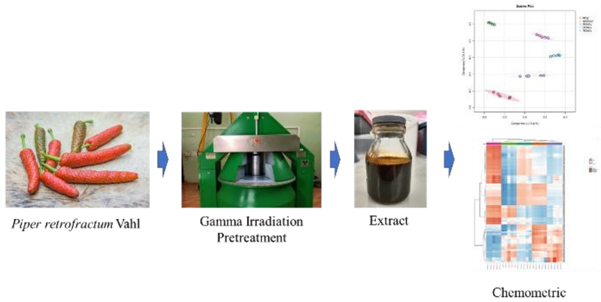

Fig. 1. Process flowchart.

2.2 Extraction process

The control and irradiated pepper powders were extracted using solvent maceration. Ten grams of powder were soaked in 100 mL of 96% ethanol at room temperature for 24 hours with occasional stirring. The mixture was then filtered through Whatman no. 1 filter paper, and the filtrate was evaporated under reduced pressure at 40 °C using a rotary evaporator to obtain a concentrated extract. The thick extract was transferred to a dark glass vial and stored at 4 °C until analysis. Before spectroscopic measurements, the extract was re-dissolved in ethanol to a concentration of 1 mg/mL.

2.3 UV-Vis spectral measurement

The UV-Vis spectrum of each extract sample was recorded using a UV-Vis spectrophotometer (Thermo Scientific Aquamate 8100, Germany). Spectra were acquired over the 200–700 nm wavelength range with a 2 nm resolution interval. Liquid samples were placed in a quartz cuvette with a 1 cm path length. Each sample was scanned three times, and the absorbance readings were averaged. To minimize instrument-related variations, the raw spectra were normalized (e.g., by baseline correction or autoscaling) before chemometric analysis. The spectral data (in digital .csv format) were then utilized for multivariate analysis.

2.4 Chemometric analysis (PLS-DA)

Chemometric analysis was performed using the Partial Least Squares–Discriminant Analysis (PLS-DA) method. A PLS-DA model was constructed to classify samples based on their UV-Vis spectra (predictor variables X) in relation to the irradiation dose categories (response variable Y). Computations were done using the MetaboAnalyst 6.0 software platform (accessible online at https://www.metaboanalyst.ca/). The absorbance at each recorded wavelength (200–700 nm, 2 nm interval) was used as input X variables, yielding hundreds of spectral variables per sample. Before modeling, the X data were preprocessed by autoscaling to normalize the variable scales. Internal model validation was performed via cross-validation. Model performance was evaluated by the classification accuracy metric and by examining the score plots for class separation.

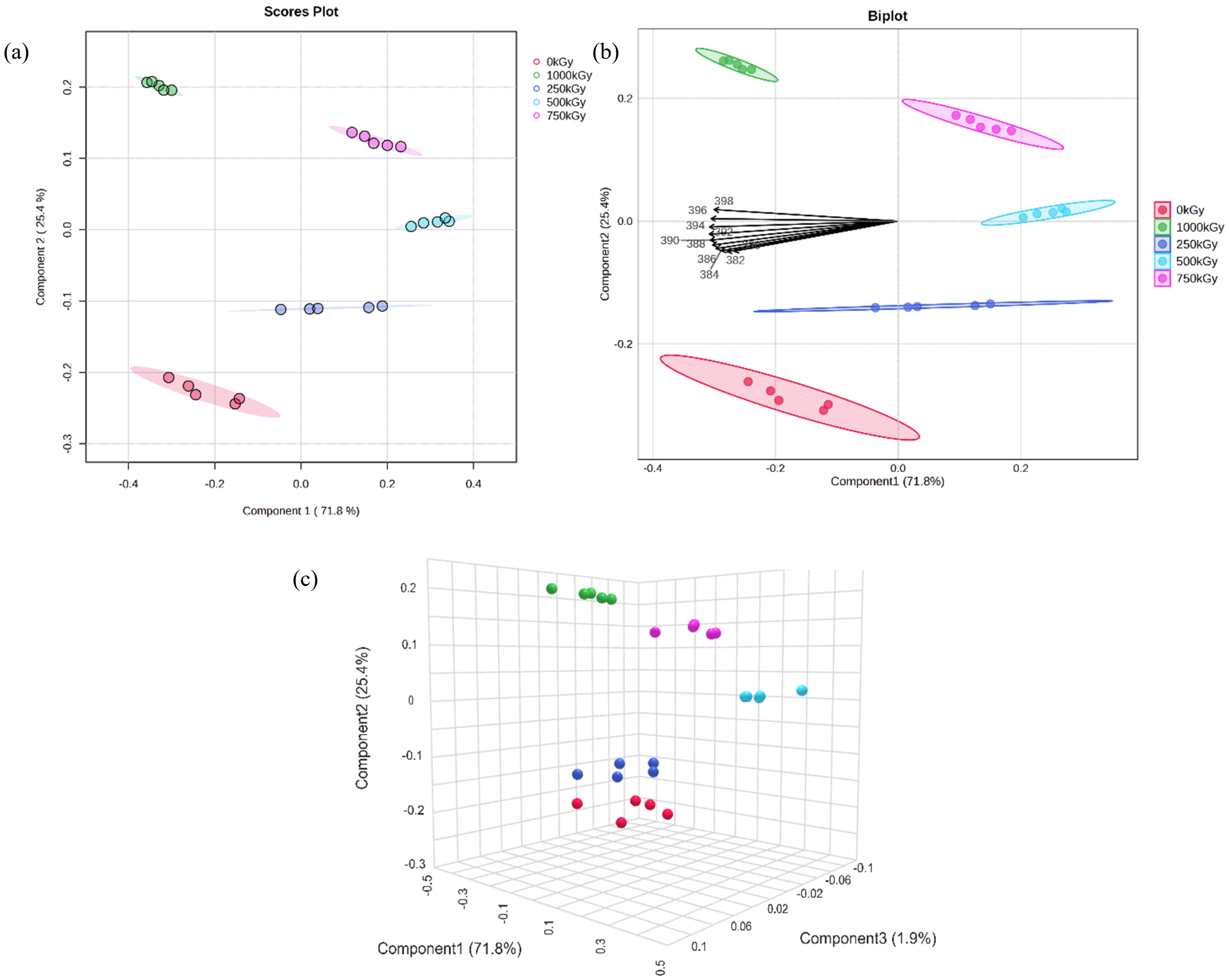

Fig 2. Chemometric analysis results for Piper retrofractum extract at various gamma irradiation doses: (a) 2D PLS-DA score plot (Component 1 vs Component 2); (b) PLS-DA biplot showing the contribution of wavelength variables; (c) 3D PLS-DA score plot (Component 1 vs. Component 2 vs. Component 3).

2.5 Cluster analysis and heatmap visualization

In addition to PLS-DA, a Hierarchical Cluster Analysis (HCA) was conducted to group samples based on spectral similarity. HCA was applied to the same spectral dataset (200–700 nm) using Ward's linkage as the clustering method and Euclidean distance as the similarity measure. The HCA results were visualized as a dendrogram and a two-dimensional heatmap, illustrating the proximities or differences among samples at different irradiation doses. The heatmap was generated by mapping the normalized absorbance intensities to a color scale, where color differences reflect relative differences in absorbance values between samples at each wavelength. This combination of clustering and heatmap analysis facilitates the identification of clusters of samples with similar spectral profiles, thereby supporting the classification results obtained from the PLS-DA model.

3 Results and discussion

3.1 UV-Vis spectral analysis and PLS-DA discrimination patterns

The UV-Vis spectra of *P. retrofractum* extracts across 200–700 nm revealed distinct absorbance profiles for control (0 kGy) versus gamma-irradiated samples. Notably, the irradiated extracts showed reduced absorbance intensities at specific wavelengths compared to the control, with differences becoming more pronounced as the irradiation dose increased. In particular, the spectral region around 280–400 nm exhibited the most notable differences between treatments. The near-UV and visible regions correspond to electronic transitions of aromatic compounds (e.g., $\pi \rightarrow \pi$ transitions in the aromatic rings of piperine and phenolics). The decrease in absorbance intensity at higher doses (750–1000 kGy) suggests that the high-energy radiation partially degraded some aromatic constituents in the extract. This observation is consistent with reports that gamma irradiation can generate radicals that break chemical bonds and reduce levels of compounds like piperine (1).

Evaluation of discrimination ability based on UV-Vis spectral data, PLS-DA analysis applied to spectral datasets. The PLS-DA scores plot in two dimensions (Figure 2a) shows that samples are clearly segregated according to irradiation dose. The first latent component (PLS1) accounted for approximately 71.8% of the variance, and the second component (PLS2) accounted for an additional 25.4%, resulting in a total of 97.2% of the variance explained by the model. The control samples (0 kGy) cluster far apart from the irradiated samples, and there is a graded shift in cluster positions corresponding to increasing dose. The 250 kGy samples cluster relatively close to the control, whereas the 750–1000 kGy samples are farthest. This pattern indicates a dose-dependent gradation in chemical composition changes, with higher doses inducing progressively larger differences. Overall, the PLS-DA model accurately classified all samples into their respective dose groups, achieving 100% classification accuracy and demonstrating excellent discriminative ability. The clear separation of clusters supports the premise that gamma irradiation induces consistent and detectable changes in the spectral profiles that multivariate analysis can capture.

Changes in chemical composition due to irradiation must be considered, especially at high doses. The results of research by Navehsi et al., (9) found that low gamma doses (2.5 kGy) were able to increase the essential oil content and its main components (p-cymene, carvacrol) in Satureja mutica herbs, while maximizing total phenol content and antioxidant capacity. However, higher doses (7.5–10 kGy) reduced the percentage of several important volatile compounds (e.g., a decrease in p-cymene and carvacrol at 10 kGy). They reduced the total essential oil content compared to low doses (9). The concentration of thymol, one of the main phenolics, was even higher in the non-irradiated sample (9). This decrease in active components at excessive doses is in line with the observations of Yang et al., (10) on Tibetan Pazhu Powder, which showed a reduction in the content of terpenes, alkanes, amides, and alcohols in volatile components, along with all non-volatile components, indicating a decrease that was directly proportional to the increase in dose (up to 100 kGy). Interestingly, however, the total antioxidant activity of Pazhu Powder extract actually increased after irradiation (10). Researchers speculate that high radiation triggers the degradation of complex molecules into simpler compounds with higher antioxidant activity, even though the total amount of the initial compounds is reduced (10). Consequently, caution is required in determining the optimal irradiation dose, as irradiation doses can enhance some beneficial components, while excessive doses have the potential to damage certain active compounds (10).

3.2 Component interpretation and variable contributions (Biplot Analysis)

The PLS-DA biplot (Figure 2b) was examined to identify which wavelengths contribute most strongly to the group separation. The loading vectors in the biplot indicate that the wavelength region around 384–396 nm had a dominant influence on discriminating the dose groups. This 385 nm region likely corresponds to an absorption feature of piperine or its irradiation-induced derivatives. Pure piperine typically has a maximum absorbance around 342 nm in alcoholic solution (2). A shift of absorbance toward 384 nm in the high-dose irradiated extracts may be due to the formation of degradation products or structural changes (e.g., oxidation yielding new chromophores) during irradiation. The direction of the loading vectors for high-dose groups (750–1000 kGy) suggests increased relative absorbance in the mid-visible region (380 nm), whereas the control group’s vectors emphasize the UV region (250–300 nm). This implies that high-dose irradiation shifted the extract’s absorption profile toward longer wavelengths, possibly associated with radiation-induced transformation of the bioactive molecules' conjugation systems. These findings support the notion that gamma irradiation can modify the chromophore structures of the pepper’s constituents, leading to altered UV-Vis absorption patterns.

Moreover, inclusion of the third PLS component (Figure 2c) showed that the group separation remains consistent in three-dimensional score space. The third component accounted for 1.9% of variance, bringing the explained variance to approximately 99.1%. The stability of group clustering in the 3D plot attests to the robustness of the PLS-DA model. The PLS-DA results indicate that the UV-Vis spectroscopic approach combined with chemometrics is highly practical for detecting and separating subtle changes in herbal extracts caused by gamma irradiation. The model’s success in classifying samples by dose (with high accuracy) suggests that similar techniques could be applied to monitor the quality of natural products subjected to other physical treatments.

The use of PLS-DA has been effectively utilized to authenticate the geographical origin and processing methods of black pepper. The combination of GC-HRMS fingerprinting with PLS-DA is capable of distinguishing samples from different regions and processing conditions with high accuracy (11). The use of UV-Vis and FT-IR spectra combined with PLS-DA to classify and authenticate Iranian herbs and spices is fast, easy, economical, and highly accurate (12). PLS-DA is applied to analyze non-polar phytochemicals in the dried roots of several medicinal herbs. This technique helps in the classification and grouping of different herbs based on their chemical profiles (13). The use of PLS-DA was used to determine the impact of processing on the pharmacological properties of herbs in traditional Chinese medicine (14).

3.3 Cluster analysis and heatmap visualization

The HCA results are presented as a dendrogram accompanied by a heatmap in Figure 3. The dendrogram reveals that the samples clustered according to their irradiation dose, with each dose group forming a distinct cluster. The distance between clusters (Euclidean distance) is considerable, indicating significant differences between treatments. The 0 kGy and 250 kGy samples form the closest clusters, consistent with earlier observations that the chemical composition difference between control and 250 kGy is relatively small. In contrast, the 750 kGy and 1000 kGy samples form clusters farthest from the control, indicating the most drastic compositional changes occurred at the highest doses. This HCA clustering trend aligns with the PLS-DA results, confirming that spectral similarity between samples decreases as the irradiation dose increases.

The heatmap provides a detailed visualization of all samples' absorbance intensity differences at each wavelength. In the heatmap, red-colored segments indicate relatively higher absorbance intensity, whereas blue segments indicate lower intensity than the overall mean. The heatmap patterns for control vs. irradiated groups show distinct color differences in specific wavelength regions. For example, the 1000 kGy group exhibits extensive blue segments in the 280–350 nm range, indicating a reduction in absorbance (likely due to the degradation of UV-absorbing compounds). Conversely, reddish segments around 380 nm for the high-dose samples are consistent with the biplot finding of increased contribution at that wavelength. The differing color distributions for each dose group demonstrate that gamma irradiation significantly affects the spectral composition of the pepper extracts.

In general, the combined PLS-DA and heatmap analyses in this study yield mutually reinforcing results. PLS-DA provides a predictive model that can accurately discriminate samples based on their spectra. In contrast, HCA/heatmap offers an unsupervised visualization that confirms that samples with similar treatment clusters are together and apart from others. Similar multivariate fingerprinting approaches have been successfully used in herbal and spice authentication (7,8). Our findings confirm that these methods are also effective for detecting the effects of physical treatments (irradiation) on herbal extracts. In other words, the UV-Vis spectral fingerprinting technique coupled with chemometrics can rapidly verify whether an herbal product has been irradiated and potentially to what dose. This information is valuable for quality control purposes, given that international regulations generally limit the maximum irradiation dose for food/herbal products to ensure safety and quality (1,4).

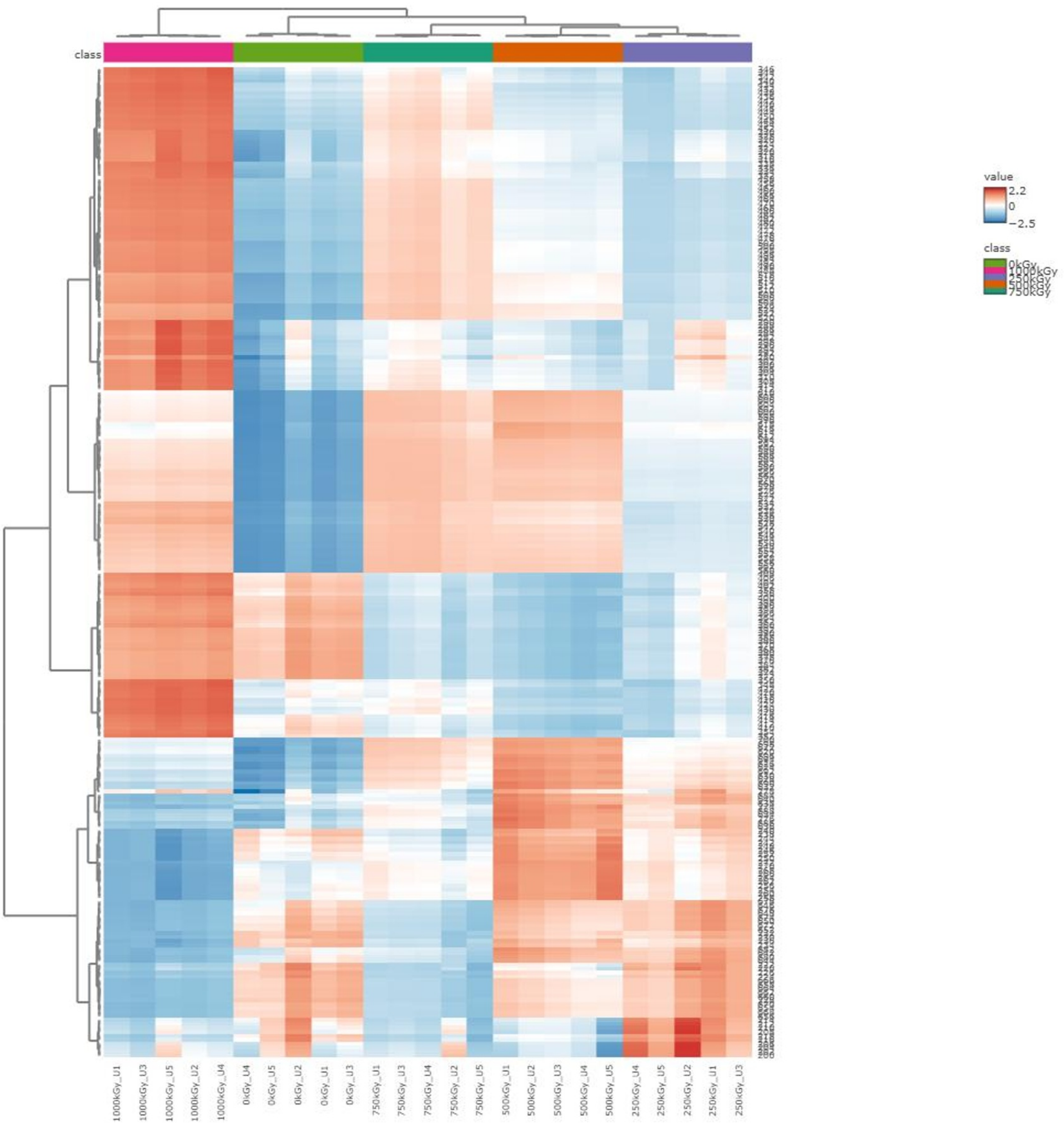

Fig. 3. Hierarchical Cluster Analysis (HCA) dendrogram and spectrum heatmap.

Cluster analysis of herbal plant extracts can be performed to group plant extracts based on their chemical composition, spectral data, and geographical origin. HCA has been used to classify herbal plant extracts from various geographical regions based on their chemical composition and spectral data (15). HCA has also been used to classify methanol extracts from Sedum taxa based on their phenolic content (16). The classification of medicinal plants using HCA based on HPTLC fingerprinting is able to detect similarities and differences between plants from different geographical regions (17). UV-Vis, FTIR, and NMR spectral data are often used to assist in the classification of plant extracts (15,18–20).

4 Conclusion

This study proves that the combination of UV-Vis spectroscopy with chemometric analysis (PLS-DA and HCA) is effective in distinguishing samples of Javanese long pepper (*Piper retrofractum* Vahl.) that have been exposed to gamma irradiation at various doses (0–1000 kGy). Gamma irradiation causes significant changes in the UV-Vis spectral profile, characterised by a decrease in absorbance intensity in the 280–350 nm region and a shift in contribution around 384–396 nm, indicating structural modifications in bioactive compounds such as piperine and phenolics due to oxidation or degradation processes. The PLS-DA model showed high classification ability, while the HCA results confirmed consistent grouping between doses, confirming the sensitivity of the chemometric approach in detecting chemical changes due to irradiation. Thus, the UV-Vis spectral fingerprinting method based on multivariate analysis has the potential to be developed as a fast, accurate, and non-destructive tool for the authentication and quality control of irradiated herbal products, while also supporting the application of this technology in the herbal medicine and phytopharmaceutical industries.

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