Biotech Studies 33(1), 1-12
http://doi.org/10.38042/biotechstudies.1383424

PROOF
RESEARCH PAPER

BIOTECH STUDIES

Evaluation of anticancerogenic effect of flavonoid rich *Verbascum gypsicola* Vural & Aydoğdu methanolic extract against SH-SY5Y cell line

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Article History
Received 05 Semptember 2023
Accepted 30 October 2023
First Online 30 October 2023

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Keywords
Apoptosis
Flavonoid
Mitochondrial membrane potential
Reactive oxygen species

Abstract

Neuroblastoma (NB) is an embryonal neoplasm affecting the autonomic branch of the nervous system; it is the most commonly detected cancer type in children. NBs affecting children mostly present with metastatic disease that is hardly treatable with intensive multimodal therapy and portends a poor prognosis. Therefore, the likelihood of children with high-risk NB relapse remains extremely high, which calls for urgent action to discover novel treatment options to improve survival. Assessing the anti-cancer properties of known natural compounds may offer novel therapeutic options against NB. In this study we aimed to investigate the anti-cancer properties of the *Verbacum gypsicola* methanol extract (VGME) rich in flavonoids on SH-SY5Y cell line. For this purpose, we used LC-MS analysis to investigate the flavonoid composition of VGME, MTT analysis to investigate its effect on cell viability, and flow cytometry and qRT-PCR analyses to investigate its effect on apoptosis. VGME had a high flavonoid content. Its IC_{50} dose was 50 µg/mL at 48 hours. It significantly increased intracellular ROS level, apoptotic cells' percentage, and mitochondrial disruption. The capacity of VGME to block cancer growth via an intrinsic apoptotic route implies that it might be a classic option for anticancer drug creation.

Introduction

Neuroblastoma (NB) is a prevalent malignancy that originates from the neural crest in children and represents a substantial challenge in pediatric oncology, as highlighted by Anoushirvani et al. (2023). This aggressive cancer is responsible for a significant proportion of childhood cancer-related mortality, with roughly 15% of pediatric cancer-related deaths attributed to NB. What's particularly concerning is that a substantial portion of new NB cases, close to 40%, present with high-risk criteria, and many of these cases have already metastasized at the time of diagnosis, as reported by Jacobson et al. (2023). The inherent aggressive nature and advanced disease stage at diagnosis pose significant hurdles in effectively managing this condition. The current therapeutic arsenal for NB includes a combination of treatments such as surgical removal, chemotherapy, stem cell transplantation, radiotherapy, and immunotherapy. Despite these intensive multimodal therapeutic approaches, the relapse risk in NB remains notably high. Distressingly, when the disease does recur, it often exhibits poor responsiveness to subsequent therapeutic interventions, as highlighted by Gao et al. (2023). It is noteworthy that over two-fifths of NB cases with high-risk characteristics experience relapse, underscoring the pressing need for the discovery of novel therapeutic targets and the exploration of natural therapeutic compounds, especially those derived from herbal sources, as emphasized by Hagemann et al. (2013). Additionally, in NB, imbalances in the gene and protein levels of *BAX* (BCL2 associated X), *BCL2* (B-cell CLL/lymphoma 2), *CASP3* (caspase-3), *CASP7* (caspase-7), *CASP8* (caspase-8), *CASP9* (caspase-9), and *CYCS* (cytochrome C), which are involved in the intrinsic apoptotic pathway, can lead to a predisposition of cells favoring survival over programmed cell death. This contributes to the growth of the tumor and its resistance to treatments. Understanding how the

intrinsic apoptotic pathway is impacted in NB treatment is crucial for the development of herbal treatment methods. Specifically, targeting these genes and proteins in the pathway could be a potential strategy to eradicate tumor cells (Ataur Rahman et al., 2012; Chen et al., 2014a; Rahman et al., 2013). The pursuit of innovative treatment options and the investigation of herbal compounds hold promise for improving the outlook for NB patients and addressing the unique challenges posed by this complex pediatric cancer.

The *Verbascum* genus, belonging to the Scrophulariaceae family, encompasses a rich diversity of approximately 360 species worldwide. However, it's in Turkey where *Verbascum* truly flourishes, with a remarkable 239 species and 107 hybrids, and notably, a staggering 200 of them are exclusive to this region, as highlighted by Keser et al. (2023). These plants have found a prominent place in traditional remedies and have been attributed with a wide range of medicinal properties. *Verbascum* species have been historically harnessed for their medicinal potential, serving as remedies for a plethora of health concerns. They have been valued for their anticancer, cytotoxic, immunomodulatory, antiulcerogenic, antihepatotoxic, antihyperlipidemic, antitussive, antiviral, antimicrobial, antimalarial, antioxidant, antiinflammatory, antinociceptive, antitumor, and antigermination properties, as documented by Alkowni et al. (2023) and Tatlı and Akdemir (2006). This extensive range of uses attests to the rich pharmacological potential inherent in *Verbascum* species. Furthermore, *Verbascum* species are a reservoir of diverse chemical compounds, including flavonoids, phenolics, tannins, coumarins, cardiac glycosides, quinones, and flavanones as indicated by Amiri et al. (2023), Aydın et al. (2020), Gose and Hacioglu Dogru (2021), Pourmoslemi et al. (2023), Tatlı et al. (2008). This diverse chemical profile contributes to their multifaceted therapeutic properties. Previous research, such as the work by Taşkaya et al. (2023), has unveiled the significant anticancer potential of *Verbascum napifolium*, particularly in relation to cell viability in CaCo-2 and L929 cell lines. Similarly, Demirci et al. (2023) delved into the anticancer activities of the methanol extract of *V. speciosum*, which is rich in iridoid glucosides and phenyl ethanoids. Research on the leaves of *V. sinaiticum* has led to the discovery of two flavolignans: hydrocarpin and the new compound sinaiticin. Additionally, two flavones, chrysoeriol and luteolin, were identified. All these compounds demonstrated dose-dependent cytotoxic effects on cultured P388 cells, as reported by Afifi et al. (1993). Fifty-one extracts from various parts of 14 different plants were analyzed for their cytotoxic properties using the MTT assay. Among them, the ethanol extract from the flowers of *V. sinaiticum* demonstrated cytotoxic effects against the Vero cell line, as reported by Talib and Mahasned (2010a). The *in vitro* antiproliferative effects of *V. sinaiticum* were studied against Hep-2 and MCF-7 cell lines. The ethanol extract of *V. sinaiticum* showed strong antiproliferative capabilities against these cell lines. Notably, the extract from the flowers of *V. sinaiticum* was more potent than that from its aerial parts, as documented by Talib and Mahasneh (2010b). Luteolin and 3-O-fucopyranosylsaikogenin F, extracted from *V. thapus*, have been identified to exhibit potent antiproliferative properties. Specifically, they induced apoptosis in A549 lung cancer cells, as documented by Zhao et al. (2011). Notably, the study at hand is pioneering in its investigation of the anticancer activity of *V. gypsicola* species on the SH-SY5Y cell line. The choice of SH-SY5Y cells as the experimental model is well-justified for several compelling reasons: First, it's closely associated with NB, a cancer type commonly found in children that originates from nerve cells. Hence, using SH-SY5Y cells provides a logical choice to assess the potential effects against this specific cancer type, as outlined by Kovalevich and Langford (2013). Second, these cells are of human origin, which aligns well with clinical applications for treating NB. Third, SH-SY5Y cells have a strong basis in prior research and are a widely recognized model in the fields of NB and cancer research, as evidenced by the considerable body of literature that has utilized this cell line, as underscored by Mellado et al. (2018), Pk et al. (2023), Richeux et al. (1999), and Tai et al. (2011) and Vetter et al. (2012). Lastly, SH-SY5Y cells may share similar characteristics with NB cells, thus providing a more specific model for understanding the underlying mechanisms related to NB, a feature underscored by Ataur Rahman et al. (2012). By choosing SH-SY5Y cells for this study, it not only opens the door to exploring the potential anticancer properties of *V. gypsicola* but also allows for a more nuanced understanding of the mechanisms underpinning its effects, thereby offering promising avenues for the advancement of NB research and treatment strategies.

The primary objective of the current study was to assess the potential anti-cancer properties of the flavonoid compounds present within the chemical composition of *V. gypsicola* methanolic extract (VGME). To achieve this, LC-MS/MS analysis was employed to precisely identify the specific flavonoids residing within VGME. Subsequently, VGME was evaluated for its cytotoxic effects against human neuroblastoma cancer cells (SH-SY5Y). In an effort to unravel whether VGME's cytotoxicity could be attributed to the flavonoids it contains, this study delved further into a comprehensive investigation that encompassed, for the first time, the examination of several crucial aspects. These included the assessment of intracellular ROS levels, the quantification of apoptotic cells, the determination of disrupted mitochondria, and an exploration of the influence on gene expression levels related to the apoptosis pathway. This holistic approach allowed us to not only identify the presence of flavonoids within VGME but also to dissect the precise mechanisms through which VGME exerts its cytotoxic effects on

cancer cells, shedding light on potential therapeutic avenues in the quest to combat cancer.

Material and Methods

Plant material

Verbascum gypsicola Vural & Aydoğdu, a distinctive plant species, is known for its natural habitat within an area rich in saline salts, nestled in the scenic region of Beypazarı, located in Ankara, Turkey. This remarkable plant was thoughtfully sampled during the month of August in the year 2011, precisely when it was in full bloom, showcasing its vibrant and characteristic flowers. The precise identification and authentication of this plant specimen were expertly conducted under the discerning eye of Prof. Dr. Zeki AYTAÇ, a distinguished authority in botanical studies affiliated with Gazi University. This rigorous verification process ensured the accurate classification and taxonomy of *Verbascum gypsicola*. To further validate its identity and as a testament to its existence, a voucher specimen was meticulously preserved within the Herbarium of Gazi University. This voucher specimen bears the distinctive identifier "ZA-10440" and serves as a tangible record of *Verbascum gypsicola*'s presence in this unique ecological niche. This diligent documentation and verification process not only contribute to our understanding of this plant species but also provide valuable insights into the biodiversity and botanical richness of the Beypazarı region in Ankara, Turkey.

Preparation of the methanolic extract

The preparation of the *V. gypsicola* methanolic extract (VGME) was carried out with precision and attention to detail. Initially, 30 grams of dried and powdered *V. gypsicola* were carefully used for the extraction process. The extraction was performed at a controlled temperature of 60 °C, and it extended over a period of four h. To facilitate this process, a specialized Soxhlet apparatus was thoughtfully employed. Following the extraction, the VGME was meticulously filtered and concentrated under vacuum conditions at 80 °C using a rotary evaporator from Heidolph (Schwabach, Germany). This step ensured the removal of excess solvent and the concentration of the extract, resulting in a more potent and manageable form. Subsequently, a crucial freeze-drying process was carried out, further enhancing the stability and preservation of the extract. The extract was then carefully stored at a controlled temperature of 4 °C for a maximum period of one week, during which it remained in optimal condition and ready for chemical analyses. This methodical procedure ensured the integrity and quality of the VGME, making it well-suited for the subsequent chemical analyses required for the study's objectives.

Determination of flavonoid composition

The quantification of VGME's flavonoid contents was meticulously achieved through a precise and sophisticated analytical approach. To carry out this task, an Agilent 6460 Triple Quad LC/MS (California, USA) was expertly coupled with the Agilent 1200 series high-performance liquid chromatography (HPLC) system (California, USA). This cutting-edge instrumentation allowed for the accurate determination of the concentrations of specific flavonoids present in VGME. To do so, calibration curves correlating concentration with peak area were thoughtfully generated for each individual flavonoid of interest. By skillfully comparing the peak areas of the flavonoids in VGME to the established calibration curves, the respective concentrations of these compounds within VGME were quantified. This methodical and data-driven approach ensures the precision and reliability of the obtained results regarding VGME's flavonoid content, providing invaluable insights for the study's objectives.

Determination of cell viability

In this study, human neuroblastoma cells (SH-SY5Y, ATCC CRL-2266) were thoughtfully procured from the Foot and Mouth Institute located in Ankara, Turkey. These cells were cultivated using Dulbecco's modified Eagle's medium (DMEM) sourced from Gibco (Massachusetts, USA). The growth medium was enriched with 10% fetal bovine serum (FBS) from Gibco (Massachusetts, USA) along with 1% penicillin/streptomycin and 1% L-glutamine, both also from Gibco (Massachusetts, USA). The cell culture was meticulously maintained in a humidified environment with a 5% CO_2 and 95% air mixture at a stable temperature of 37 °C. To ensure healthy cell growth, the medium was replenished three times weekly when the cells approached confluence. This careful maintenance of cell cultures helps to guarantee their vitality and reliability in subsequent experiments. The impact of VGME on cell viability was systematically assessed by cultivating SH-SY5Y cells in 96-well plates, with an initial seeding density of 10,000 cells per well. The cells were then incubated in a controlled environment with 5% CO_2 at 37 °C. Subsequently, VGME was introduced to the wells at varying concentrations (ranging from 0 to 1000 µg/mL) and for different durations of 24, 48, and 72 h. Following the designated incubation periods, VGME was carefully removed from the wells, and the cells were thoroughly washed with phosphate-buffered saline (PBS) sourced from Merck (Pennsylvania, USA). Fresh media were then added to the wells. To assess cell viability, the widely-used MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed. Specifically, 20 µL of a 0.5% MTT solution from Sigma-Aldrich (Massachusetts, USA), prepared in PBS, was added to each well. After a subsequent four h incubation and removal of the medium, 200 µL of dimethyl sulfoxide (DMSO) from Sigma-Aldrich (Massachusetts, USA), was applied to dissolve the formazan crystals that had formed. The absorbance

levels were then accurately measured at 570 nm using an ELISA microplate reader from BioTek (Vermont, USA). To derive the cell viability values, the absorbance of the untreated control cells was divided by that of the treated cells. The results were expressed as percentages, providing a comprehensive view of how VGME affected the viability of SH-SY5Y cells. This rigorous experimental procedure ensured the reliability and validity of the findings in the assessment of cell viability.

Flow cytometric detection of reactive oxygen species (ROS)

In the investigation of cellular reactive oxygen species (ROS) levels, a meticulous protocol was meticulously adhered to. To assess ROS levels, the cells were deliberately treated with DCFDA (20 µM), a fluorescent probe supplied by Cayman Chemical (Michigan, USA). This step enabled the specific detection of ROS within the cellular environment. Following the introduction of DCFDA, the treated cells were subjected to a controlled incubation period at 37 °C for a duration of 30 min. During this incubation, the cells were allowed to interact with DCFDA, which is capable of detecting changes in ROS levels. After this incubation period, the cells were carefully pipetted and made ready for detailed analysis through the utilization of the ACEA NovoCyte flow cytometer (California, USA). This advanced flow cytometry system was instrumental in the systematic examination of the cells, providing a comprehensive view of ROS levels and their fluctuations. For each sample, an impressive 10^4 cells were analyzed, ensuring a statistically robust dataset. The ACEA NovoExpress software (California, USA), a powerful tool for flow cytometry data analysis, was then employed to process and record the data. The software allowed for the precise determination of the mean change in ROS levels within the cell population, shedding light on how the experimental conditions influenced the cellular oxidative stress. This information is crucial in understanding the impact of the study variables on cellular responses and oxidative processes. The careful execution of this procedure, coupled with the use of advanced equipment and software, guaranteed that the results were reliable and informative. This, in turn, greatly contributed to the comprehensive understanding of the dynamics of ROS levels in the context of the study.

Flow cytometric detection of apoptotic cells

In the meticulous evaluation of cell apoptosis, a well-thought-out procedure was meticulously followed. Specifically, a total of 5 µL of annexin V-FITC and an additional 5 µL of propidium iodide (PI), both sourced from Abcam (Cambridge, UK), were thoughtfully introduced into each 100 µL of the cell suspension, encompassing approximately 1×10^6 cells. These vital fluorescent probes were instrumental in distinguishing and quantifying different cell populations based on their membrane integrity and apoptotic status. Following the addition of annexin V-FITC and PI, the cells were subjected to a controlled incubation period in the dark, carried out at room temperature, for a duration of 15 min. This incubation allowed for the binding of annexin V-FITC and PI to the cells, enabling their distinct identification in terms of apoptotic features. After the incubation, 400 µL of 1x annexin-binding buffer was meticulously added to the cell suspension. This step served to stabilize and prepare the cells for precise analysis. The cell analysis was conducted using the advanced ACEA NovoCyte flow cytometry system (California, USA). This state-of-the-art equipment enabled the systematic examination of the stained cells, providing detailed insights into their apoptotic status and membrane integrity. For each sample, an extensive dataset was generated by analyzing a representative sample size of 10,000 cells, ensuring that the results were both comprehensive and statistically sound. Subsequently, the ACEA NovoExpress software (California, USA), designed for flow cytometry data analysis, was employed to record and process the data. The recorded data allowed for the precise determination of the percentages of live, early apoptotic, late apoptotic, and necrotic cells within the cell population. This comprehensive analysis shed light on the dynamic processes associated with cell apoptosis and provided valuable information regarding the experimental conditions' impact on cellular responses. The rigor and attention to detail in this procedure ensured that the results were robust, reliable, and informative, contributing significantly to the understanding of cell apoptosis in the context of the study.

Flow cytometric detection of mitochondrial membrane potential ($\Delta\psi_m$)

In the assessment of mitochondrial integrity, tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a fluorescent probe provided by Cayman Chemical (Michigan, USA), was skillfully incorporated into the experimental setup. Specifically, 100 µL of JC-1 was meticulously added to a cell population comprising approximately 1×10^6 cells. This critical step ensured the staining of the mitochondria within the cells. The cells were then subjected to a controlled cell culture environment maintained at 37 °C for a duration of 30 min. This incubation period was crucial for allowing JC-1 to interact with the mitochondria within the cells, revealing crucial information about their integrity. Subsequently, the cells were systematically harvested and carefully pipetted for removal, followed by a thorough washing with PBS, performed twice. This rigorous washing process was essential to remove any excess JC-1 and to prepare the cells for precise analysis. To carry out the analysis, an ACEA NovoCyte flow cytometer (California, USA) was skillfully employed, a powerful instrument for conducting flow cytometry. This high-tech equipment facilitated the assessment of

mitochondrial integrity by capturing and analyzing data from the stained cells. A substantial dataset was generated by analyzing 10,000 cells per sample, ensuring a comprehensive and representative assessment. The ACEA NovoExpress software (California, USA), a sophisticated tool designed for flow cytometry data analysis, was then utilized to process the data. Through the application of this software, the percentages of intact and disrupted mitochondria were meticulously determined. This analysis provided valuable insights into the state of the mitochondria within the cell population, shedding light on their structural integrity and any alterations induced by the experimental conditions. This meticulous procedure not only offers precise quantitative data but also ensures the reliability and validity of the results in the context of mitochondrial integrity assessment, contributing to a deeper understanding of cellular responses.

qRT-PCR

In the pursuit of investigating the molecular alterations induced by VGME, a meticulously designed series of laboratory techniques was employed. Initially, total RNA extraction from SH-SY5Y cells was carried out with the highly regarded RNeasy mini kit from Qiagen (Hilden, Germany), ensuring the efficient isolation of RNA. Subsequently, the quantitect reverse transcription kit, also from Qiagen (Hilden, Germany), was conscientiously utilized to perform reverse transcription following the manufacturer's recommended protocols. This step allowed for the conversion of RNA into complementary DNA (cDNA), a pivotal transformation in molecular biology research. To delve into the VGME-mediated changes in gene expression, a quantitative real-time polymerase chain reaction (qRT-PCR) technique was executed. For this purpose, carefully selected primers tailored to each specific gene of interest, including *BAX*, *BCL2*, *CASP3*, *CASP7*, *CASP8*, *CASP9*, and *CYCS*, were thoughtfully employed. These primers facilitated the precise quantification of gene expression levels. To ensure accurate comparisons and normalize the data, *β-actin* was chosen as a reference gene, which served as a stable point of reference for the analysis. This normalization step is essential to mitigate variations in the experimental process. The qRT-PCR protocol was executed with the following settings: a preliminary 5-min denaturation step at 95 °C, followed by 40 cycles comprising 10 sec of denaturation at 95 °C, 30 sec of annealing at 60 °C, and 30 sec of extension at 72 °C. This meticulously designed protocol allowed for the precise amplification and quantification of the target genes. To determine the relative changes in gene expression levels, the $2^{-\Delta\Delta CT}$ method was meticulously employed, a widely accepted approach in qRT-PCR analysis. This method provides a robust means of calculating fold regulation and enables a clear interpretation of the alterations in gene expression induced by VGME. Finally, the results were thoughtfully presented as fold regulations, offering a comprehensive and understandable depiction of the changes in gene expression levels attributed to VGME treatment. This methodical approach ensures the reliability and validity of the molecular insights gained in this research.

Data analysis

Each experiment was thoughtfully conducted in triplicate, ensuring rigorous and reliable data for subsequent statistical analyses. The resulting data for all variables were thoughtfully presented as mean values accompanied by their corresponding standard deviations (SD), a commonly accepted practice to convey the consistency and dispersion of the measurements. For the purpose of data analysis, all statistical procedures were meticulously carried out using SPSS version 11.0 (New York, USA), a widely used and respected statistical software tool. To assess the significance of differences between the study groups in terms of means, a one-way ANOVA test, a robust statistical method, was thoughtfully employed. This test provided a comprehensive assessment of any variations among the experimental groups. It's important to note that a threshold of statistical significance was defined as a *P*-value of less than 0.05. In accordance with this criterion, any observed results with a *P*-value below 0.05 were regarded as statistically significant, underscoring the reliability and validity of the findings in the study. This rigorous approach to experimental design and data analysis ensures that the conclusions drawn from this research are both credible and meaningful.

Results & Discussion

As a preliminary study, total phenolic (15.42 ± 0.92 mg/g), total flavonoid (117.13 ± 0.78 mg/g), ascorbic acid (2.12 ± 0.93 mg/g), β-carotene (1.89 ± 0.65 mg/g), lycopene (0.63 ± 0.23 mg/g), and total alkaloid (0.45 ± 0.11 mg/g) contents of VGME were determined. Since the total flavonoid content was the main component found in VGME, the study focused on flavonoids. In a study conducted by Selseleh et al. (2020), the total flavonoid content of 10 distinct *Verbascum* species gathered from Iran was found to range from 12 to 22 mg/g. In a study conducted by Kızıltaş et al. (2022), the total flavonoid content in *V. speciosum* Schrad extracts was measured to be in the range of 5-16 µg/mL. Similarly, Luca et al. (2019) revealed that the total flavonoid content of *V. ovalifolium* Donn ex Sims extracts, prepared using various solvents, fluctuated between 17-107 mg/g. Additionally, research by Mihalovic et al. (2016) showed that extracts from *V. nigrum*, *V. phlomoides*, and *V. thapsus* had total flavonoid contents varying from 10-53 mg/g. Our results are confirming the high total flavonoid content in VGME. Our study found a higher total flavonoid content in VGME compared to the studies conducted by Kızıltaş et al. (2022), Luca et al. (2019), Mihalovic et al. (2016), and Selseleh et al. (2020).

Extensive prior phytochemical studies, as exemplified by Gökmen et al. (2021), have previously uncovered the presence of flavonoids within the chemical composition of the *Verbascum* genus. Flavonoids, regarded as a vital category of bioactive compounds in plants, have been reported to be distributed across various plant components, a fact highlighted by Amini et al. (2021). In light of these findings, our study sought to elucidate the flavonoid composition of VGME (*Verbascum gypsicola* methanolic extract) through the employment of LC-MS/MS techniques. The outcomes of our investigation revealed the presence of seven distinct flavonoids within VGME. Notably, the analysis disclosed that apigenin exhibited the highest concentration of flavonoids within VGME, while amentoflavone was found to be the least abundant, as outlined in **Table 1.** Drawing from the research conducted by Amini et al. (2021), it is evident that certain *Verbascum* species, particularly *V. saccatum* and *V. songaricum*, are distinguished by their high overall flavonoid content, featuring compounds such as apigenin, quercetin, and rutin. Further corroborating our findings, a study by Klimek et al. (2020) demonstrated that flower samples of *V. phlomoides* are notably rich in diosmin and tamarixetin, while *V. densiflorum* predominantly contains verbascoside and luteolin. Moreover, Mahmoud et al. (2007) successfully isolated and identified luteolin and chrysoeriol within *V. sinaiticum*. In the case of *V. thapsus*, a species within the *Verbascum* genus, research by Alipieva et al. (2014) has revealed the presence of a unique bisflavonoid known as amentoflavone. Consistent with the wealth of knowledge within the existing literature, our study underscores the significant flavonoid content in *V. gypsicola*, reinforcing the potential for this plant species to serve as a valuable source of flavonoids, a feature that aligns with the established characteristics of certain other *Verbascum* species recognized for their flavonoid richness. These findings collectively emphasize the substantial value and relevance of VGME within the context of flavonoid research and its potential applications.

Table 1. Flavonoid composition and quantity of VGME

Flavonoid composition	Control (µg/g)	VGME (µg/g)
Apigenin	0.00 ± 0.00	0.47 ± 0.02*
Luteolin	0.00 ± 0.00	0.43 ± 0.03*
Quercetin	0.00 ± 0.00	0.39 ± 0.01*
Diosmin	0.00 ± 0.00	0.13 ± 0.00*
Chrysoeriol	0.00 ± 0.00	0.08 ± 0.02*
Tamarixetin	0.00 ± 0.00	0.07 ± 0.04*
Amentoflavone	0.00 ± 0.00	0.05 ± 0.04*

*P <0.05, compared with the control group

Numerous studies have delved into the pharmacological effects of plant extracts enriched with flavonoids, underscoring the increasing significance attributed to these flavonoid-rich plant sources, as noted by Shakeri et al. (2015) and emphasized by Kızıltaş et al. (2022). Consequently, the exploration of VGME's impact on cellular viability, particularly in the context of SH-SY5Y cells, becomes a critical focal point in this research. The assessment of SH-SY5Y cell viability through MTT analysis revealed a compelling and dose-dependent response to varying concentrations of VGME (ranging from 0 to 1000 µg/mL) over different time intervals (24, 48, and 72 h). Notably, an inverse relationship was observed between VGME concentrations and treatment duration, leading to a statistically significant inhibition of SH-SY5Y cell viability (P< 0.05). The calculated IC_{50} doses for VGME were found to be 96 µg/mL for 24 h, 50 µg/mL for 48 h, and 10 µg/mL for 72 h, ultimately leading to the selection of the IC_{50} dose of 50 µg/mL for 48 h as the optimal condition for subsequent investigations, as depicted in **Figure 1.** In a parallel context, Dinani et al. (2020) explored the cytotoxic properties of various saponin- and flavonoid-rich fractions derived from *V. alceoides*, a member of the *Verbascum* genus. Their study involved in vitro assessments using the MTT assay on HeLa and HUVEC cells, revealing a significant and dose-dependent inhibitory effect on cell proliferation, with components D, E, and A exhibiting IC_{50} values of 30, 39.8, and 188.6 µg/mL, respectively. Additionally, Garcia-Oliveira et al. (2022) examined the cytotoxic effects of ethanolic and infusion extracts *from V. sinuatum*, one of the traditional medicinal herbs, against four distinct cancer cell lines: MCF-7, NCI-H640, HeLa, and HepG2. Their findings demonstrated the pronounced impact of *V. sinuatum* on these cancer cell lines, with GI_{50} values ranging between 101.1 and 172.2 µg/mL for the ethanolic extract and 59.1 and 92.1 µg/mL for the infusion extract. In light of these studies, our findings are consistent with the existing literature, illustrating VGME's cytotoxic activity against SH-SY5Y cells. It is our belief that the potent cytotoxicity of VGME against SH-SY5Y cells can be primarily attributed to its abundant flavonoid content. Nevertheless, further research (to investigate the direct effect of flavanoids in VGME, it is necessary to isolate and purify these compounds from VGME) is imperative to validate and expand upon this hypothesis, shedding more light on the precise mechanisms underlying VGME's cytotoxic effects and its potential applications in the field of cytotoxicity and cancer research.

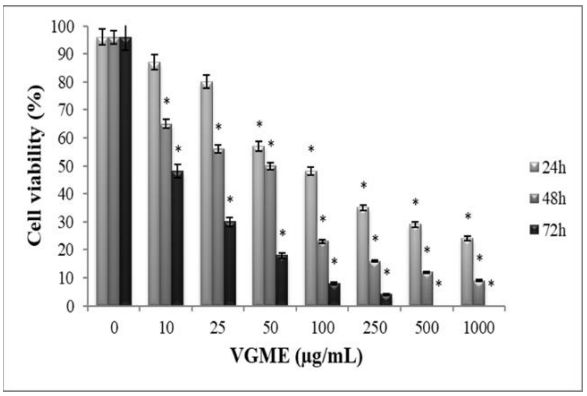

Figure 1. Effect of different VGME concentrations (0-1000 µg/mL) on cell viability for 24, 48, and 72 h in SH-SY5Y cells. *P <0.05, compared with the control group.

In specific contexts, flavonoids have been found to exhibit a paradoxical propensity towards oxidation, a phenomenon well-documented in scientific studies. This dual nature of flavonoids, which can act as both antioxidants and prooxidants, has been linked to their ability to induce oxidative stress within cells, particularly in the context of neoplastic conditions. As highlighted by Slika et al. (2022), certain flavonoids have the remarkable capacity to stimulate the accumulation of reactive oxygen species (ROS) within cells, a process that can trigger apoptosis and contribute to the reduction of tumor mass. In the context of our study, flow cytometry analysis was conducted to investigate the impact of VGME on intracellular ROS levels in SH-SY5Y cells. Our results revealed a significant reduction in ROS levels in the VGME-treated SH-SY5Y cells compared to the control cells that were not exposed to VGME ($P< 0.05$), as depicted in **Figure 2** and **Table 2.** Previous research by Shi et al. (2015) and Hu et al. (2015) has suggested that the prooxidative activity of apigenin arises from mechanisms such as partial glutathione depletion and inhibition of superoxide dismutase. Similarly, studies by Jeong et al. (2009), Gibellini et al. (2010), and Lu et al. (2006) have linked quercetin's prooxidative effects to the generation of free radicals through participation in intracellular redox reactions, as well as partial glutathione depletion and the inhibition of thioredoxin reductase. Additionally, research by Ferino et al. (2020) and Zhou et al. (2017) has demonstrated that luteolin can induce prooxidative activity by activating PI3K through oxidative stress-induced pathways, subsequently engaging the Akt/mTOR/p70S6K pathway, inhibiting Nrf2 and the prosurvival protein Snail, and activating the proapoptotic Raf kinase inhibitor protein. In consonance with the existing body of literature, our findings align well with the prooxidative activity of VGME, particularly in its ability to elevate ROS levels within SH-SY5Y cells. We posit that this prooxidative impact of VGME on SH-SY5Y cells can be attributed to its substantial flavonoid content. Nevertheless, it is crucial to emphasize that additional research (to investigate the direct effect of flavanoids in VGME, it is necessary to isolate and purify these compounds from VGME) is required to validate and expand upon this hypothesis, delving deeper into the specific mechanisms through which VGME exerts its prooxidative influence and its potential implications for applications in the context of oxidative stress and cancer research.

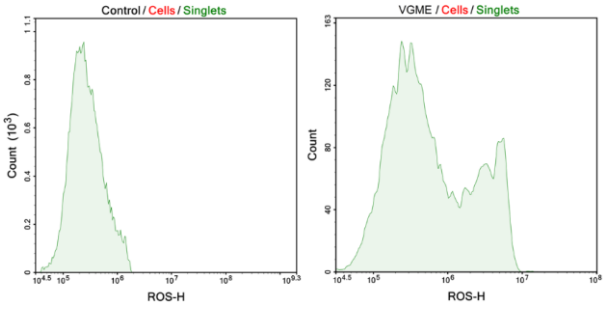

Figure 2. Effect of 50 µg/mL VGME (48 h) on intracellular ROS levels in SH-SY5Y cells.

Table 2. Effect of VGME on mean intracellular ROS levels in SH-SY5Y cells using flow cytometry

	Control	VGME
Mean ROS	192 ± 24	1360 ± 38*

*P <0.05, compared with the control group

The intrinsic apoptotic pathway, also known as the mitochondrial pathway, is one of the primary mechanisms of programmed cell death (Singh and Lim, 2022). This pathway is activated in response to intracellular stress signals such as DNA damage, lack of growth factors, or oxidative stress (Morana et al., 2022). At the heart of the intrinsic apoptotic pathway are the mitochondria. Under intracellular stress conditions, pro-apoptotic proteins like BAX and BAK permeabilize the mitochondrial outer membrane (Green, 2022a). This leads to the release of pro-apoptotic factors from the mitochondria, such as cytochrome c, into the cytosol (Lossi, 2022). Once released, cytochrome c interacts with apoptotic protease activating factor-1 (APAF-1), resulting in the formation of the apoptosome complex that activates caspase-9 (Green, 2022b). The activated caspase-9 subsequently activates caspase-3 and caspase-7, leading to controlled cell death (Gourisankar et al., 2023). On the other hand, anti-apoptotic proteins like BCL2 inhibit apoptosis by preventing the permeabilization of the mitochondrial membrane (Cetraro et al., 2022). This balance plays a critical role in determining whether a cell lives or dies and is a critical factor in many diseases, especially in cancer (Wan et al., 2022). Therefore, understanding and modulating the intrinsic apoptotic pathway forms the foundation of many therapeutic strategies (Singh et al., 2022).

Flavonoids have emerged as potent inhibitors of numerous signal transduction pathways critical to the development of cancer, exerting their influence by disrupting cell viability, angiogenesis, inhibiting distant tumor spread, and promoting apoptosis, as established by Abotaleb et al. (2018). In this context, our study focused on investigating the impact of VGME on the percentages of viable cells, cells in the early and late stages of apoptosis, as well as necrotic cells within the SH-SY5Y cell line, employing flow cytometry. The results from our study revealed a significant increase in the percentage of cells in the early and late stages of apoptosis, as well as necrotic cells when treated with VGME in comparison to the control cells that were not subjected to VGME treatment ($P< 0.05$), as illustrated in **Figure 3** and **Table 3**. Building upon these findings, Srivastava et al. (2016) conducted research on quercetin, showing that the treatment of cells led to an increase in early apoptotic cells at 6 and 12 h, while treatments over longer durations (18, 24, and 48 h) resulted in a higher percentage of late apoptotic cells. This observation strongly suggests a promotion of apoptotic pathways. Cai et al. (2011) reported a proapoptotic effect of luteolin on A549 cells, as evidenced by Hoechst 33258 and annexin V-FITC/PI staining. Flow cytometry analysis revealed a significant

population of apoptotic cells and an increased number of cells in the G2 phase. Furthermore, Souza et al. (2017) investigated the effects of apigenin and found that this compound displayed a selective cytotoxic effect, mediating apoptosis in Annexin V-marked HeLa, SiHa, CaSki, and C33A cells, while not affecting the HaCaT control cells. In accordance with the established literature, our study aligns with these findings, demonstrating that VGME has a proapoptotic impact on SH-SY5Y cells. It is our belief that VGME's proapoptotic effect against SH-SY5Y cells can be attributed to its substantial flavonoid content. However, to provide conclusive evidence, further research (to investigate the direct effect of flavanoids in VGME, it is necessary to isolate and purify these compounds from VGME) is essential to delve deeper into the precise molecular mechanisms through which VGME induces apoptosis and to explore its potential applications in cancer research and therapy.

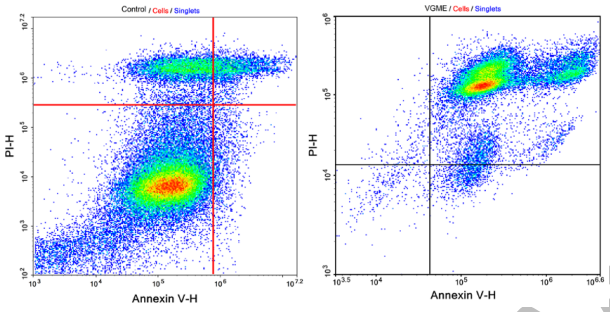

Figure 3. Effect of 50 µg/mL VGME (48 h) on live, apoptotic, and necrotic cells in SH-SY5Y cells.

Table 3. Effect of VGME on live, apoptotic, and necrotic cells (%) in SH-SY5Y cells using flow cytometry

Type of Cells	Control (%)	VGME (%)
Live	83.43 ± 5.43	3.13 ± 0.75*
Early apoptotic	2.35 ± 0.96	6.35 ± 3.23*
Late apoptotic	5.68 ± 1.23	89.78 ± 7.65*
Necrotic	8.54 ± 3.54	0.74 ± 0.50*

*P <0.05, compared with the control group

Flavonoids have been recognized for their ability to promote apoptosis in cancer cells, and this effect is often associated with their capacity to increase the generation of mitochondrial reactive oxygen species (ROS), including superoxide and hydrogen peroxide. This increase in ROS production is typically followed by the creation of mitochondrial permeability transition pores and the subsequent release of cytochrome c (*CYCS*), a pivotal event in the apoptosis process, as noted by Kachadourian and Day (2006). In light of this knowledge, our study employed flow cytometry analysis to evaluate VGME's impact on the percentage of intact and disrupted mitochondria within SH-SY5Y cells. Notably, VGME led to a substantial increase in the percentage of disrupted mitochondria in SH-SY5Y cells when compared to control cells that did not receive VGME treatment (P< 0.05), as visualized in **Figure 4** and outlined in **Table 4.**

Reinforcing our findings, Chen et al. (2014b) reported that apigenin has the ability to reduce the mitochondrial membrane potential of gastric carcinoma cells, as demonstrated through JC-1 staining. Similarly, in an event reliant on mitochondrial membrane potential, Shen et al. (2016) revealed that quercetin triggered apoptosis in cells. Moreover, Chen et al. (2017) observed that luteolin induced a reduction in mitochondrial membrane potential, indicating intrinsic apoptosis mediated by JC-1. Our study aligns with the existing literature by demonstrating that VGME induces mitochondrial depolarization in SH-SY5Y cells. It is our perspective that VGME exerts its activity by causing mitochondrial depolarization in SH-SY5Y cells, likely attributed to its enriched flavonoid content. Nevertheless, further in-depth investigation (to investigate the direct effect of flavanoids in VGME, it is necessary to isolate and purify these compounds from VGME) is essential to validate and expand upon this hypothesis, unraveling the precise mechanisms through which VGME induces mitochondrial depolarization and exploring its potential implications for cancer research and therapeutic development.

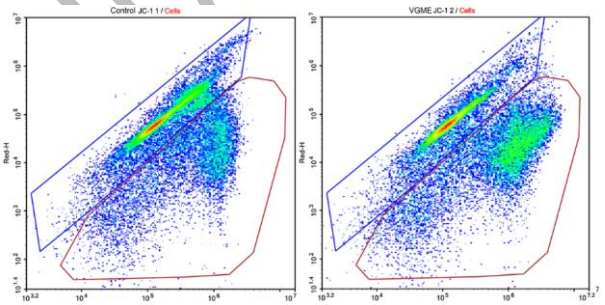

Figure 4. Effect of 50 µg/mL VGME (48 h) on intact and disrupted mitochondria in SH-SY5Y cells.

Table 4. Effect of VGME intact and distrupted mitochondria (%) in SH-SY5Y cells using flow cytometry

Types of mitochondria	Control (%)	VGME (%)
Intact	84.76 ± 5.67	57.57 ± 3.45*
Distrupted	14.97 ± 4.43	42.25 ± 2.12*

*P <0.05, compared with the control group

Flavonoids have been identified as influential agents capable of initiating events that lead to cell death by modulating the apoptotic signaling cascade, as established by Kopustinskiene et al. (2020). Our study aimed to elucidate the impact of VGME on SH-SY5Y cells by assessing its effect on genes involved in the intrinsic apoptosis pathway through quantitative real-time polymerase chain reaction (qRT-PCR). The results were striking, indicating that VGME significantly increased the expression levels of key apoptotic genes, including *BAX*, *CASP3*, *CASP7*, *CASP8*, *CASP9*, and *CYCS*, while simultaneously reducing the expression of the anti-apoptotic gene *BCL2* in SH-SY5Y cells when compared to control cells that were not exposed to VGME (P< 0.05),

as depicted in **Figure 5**. Expanding upon these findings, it's worth noting that luteolin, as reported by Lin et al. (2008), has been shown to induce DNA damage and activate p53, thereby promoting the intrinsic apoptosis pathway. This is achieved through its ability to impede the proper functioning of DNA topoisomerases. Similarly, in a study conducted by Teekaraman et al. (2019), quercetin was found to promote apoptosis in the PA-1 cell line by upregulating the expression of key apoptotic genes, including *CYCS*, *CASP9*, and *CASP3*. This suggests the activation of caspase-9 and caspase-3 via the release of cytochrome c from mitochondria, a process that may be mediated by the intrinsic pathway due to a reduction in ROS levels and the ensuing apoptosis. Moreover, apigenin, as reported by Wang and Zhao (2017), has been shown to enhance apoptosis by triggering increased intracellular ROS production, calcium release, mitochondrial membrane damage, and upregulation of gene expression related to various apoptotic factors. This cascade of events ultimately leads to cell apoptosis. In accordance with the wealth of data within the existing literature, our study demonstrates that VGME exhibits a pronounced proapoptotic effect on SH-SY5Y cells. We believe that VGME's proapoptotic activity on SH-SY5Y cells can be attributed to its rich flavonoid content. However, it is imperative to emphasize the necessity for further research (to investigate the direct effect of flavanoids in VGME, it is necessary to isolate and purify these compounds from VGME) to validate and expand upon this hypothesis, gaining a deeper understanding of the specific molecular mechanisms through which VGME induces apoptosis and exploring its potential applications in the context of cancer research and therapeutic development. While studies involving plant extracts provide valuable insights into potential health benefits or medicinal properties, it's essential to acknowledge the limitations and complexities associated with such studies. Researchers often utilize these initial findings as a springboard for more targeted investigations to identify and comprehend the specific compounds responsible for the observed effects.

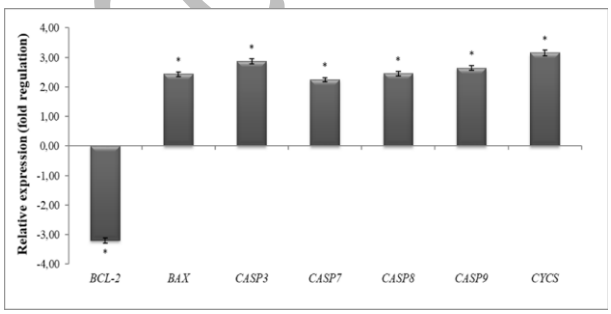

Figure 5. Effect of VGME on relative expression levels of genes involved in the intrinsic apoptosis pathway on SH-SY5Y cells. **P* <0.05, compared with the control group.

Conclusion

In summary, this comprehensive study has provided compelling evidence that VGME, rich in flavonoids, plays a pivotal role in the generation of a significant quantity of reactive oxygen species (ROS). This surge in ROS levels triggers a cascade of events within SH-SY5Y cells, including the disruption of mitochondrial membrane potential (ΔΨM) and activation of the intrinsic apoptotic pathway. Consequently, these cellular changes culminate in a noteworthy reduction in the longevity of SH-SY5Y cells. The remarkable ability of VGME to use the intrinsic apoptotic pathway as a mechanism to inhibit cancer growth has been demonstrated for the first time in this study, demonstrating its potential as a promising candidate in the development of new anticancer drugs. This research underscores the significance of exploring VGME and its flavonoid content as a potential therapeutic avenue for combating cancer, further emphasizing the need for continued investigation and development in this exciting field of study.

Ethical Statement

Not applicable.

Funding Information

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Author Contributions

Conceptualization: SŞ, Data Curation: SŞ, Formal Analysis: SŞ, Investigation: SŞ, Methodology: SŞ, Supervision: SŞ, Visualization: MDO, Writing-original draft: SŞ, Writing -review and editing: SŞ.

Conflict of Interest

The author declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Acknowledgements

I would like to thank Prof. Dr. Belma Aslım for providing *Verbascum gypsicola* Vural & Aydoğdu methanolic extract.

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