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Original Research Report

Isolation, Characterization, and Analytical Validation of Key Bioactive Compounds in Vaccinium bracteatum Thunb Leaves

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Abstract: *Vaccinium bracteatum Thunb*. (VB) is a species of Vaccinium with a high flavonoid content and broad pharmacological potential, including antioxidant, anti-inflammatory, and neuroprotective activity. This study aims to isolate and characterize the main bioactive compounds from VB leaf water extract, as well as to develop and validate analytical methods for quantification of marker compounds as the basis for standardization of extracts. VB leaves are collected from various locations in South Korea and extracted using the aqueous extraction method. The compounds were then separated through silica gel column chromatography and HPLC preparations, and identified using UV-Vis, FTIR, 1D/2D NMR, and LC-MS/MS. HPLC-PDA AND LC-MS/MS methods were validated according to ICH guidelines (linearity, accuracy, precision, LOD, LOQ, specificity). The isolation results showed five main compounds: the flavonoids orientin (2.35%) and isoorientin (1.87%), phenolic acid vanillic acid (0.95%) and protocatechuic acid (1.12%), as well as the glycoside (0.78%). Validation of the analytical method showed a linearity of $R^2 > 0.999$, an accuracy of 98.9–101.2%, a precision of <2%, and a low LOD/LOQ, supporting the sensitive quantification of major and minor compounds. Spectroscopic and LC-MS/MS characterizations confirm the chemical structure of the compounds, confirming the purity of the isolate. These findings provide a scientific basis for the standardization of VB leaf extracts, supporting the development of herbal or nutraceutical products based on flavonoids and glycosides. Further research is recommended to explore in vivo activity, minor metabolite interactions, as well as the relationship of biosynthetic gene expression with phytochemical profiles to maximize the therapeutic potential of VB.

Keywords: Flavonoid, Glycoside, LC-MS/MS, Nutraceutical, Vaccinium Bracteatum.

1. Introduction

Vaccinium bracteatum Thunb. (VB) is a shrub or small tree plant of the genus *Vaccinium* (family Ericaceae), with a wide geographical distribution in East Asia, including in South Korea, Japan, and China [1]. Genomic research on *V. bracteatum* showed the genetic mechanisms that support flavonoid synthesis in this species: in whole-genome analysis, 298 candidate genes associated with flavonoid synthesis and regulation of *MYB* transcription factors were found through evolutionary comparisons, explaining why VB has a relatively high flavonoid content compared to some other *Vaccinium* species [2].

In South Korea, VB has been used in local traditional medicine, and modern laboratory research has confirmed that VB leaf or fruit extract has real pharmacological potential, including antioxidant, anti-inflammatory, and hypoglycemic effects [3]. For example, cellular and in vivo tests show that VB extract can suppress oxidative stress and regulate antiapoptotic pathways, supporting its traditional use claims. These pharmacological properties are particularly relevant for the development of plant-based supplements due to their ability to target a variety of cellular pathways.

In terms of chemical components, VB exhibits a very rich and diverse profile of secondary metabolites. Phytochemical analysis has revealed the presence of flavonoids such as orientin and isoorientin, phenolic acids such as vanyl and protocatescorice, iridoid glycosides (e.g. divaccinoside, vaccinoside), as well as lignans and polysaccharides [4]. The presence of these compounds provides the molecular basis for a wide range of reported biological activities.

In a recent isolation and characterization study, Wang, Zhang, Wang, Tian, Li, Ma, Yin, Bao, and Zhang successfully identified 32 small molecular compounds from VB leaves, including ten new compounds and ten enantiomer pairs, using advanced spectroscopy (NMR, ECD) methods [5]. Some of the new compounds exhibit antioxidant (anti-radical DPPH/ABTS activity), neuroprotective activity on PC12 cells, as well as antidiabetic (α-glucosidase inhibition) and anti-inflammatory (suppression of NO production).

A special focus on the flavonoids orientin and isoorientin is essential because they both show significant functional activity. Studies by Lee and Bae (reported in the literature) have shown that orientin and isoorientin prolong blood clotting time (aPTT, PT), inhibit thrombin and factor Xa activity, as well as inhibit platelet aggregation through inhibition of PKC activation and calcium mobilization, effects that were also demonstrated in animal models and lowered the PAI1/tPA ratio signaling strong anticoagulant potential [5].

In addition, VB is also explored in the context of antiviral activity: Kim, Kwon, Yang, Li, Hwang, Kim, Pak, Go, and Choi reported that VB extract from leaves is able to suppress HSV-1 (*Herpes Simplex Virus Type 1*) infection in neuronal cells by regulating endoplasmic reticulum stress (ER stress) and apoptosis pathways [6]. They found that after HSV-1 infection, treatment with VB extract lowered the expression of *the VP16* and *IE viral genes*, inhibited the induction of ER stress (PERK, ATF4, CHOP), as well as reduced pro-apoptotic proteins, suggesting that the mechanism of neuronal cell protection by VB involves modulation of cellular stress pathways and cell death [7].

Further chemical studies also uncovered other unique glycoside compounds: Wang, Li, Wei, Wang, Zhang, Yan, and Zhang isolated two glycoside neolignans (one new), seven iridoid glycosides, and nine flavonoid glycosides from VB leaves through NMR and ESIMS analysis [8]. Some of these compounds exhibit antiradical activity (against ABTS/DPPH), as well as protective activity against ROS in RAW264.7 cells and PC12 cells, confirming their potential biological role [9].

Furthermore, on the genome side, genome assembly at the chromosomal level for *V. bracteatum* opens up new insights into the genetic basis of biosynthesis of bioactive compounds [10]. With hundreds of predicted genes (36,756 prediction genes), the analysis showed an expansion of the gene family involved in flavonoid biosynthesis as well as the regulation of MYB transcription factors, which may explain the high concentration of flavonoids in this species [11].

In addition to the core genome, mitogenome analysis *of V. bracteatum* has also been performed: Zhou, P., and colleagues completed a complete mitogenome sequence using PacBio HiFi data, and found differences in gene structure and content compared to other Ericales species, but functional genes remained relatively conservative [12]. These data not only strengthen the genome reference but also provide a foundation for the future molecular breeding and utilization of these plants [13].

Overall, the chemical and pharmacological profile *of Vaccinium bracteatum* is very complex and promising. The combination of metabolites such as flavonoids, iridoids, neolignans and phenolates, along with evidence of anti-coagulant, neuroprotective, and antiviral activity, makes VB a strong candidate for the development of nutraceutical or herbal medicines. However, in order for this

potential to be harnessed safely and effectively, accurate and validated analytical methods are needed for the quantification of active components, a need that underlies further development in phytochemical research and large-scale production [14].

The purpose of this study was to isolate key phytochemical components, including flavonoids, phenolic acids, and glycoside compounds, from the water extract of *Vaccinium bracteatum* Thunb. leaves grown in South Korea; develop and validate analytical methods, such as HPLCPDA and/or LC-MS/MS, for the quantification of these compounds according to ICH guidelines (including linearity, accuracy, precision, LOD, LOQ, and specificity); and provide chemical data that can be the basis for standardization of VB extract, supporting the development of herbal and nutraceutical pharmaceutical products [15].

This study is of scientific and practical significance because it can enrich the phytochemical knowledge *of Vaccinium bracteatum* by identifying and quantifying previously underexplored key compounds in the South Korean population, while providing a reliable validated analytical method for quality control and standardization of VB herbal extracts, important for the production of supplements or plant-based drugs; in addition, the results of this study support development of VB-based nutraceutical or pharmaceutical products with strong chemical evidence, enhancing the commercial and clinical potential of VB leaf extracts, as well as bridging the gap in the literature between the biological potential of VB and the application of advanced analytics, as well as being the basis for further research such as in vivo activity tests or the development of drug/herbal formulas [16].

2. Literature Review

One of the pillar studies in the phytochemistry *of Vaccinium bracteatum* came from Lee, S.-G., Ko, H., Choi, E.-J., Oh, D.-R., Bae, D., and Choi, C., who successfully isolated five major compounds from VB leaf water extract, namely vaccinoside, vanillic acid, protocatechuic acid, orientin, and isoorientin. They used column chromatography and HPLC preparations for purification, then developed a reverse phase HPLC method validated according to ICH (International Conference on Harmonisation) guidelines for orientin and isoorientin analytics [17], [18]. Their validation includes essential parameters such as linearity ($R^2 \geq 0.9999$), precision (intra and inter day RSD-< 2%), accuracy (recovery approximately 98–102%), detection limit (LOD) and quantification limit (LOQ), which suggests that the method is highly sensitive and reliable for the analysis of flavonoid markers within VB extract [19] [20]. This method provides a critical analytical foundation for follow-up studies as it provides a validated quantitative tool for marker compounds, which is essential in the standardization of herbal extracts.

The isolation of vanillic acid and protocatechuic acid by Lee et al. is significant because these two compounds have not previously been identified in VB leaf water extract [21]. Vanillic acid and protocatechuic acid are phenolic acids that have been widely associated with antioxidant activity as well as the ability to counteract oxidative stress in cells [8]. Their discovery as a key component adds pharmacological weight to the VB profile, especially given that phenolic acids may contribute to therapeutic effects through the mechanism of free radicals and modulation of cellular signaling pathways. In addition, the isolation of vaccinoside (iridoid glycosides) with flavonoids (orientin, isoorientin) suggests that VB has a complex chemical structure, making it a strong candidate plant for the development of standardized supplements or herbal remedies, provided that its analytics are truly reliable and reproducible.

Research significantly expands the chemical insights *of V. bracteatum* by identifying 32 small molecules in leaves through NMR and ECD techniques. Of the 32 compounds, 10 were novel compounds and there were 10 pairs of enantiomeres, a finding that confirms the heterogeneous molecular stereochemistry of VB [22]. The study not only showed a complex chemical profile but also linked the compounds to biological activity: some compounds showed antioxidant activity (inhibition of DPPH and ABTS), neuroprotective activity in PC12 cells, as well as antidiabetic (α-glucosidase inhibition) and anti-inflammatory (NO production inhibition) abilities [9] [10]. This relationship is very important because it shows that small compounds of VB can directly explain their traditional functional effects and potential as functional foods or pharmaceutical ingredients.

The finding of enantiomer pairs in the study of Wang et al. provides a very important insight: stereoisomerism in natural molecules often determines biological activity, toxicity, and bioavailability

[23]. The identified enantiomer pairs can have pharmacodynamically different activities, so purification and stereochemical characterization are of great importance. Using the ECD (Electronic Circular Dichroism) technique, researchers were able to determine the absolute configuration of the compound, which further allowed for further structure–activity relationship (SAR) studies. Without stereochemical information, the risk of using compounds in therapeutic formulations could be higher due to potential side effects or differences in efficacy.

Genomic analysis conducted researcher provide the genetic foundation for understanding why *V. bracteatum* produces high amounts of secondary metabolites, especially flavonoids. Their genome was successfully assembled at the chromosomal level with a size of about 569.81 Mb and a predicted number of genes of 36,756 [24]. Further analysis showed gene family expansion and positive selection in 298 gene candidates, including genes involved in flavonoid biosynthesis and MYB transcription factors, which may explain the high flavonoid content in VB [25] [26]. These findings are crucial because they link molecular data (genomes) to phytochemical profiles allowing for an integrated approach in phytochemical research, which is not only chemically analyzing but also tracing the genetic basis of biosynthesis of active compounds.

From an applicative point of view, a genome with a complete chromosomal level paves the way for molecular breeding and conservation of *V. bracteatum*. As genes associated with the biosynthesis of flavonoids and bioactive metabolites are identified, researchers or breeders can target these genes to increase the content of therapeutic compounds through selective breeding or genetic engineering [11]. Since VB is also used as a rootstock in the cultivation of blueberries (Vaccinium), understanding its genome allows for two-way utilization: (1) strengthening the medicinal value of the plant, (2) improving agronomic resistance or fruit character through genome-based breeding. This makes genomic studies not only the basis of phytochemical research, but also a bridge between biotechnology research and agronomy/pharmaceutical applications.

A new study managed to construct the complete mitogenome *of V. bracteatum* using PacBio HiFi data, resulting in a 708,384 bp long circular sequence with 67 annotated genes (36 protein-coding genes, 26 tRNAs, 3 rRNAs, and 2 pseudogenes) [13]. The analysis also found thousands of dispersed, tandem, simple repeats and more than 360 RNA editing sites, demonstrating post-transcriptional complexity in the VB mitogenome [13]. The presence of DNA migration between genomes (nuclear, mitochondria, chloroplasts) exhibits significant evolutionary genetic dynamics and may contribute to the regulation of the expression of secondary metabolites.

Mastering the structure of the full mitogenome has long-term implications: (1) it provides a stronger phylogenetic basis for placing *V. bracteatum* in the clade *Ericales*, (2) it allows the identification of mitochondrial genes that may be related to energy metabolism, stress resistance, or metabolite production, (3) it provides a foundation for mitochondrial engineering or molecular breeding that considers the persistence and stability of genes in organelles. In addition, the complete mitogenome aids in primary design, molecular marking, and evolutionary biology studies, all of which are relevant to plant development as a bioactive source.

In the field of pharmacology, researcher suggests that Methanol extract *of V. bracteatum* (VBME) may inhibit NF-κB activation in LPS-induced BV-2 microglia cells. VBME significantly decreases the production of NO (nitric oxide), the expression of iNOS, COX-2, and the secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, with no obvious cell toxicity [2] [6]. They also found that VBME inhibits the phosphorylation of IκB-α and its degradation, which prevents the translocation of p65 NF-κB to the nucleus, as a major mechanism of anti-inflammatory action [6]. This discovery is particularly important because it links the anti-inflammatory effects of VB to critical cellular signaling pathways involved in many neurological and chronic inflammatory diseases.

The effects of VBME on BV-2 microglia suggest broader therapeutic potential than just peripheral anti-inflammatory: since microglia are the main immune cells in the central nervous system, modulation of their activity via NF-κB gives an indication that VB could play a role in neurodegenerative therapies, such as Alzheimer's or Parkinson's disease. The research of Kwon et al. suggests that VBME could be a candidate to be studied in *in vivo* models of neuroinflammation, although such studies are still limited [6]. In addition, the control of oxidative stress and inflammatory signals in microglia could reduce neuronal death and neurotoxicity, reinforcing the benefits of VB as a nutraceutical-neuroprotective agent.

Recent research reported that *V. bracteatum extract* may dampen the inflammatory response in LPS-induced RAW264.7 macrophages. In the study, treatment with VB extract lowered COX-2 expression and the release of pro-inflammatory cytokines in macrophages, confirming that VB's anti-

inflammatory properties are not limited to microglia cells, but are also applicable in peripheral immune cells [14]. These findings broaden the spectrum of pharmacological applications of VB, opening up the possibility for use in systemic inflammatory diseases (e.g. metabolic or autoimmune diseases).

The anti-inflammatory mechanism through the NF-κB pathway observed in *V. bracteatum* has parallels with research on other *Vaccinium species*. For example, Yang, Mi-Ok (2024) showed that blueberry extract (Vaccinium spp.) inhibits NF-κB activation as well as caspase-1 activation in LPS-induced RAW264.7 cells, reinforcing the hypothesis that Vaccinium flavonoids and polyphenols in general can suppress the inflammasome and NF-κB pathways [15], [16]. This relationship reinforces the pharmacological relevance of cross-species within the genus *Vaccinium* and supports the importance of comparative research.

In the context of isolation of antioxidant compounds, the UHPLC-Q-TOF study by Hong Kong Baptist University identified seven flavonoids in the Cb *V. bracteatum* fraction, including isoorientin, orientin, vitexin, isovitexin, isoquercitrin, quercetin-3-O-α-L-rhamnoside, and chrysoeriol-7-O-β-D-glucokopiranoside [27]. The study showed that isoorientin and orientine are the dominant contributors of measured antioxidant activity, and these compounds also provide protective effects against kidney damage in models on cells, demonstrating clinical potential in counteracting renal oxidative stress [5].

The findings of antioxidants from compounds such as isoorientin and orientin are particularly relevant considering that oxidative stress is a major causative factor in chronic diseases such as kidney, cardiovascular, and neurodegenerative diseases. By showing that specific compounds of VB provide protection against kidney damage *in vitro*, this study supports the idea that *V. bracteatum* could be a natural therapeutic source that mitigates oxidative damage through well-defined biological mechanisms [28]. It also emphasizes the importance of isolation of pure compounds and activity-guided fractionation in VB phytochemical research.

Animal studies by other researchers reported that polyphenol-rich *V. bracteatum* fruit extract may reduce weight gain in rats fed a high-fat diet, while modulating the composition of the gut microbiota [29]. They used LC-ESI-MS/MS to analyze the polyphenol components in the extract and found about 36 types of polyphenols [12]. Reported changes in the microbiota include an increase in bacterial genus negatively related to glucose and lipids, such as *Akkermansia*, and a decrease in *Escherichia-Shigella bacteria*, which are positively correlated with insulin resistance [12]. These findings are particularly important because they link the systemic metabolic effects of VB to the modulation of gut microbes a mechanistic pathway that is gaining increasing attention in modern nutraceutics.

The ability *of V. bracteatum* to modulate the gut microbiota in obesity models shows dual therapeutic potentials: (1) direct effects through polyphenols as bioactive compounds, and (2) indirect effects through changes in microbiota communities that then affect glucose, lipid, and insulin resistance metabolism [12]. This kind of strategy is in line with the trend of modern nutraceutical research where plant-based supplements are geared towards influencing the gut microbiota as a mediator of health effects. It also provides a scientific justification for the traditional use of VB as a dietary supplement in the management of obesity and metabolic disorders.

The phytochemical isolation method in the study of researcher is highly representative of common practice in natural chemistry: they used silica column chromatography for coarse separation, then HPLC preparations for the final purification of compounds such as vaccinoside, orientin, and isoorientin [1]. This strategy is simple but effective, as the combination of normal-phase and reversed-phase columns allows separation based on polarity and interaction with the stationary phase. This approach is a classic method but remains relevant in modern phytochemical research, as silica columns and HPLC reverse phases are still the standard for the isolation of pure natural compounds.

However, such methods also have limitations: isolation through columns and HPLC preparations can be very time-consuming and material-intensive, especially if the target compound is in low concentrations. Additionally, this process requires a lot of organic solvents, which has environmental and cost implications. In the context of VB, where minor compounds such as enantiomers have just been discovered (Wang et al.'s study), activity-guided fractionation methods and advanced techniques such as SPE (solid-phase extraction) or multidimensional chromatography may be more efficient in terms of time and resources.

The analytical validation used by Lee et al. follows the ICH guidelines, which establish parameters such as specificity, linearity, precision, accuracy, LOD, LOQ, and stability [30]. Adherence to these guidelines is essential in the context of pharmaceutical and nutraceutical research, as it produces quantitative data that is reliable and regulatedly acceptable. Without proper validation, the

quantification results of flavonoids or phenolic acids from extracts can vary between batches, making standardization and reproducibility difficult.

For comparison, Urbstaite, Rima; Raudone, Lina; Liaudanskas, Mindaugas; and Janulis, Valdimaras (2022) developed and validated the UPLC-DAD method for phenolic compound analysis in *Vaccinium macrocarpon* (cranberry), with the parameters of linearity ($R^2 > 0.999$), precision (%RSD < 2%), LOD/LOQ, and recovery according to ICH guidelines [31]. This suggests that such validation methods are well established in the genus *Vaccinium*, and a similar approach can be adapted for VB so that the quality of the extract can be properly standardized.

Although Lee et al. have validated HPLCs for orientin and isoorientin, there are still gaps related to other compounds from VB, specifically the new compounds identified by Wang et al. (e.g., iridoids, neoligans, enantiomers). Because these compounds may have different chromatography and UV properties, analytical methods already validated for flavonoids may not be directly suitable for the new compounds. Therefore, it is necessary to develop additional analytical methods (e.g. LC-MS/MS, UPLC-QToF) validated for the complete compound profile of VB.

Developing modern methods such as LC-MS/MS or UPLC-QToF will not only facilitate the quantification of minor VB compounds, but also allow the detection of enantiomers, conversion metabolites, and degradation products during extract processing. With complete validation (linearity, precision, accuracy, LOD/LOQ, specificity), this method can be used for quality control (QC) of herbal extracts in clinical-scale production or supplements. This is especially relevant in pharmaceutical or nutraceutical production frameworks where batch consistency and safety are essential [32].

The standardization of herbal extracts based on chemical markers (markers) is a crucial element in ensuring product safety, efficacy, and consistency. In the case of VB, compounds such as orientin, isoorientin, and vaccinoside can be used as primary markers because their concentration and bioactive activity have been established [33]. However, with the discovery of new compounds from Wang et al.'s research, it is necessary to consider additional markers to reflect the full chemical profile of VB extract, especially if the intended use is as a functional supplement or herbal medicine.

Although research on isolation, bioactive analysis, and genomics of VB is quite advanced, there are still significant gaps. First, there are no modern analytical methods that are comprehensively validated for new and minor VB compounds. Second, although there are in vitro activity data (antioxidant, anti-inflammatory, neuroprotective), *in vivo studies* linking specific compounds to therapeutic effects are still limited. Third, the relationship between genetic data (biosynthetic genes) and metabolite expression under physiological or stress conditions has not been widely explored. Therefore, further research is urgently needed to holistically bridge the chemical, biological, and genetic aspects of VB [34].

Overall, the literature review shows that *Vaccinium bracteatum* is a species with a rich chemical and pharmacological profile: the isolation of classical compounds by Lee et al., the discovery of new compounds via NMR/ECD by Wang et al., and the genetic underpinnings of genome analysis by Yang et al. provide a solid basis for therapeutic development. Anti-inflammatory activity (via NF-κB), oxidative protection, and modulation of the gut microbiota also show multifunctional potential. However, to bridge this potential into real applications (nutraceutical, supplements, or herbal remedies), the development and validation of modern analytical methods, *in vivo studies*, and integrative approaches between genomics, chemistry, and pharmacology are needed. Further research in this direction will be significant to optimize the utilization of VB as a safe and effective bioactive source.

3. Methodology

3.1. Materials and Samples

The leaves *of Vaccinium bracteatum* Thunb. were collected from several locations in South Korea between March 2024 and March 2025. Sampling locations include Jeju Island, Gyeonggi-do, Gangwon-do, and the forest areas of Mount Jiri, which are known to have natural populations of VB with high flavonoid content. Harvesting is carried out in spring to early fall, when the leaves show optimal levels of secondary metabolites.

The reagents and solvents used include deionized water for aqueous extraction, 70–95% ethanol for hydroalcoholic extraction, methanol analysis grade, pH 7 phosphate buffer, as well as chromatographic solvents such as n-hexane, ethyl acetate, and methanol for the separation of

bioactive compounds. All reagents are grade analysis and obtained from Sigma-Aldrich (Seoul, South Korea).

3.2. Extraction Procedure

Extraction was carried out using the aqueous extraction method, in accordance with the standard research practice of phytochemistry of the Vaccinium plant. The fresh leaves are washed thoroughly, dried at 40 °C to a moisture content of <10%, then ground into a fine powder. Leaf powder (100 g) is extracted with hot water (1 L) at 80 °C for 3 hours with constant stirring. The suspension is extracted twice to maximize yield, then filtered using Whatman No. 1 filter paper and dried with a rotary evaporator at 45 °C to obtain a viscous extract. The extract is stored at −20 °C until further analysis.

3.3. Isolation and Separation of Compounds

Compound separation was carried out using a combination of gel silica column chromatography and HPLC preparations. The viscous extract is dissolved in methanol, then loaded on a column of silica gel (60–120 mesh) and elution is performed using the n-hexane: ethyl acetate: methanol gradient. The fractions containing flavonoids, phenolic acids, and glycosides were collected based on TLC (Thin Layer Chromatography) observations with UV detection at 254 and 366 nm.

Furthermore, the active fraction is disfractionated using HPLC C18 preparatives with a water gradient: methanol containing 0.1% formic acid, a flow of 5 mL/min, to obtain a pure compound. Separation criteria included purity of >95% (determined by HPLC-DAD) and consistent retention in repeat analysis.

3.4. Characterization and Identification of Compounds

Identification of compounds is carried out using combination spectroscopy:

- UV-Vis to determine the max λ of flavonoids and phenolics, respectively.
- FTIR to detect the main function cluster.
- 1D and 2D NMR (^{1}H, ^{13}C, COSY, HSQC, HMBC) to determine the molecular structure.
- LC-MS/MS for molecular mass confirmation, fragmentation, and enantiomer identification. Quantitative analysis was performed using HPLC-PDA and LC-MS/MS to determine the concentrations of orientin, isoorientin, vaccinoside, vanillic acid, protocatechuic acid, and other minor compounds.

3.5. Validation of Analytical Methods

The analytical method was validated according to ICH guidelines for parameters: linearity ($R^2 \geq 0.999$), accuracy (recovery 98–102%), precision (intra- and inter-day RSD <2%), LOD, LOQ, specificity, and sample stability. Retests are performed on different batches and at low, medium, and high concentrations to ensure reproducibility and consistency of quantification. Validation was performed for each marker compound, including flavonoids, phenolic acids, and iridoid glycosides.

3.6. Data Analysis

Quantitative data were analyzed by making a calibration curve from the pure standard of the target compound (orientin, isoorientin, vanillic acid, protocatechuic acid, vaccinoside). The concentration of compounds in the extract is calculated based on the calibration curve and the response correction factor. Comparisons between methods (HPLC vs. LC-MS/MS) were made to ensure the suitability and validity of the quantification. The data is processed using GraphPad Prism 9 software and Microsoft Excel for descriptive statistical analysis, including mean, SD, and RSD, to ensure the reproducibility and precision of the method.

4. Finding and Discussion

4.1. Isolation Results

Water extracts from the leaves *of Vaccinium bracteatum* (VB) reveal quite diverse phytochemical profiles: two flavonoids (orientin and isoorientin), two phenolic acids (vanillic acid and protocatechuic acid), and one glycoside (vaccinoside). From Table 1, orientin has the largest yield, which is 2.35%, followed by isoorientin at 1.87%. In contrast, phenolic acid showed lower yields (0.95% for vanillic acid and 1.12% for protocatechuic acid), while vaccinoside was at the lowest level at 0.78%. This pattern indicates that the flavonoids in VB leaves are available in relatively high

amounts and are easily extracted by the water method, while phenolic acids and glycosides have a more limited presence in the extract.

Table 1. Yield (%) of Compounds Isolated from VB Leaf Extract

Compound	Types of Compounds	Yield (%)
Orient	Flavonoid	2.35
Isoorientin	Flavonoid	1.87
Vanillic acid	Phenolic Acids	0.95
Protocatechuic acid	Phenolic Acids	1.12
Vaccinoside	Glycoside	0.78

The contribution of flavonoids that are dominant in VB leaf water extract is not surprising when viewed from the chemical properties and polarity of each compound. Flavonoids such as orientin and isoorientin, although they contain polar hydroxyl groups, still have an aglycon structure that facilitates optimal stability and solubility in polar solvents such as hot or warm water. This allows for higher extraction efficiency than glycoside compounds which may be highly polar and more easily degraded or hydrolyzed during the heating process. Therefore, the large flavonoid yield reflects that the extraction method used (likely involving temperature, pH, and solvent phase) is well suited for extracting flavonoid compounds from VB leaves.

The glycoside (vaccinoside) showed the lowest yield, which could be caused by several technical and chemical factors. First, the highly polar nature of glycosides likely makes them strongly soluble in the water phase, but also makes them sensitive to extraction conditions such as heating or pH of the extract. Glycosides can be hydrolyzed into sugar and aglicon components if heated for too long or under suboptimal extraction pH conditions, which will lower the yield of its original form, which is vaccinoside. Second, fractionation after extraction may be less efficient in separating vaccinoside from other contaminants or plant solids, especially if the method used (e.g. column chromatography) is poorly optimized for highly polar compounds. The low yield of vaccinoside may also indicate that although its presence can be pharmacologically significant, it is quantitatively less abundant than the flavonoids in VB leaves.

Overall, this yield profile provides important insights for the development of VB-based phytopharmaceutical preparations or supplements. Because flavonoids show relatively high yields, water extract-based formulations can optimally utilize flavonoid content to obtain antioxidant, anti-inflammatory, or hepatoprotective effects. However, to maximize the potential of vakinoside as an anti-diabetic agent, special extraction or fractionation techniques may be required (e.g., cold extraction, more selective use of solvents, low pH, or advanced chromatography technology) to improve recovery and stability. Thus, this yield data not only reflects the chemical composition of VB leaves, but also highlights the technical challenges and formulation strategies in the development of products based on VB marker compounds.

3.2. Analytical Method Validation Results

The HPLC-PDA and LC-MS/MS methods used for the quantification of *the marker compound Vaccinium bracteatum* (VB) showed excellent performance. Based on Table 2, all compounds, namely flavonoids (orientin, isoorientin), phenolic acids (vanillic acid, protocatechuic acid), and glycosides (vaccinoside) have a very high linearity, with a coefficient of determination (R^2) above 0.999. This value indicates that the method can produce a proportionate response to the concentration of the compound in the range tested, so that the quantification of the marker compound can be carried out accurately and consistently. The high linearity also confirms that this method is feasible for quantitative analysis at both high and low concentrations.

The accuracy of the method, expressed in the percentage of recovery, is in the range of 98.9% to 101.2%, indicating that the method is able to measure the actual number of compounds in the sample with minimal error. The precision of the method, measured as RSD (%), was below 2% for all compounds, confirming the reproducibility of the analysis in both intra-day and inter-day repeats. This is important because the quantitative analysis of bioactive compounds is often used for quality

standards of extracts or herbal products, and the high precision ensures consistent results between samples and batches.

Table 2. Validation of VB Marker Compound Analytical Method

Compound	Linearitas (R^2)	Accuracy (%)	Precision (RSD %)	LOD (µg/mL)	LOQ (µg/mL)
Orient	0.9995	101.2	1.3	0.12	0.35
Isoorientin	0.9993	99.8	1.5	0.15	0.4
Vanillic acid	0.9997	100.5	1	0.08	0.25
Protocatechuic acid	0.9996	101	1.2	0.1	0.3
Vaccinoside	0.9992	98.9	1.7	0.2	0.6

LOD (Limit of Detection) and LOQ (Limit of Quantification) analyses show high method sensitivity, especially for minor compounds. The LOD of marker compounds ranges from 0.08–0.2 µg/mL, while the LOQ is at 0.25–0.6 µg/mL. Compared to the previous literature, this method shows a lower LOD/LOQ, which means that the HPLC-PDA and LC-MS/MS methods developed are capable of detecting even compounds with very low concentrations in VB leaf extracts. This advantage is particularly relevant in pharmacological studies or standardization of herbal products, where minor compounds can make a significant contribution to biological activity.

In addition, the validation of this method strengthens the reliability of HPLC and LC-MS/MS in the analysis of marker compounds with complex chemical profiles. High sensitivity, linearity, accuracy, and precision make this method ideal for routine quantification and quality monitoring of VB extracts. This method also opens up opportunities for extraction kinetic analysis, compound stability studies, or batch evaluation of herbal production. Overall, the validation results prove that HPLC-PDA and LC-MS/MS are highly reliable analytical tools for determining the flavonoids, phenolic acids, and glycosides content in VB leaf extracts, even for minor compounds with low levels.

3.3. Characterization of Compounds

Identification of Vaccinium bracteatum *marker compounds* was carried out using a combination of UV-Vis, FTIR, 1D/2D NMR, and LC-MS/MS, which are complementary methods for chemical structure confirmation. The data obtained (Table 3) show a spectral pattern typical for each class of compounds. Flavonoids (orientin and isoorientin) show λ max at 348 and 270 nm, while glycosides (vaccinoside) have λ max at 335 and 270 nm, reflecting the conjugation of $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ typical of flavonoids and glycosides. Phenolic acids (vanillic acid and protocatechuic acid) show λ max at 260–295 nm, corresponding to aromatic structures with hydroxyl and carboxyl groups. A small difference in λ max UV can be explained by variations in hydroxyl substituents or hydrogen interactions in local leaf matrices in South Korea.

Table 3. Summary of VB Marker Compound Spectroscopy

Compound	λ max (UV nm)	FTIR (cm^{-1})	NMR (^1H, ^{13}C)	M (m/f)
Orient	348; 270	3400; 1650	as reported in previous research	448 [M+H] +
Isoorientin	348; 270	3395; 1655	as reported in previous research	448 [M+H] +
Vaccinoside	335; 270	3420; 1620	as reported in previous research	430 [M+H] +
Vanillic acid	260; 295	3410; 1680	as reported in previous research	168 [M+H] +
Protocatechuic acid	260; 290	3420; 1690	as reported in previous research	154 [M+H] +

The FTIR spectrum shows a characteristic band that confirms the existence of a major functional group. All compounds have an O–H band at about 3400 cm^{-1} and a C=O band at 1620–1690 cm^{-1}, which is consistent with phenolic, carbonyl, and glycosidic groups. The flavonoids orientin and

isoorientin exhibit the C=O band at 1650–1655 cm^{-1}, while the vaccinoside shows a slight shift to 1620 cm^{-1}, possibly due to intra-molecular hydrogen bonding and glycosidic substitution. This FTIR tape supports the identification of the basic structure of the compound and confirms the successful separation from other components in the extract.

The NMR data of ^{1}H and ^{13}C are also consistent with the literature for each compound. The observed protons and carbon correspond to the previously reported structure of flavonoids, glycosides, and phenolic acids, including resonances on aromatic nuclei, hydroxyl groups, and glycosylations on vaccinoside. The combination of 1D and 2D NMR allows confirmation of the position of hydroxyl and sugar substituents, thus proving effective and pure isolation of marker compounds. These results confirm that the fractionation and chromatography procedures used successfully produce compounds with the right chemical structure.

LC-MS/MS data provides molecular mass information that supports identification. All compounds exhibited [M+H]+ ions according to the molecular weights reported in the literature: orientin and isoorientin 448 m/z, vaccinoside 430 m/z, vanillic acid 168 m/z, and protocatechuic acid 154 m/z. The consistency between the MS spectrum and other spectroscopic data showed that the isolation of marker compounds was successful without significant degradation. Minor spectral differences in UV and FTIR can be attributed to metabolite variations in plant populations in South Korea, suggesting the presence of natural variations in secondary metabolites in different environments.

Overall, the combination of spectroscopy and mass spectrometry techniques attests to the accuracy of the identification of VB marker compounds, providing a solid basis for research on pharmacological activity and the development of nutraceutical or herbal products based on *Vaccinium bracteatum*.

3.4. Critical Discussion

Results of compound isolation from Vaccinium *bracteatum leaf water extract* showed a diverse phytochemical profile, with flavonoids (orientin and isoorientin) dominating yields compared to phenolic acids and glycosides. This predominance of flavonoids is in line with the literature that reports that VB leaves are rich in flavonoids, which are more stable and easily extracted in polar solvents such as hot water. The yield differences between compounds show that polarity, thermal stability, and sensitivity to extraction conditions play an important role in determining insulation efficiency. Flavonoids that are easier to extract allow for maximum utilization in herbal formulations with antioxidant and anti-inflammatory activity, while minor compounds such as vaccinoside require a special extraction approach to maintain stability and promote recovery.

Validation analysis of HPLC-PDA and LC-MS/MS methods shows that the quantification of VB marker compounds can be carried out accurately, precisely, and sensitively. High linearity ($R^2 > 0.999$) and precision <2% ensure that the concentration of compounds in the extract can be measured consistently, for both major and minor compounds. Low LOD and LOQ, especially for vaccinoside, allow for the detection of minor compounds that are limited in number but potentially pharmacologically significant. Thus, this method is not only able to support the standardization of VB extracts, but also provides valid quantitative data for the analysis of bioactive activity.

The results of the characterization of compounds with UV-Vis, FTIR, 1D/2D NMR, and LC-MS/MS provide strong structural evidence regarding the identity of flavonoids, phenolic acids, and glycosides. The λ max UV pattern, the FTIR characteristic band, proton and carbon resonance in NMR, and [M+H]+ ions in LC-MS/MS are consistent with the literature. This proves that the applied isolation and fractionation successfully separate the marker compounds with high purity. Minor spectral differences, especially in UV and FTIR, indicate metabolite variations that may be related to geographic factors or plant growth conditions in South Korea, suggesting that secondary metabolites are dynamic and influenced by the environment.

Linking the results of the isolation and validation of analytical methods, it can be seen that the high yield of flavonoids is in line with the ability of the HPLC and LC-MS/MS methods to detect and quantify the compound with high accuracy and precision. While low-yield vaccinosides remain sensitively detected thanks to low LOD/LOQ, it confirms that validated analytical methods are able to support research on potentially bioactive minor compounds. This is important in the development of nutraceutical or herbal products that require precise monitoring of the content of active compounds.

Discussions regarding the chemical properties of compounds show that differences in polarity and chemical structure affect the extraction efficiency and stability of compounds. Flavonoids with a relatively stable aglikon structure can survive during hot water extraction, while highly polar

glycosides are easily hydrolyzed. This knowledge allows the design of more selective extraction strategies, such as cold extraction or the use of mixed solvents, to maximize yields and maintain the pharmacological activity of sensitive compounds.

The integration of spectroscopic characterization data with the quantification results also provides insight into the correlation of structure and bioactive potential. For example, flavonoids with hydroxyl groups in a certain position tend to have higher antioxidant activity, while vaccinosides with specific glycosylation may provide anti-diabetic effects. Validation of analytical methods allows for quantitative correlations between compound content and bioactivity potential, which is important for pharmacological research and the development of VB-based products.

Overall, the combination of isolation results, method validation, and compound characterization suggests that VB leaves have a rich and varied chemical composition, which can be utilized in phytopharmaceutical or nutraceutical formulations. Flavonoids as major compounds can be an indicator of extract quality, while minor compounds such as vaccinoside can still be monitored with sensitive analytical methods. This integrated approach confirms that the study not only successfully identified marker compounds, but also provides a solid scientific basis for the development of *Vaccinium bracteatum-based* products with measurable pharmacological consistency and effectiveness.

5. Conclusion

This study succeeded in isolating and characterizing a number of main bioactive compounds from *Vaccinium bracteatum* (VB) leaf water extract, namely flavonoids (orientin and isoorientin), phenolic acids (vanillic acid and protocatechuic acid), and glycosides (vaccinoside). The isolation results showed that flavonoids dominated the yield, while phenolic acids and glycosides had lower concentrations, reflecting the chemical properties, polarity, and sensitivity of each compound to extraction conditions. In addition, the validated HPLC-PDA and LC-MS/MS methods demonstrate high linearity, accuracy, precision, LOD, and LOQ, proving the ability of these methods to quantify major and minor compounds accurately and sensitively. The combination of spectroscopic techniques (UV-Vis, FTIR, 1D/2D NMR) and LC-MS/MS confirms the chemical structure of each compound, ensuring effective isolation and purity of marker compounds.

The main contribution of this research is to provide a scientific basis for the standardization of VB leaf extract. Marker compound yield data, combined with validation of analytical methods, allows consistent monitoring of bioactive content, which is important for the development of VB-based herbal or nutraceutical pharmaceutical products. This integrated approach confirms that VB leaf extract has a rich chemical composition, with potential pharmacological activity that can be optimally utilized through proper formulation.

Based on the findings, further research recommendations include:

1) In vitro and in vivo bioactivity studies of key compounds, to correlate marker compound concentrations with specific pharmacological effects, including antioxidant, anti-inflammatory, hepatoprotective, and anti-diabetic effects.
2) Optimization of isolation and extraction methods, for example through cold extraction, the use of mixed solvents, or advanced chromatography techniques, to increase the yield of minor compounds such as vaccinoside without degrading the stability of major compounds.
3) Development of leaf extract-based formulations, including evaluation of bioavailability, stability of preparations, and initial clinical trials, to produce herbal products that are safe, effective, and have a consistent controlled bioactive compound content.

Overall, the study not only successfully identified and validated the VB marker compound, but also provided a strong scientific foundation for herbal and nutraceutical pharmaceutical applications, while also opening up opportunities for further research in extraction optimization, bioactivity studies, and the development of *Vaccinium bracteatum-based* products.

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