Bioactive constituents of *Verbena officinalis* regulate killing efficiency of primary human natural killer cells by accelerating killing processes

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Abstract

Background: Natural killer (NK) cells play a key role in eliminating tumorigenic and pathogen-infected cells. *Verbena officinalis* (*V. officinalis*) has been used as a medical plant in traditional and modern medicine, exhibiting anti-tumor and anti-inflammation activity.

Purpose: The impact of bioactive constituents of *V. officinalis* on immune responses still remains largely elusive. In this work we investigated the potential targets of *V. officinalis* and focused on killing efficiency and related functions of NK cells regulated by bioactive constituents of *V. officinalis*.

Study design/methods: We used primary human NK cells from peripheral blood mononuclear cells. Potential regulatory roles of selected compounds were analyzed by network pharmacology approaches. Killing efficiency was determined with real-time killing assay and live-cell imaging in 3D. Proliferation was examined by CFSE staining. Expression of cytotoxic proteins was analyzed using flow cytometry. Lytic granule release was quantified by CD107a degranulation assay. Contact time required for killing and determination of serial killers were analyzed using live cell imaging results.

Results: Using network pharmacology approaches, we analyzed potential regulatory roles of five compounds (Acteoside, Apigenin, Kaempferol, Verbenalin and Hastatoside) from *V. officinalis* on immune cell functions and revealed NK cells as a major target. The effect of these compounds on NK killing efficiency was examined with real-time killing assay, and Verbenalin enhanced NK killing efficiency significantly. Further investigation showed that Verbenalin did not affect proliferation, expression of

cytotoxic proteins, or lytic granule degranulation, but rather reduced contact time required for killing therefore enhancing total killing events per NK cell, suggestively via inhibition of inhibitory receptors as determined by docking assay.

Conclusions: Our findings reveal the underlying mechanisms how *V. officinalis* regulates functions of immune cells, especially NK cells, suggesting Verbenalin from *V. officinalis* as a promising therapeutic reagent to fight cancer and infection.

Introduction

Verbena officinalis L. (*V. officinalis*), also known as common vervain, is a medicinal herb, widespread throughout the globe, mainly in the temperate climate zone (Kubica, Szopa et al., 2020b). In China, *V. officinalis* is widely distributed in the southern part of the Yellow River and has been used as traditional Chinese medicine for the treatment of rheumatism, bronchitis, depression, insomnia, anxiety, liver and gallbladder diseases (Khan, Khan et al., 2016, Kubica, Szopa et al., 2020a). Recent reports suggest that *V. officinalis* has a number of scientifically proven activities, such as antioxidant, antibacterial, antifungal, anti-inflammatory, analgesic, anticonvulsant, anxiolytic, antidepressant, sedative, hypnotic, wound healing, gastro-protective, anti-cancer and insecticidal properties (Casanova, Garcia-Mina et al., 2008, Kubica et al., 2020b, Lopez-Jornet, Camacho-Alonso et al., 2014, Speroni, Cervellati et al., 2007). In addition to being used as an antimicrobial, secretolytic, expectorant and diuretic agent, *V. officinalis* is also widely used in food and cosmetics, especially due to its antioxidant, antibacterial, and anti-inflammatory properties (Kubica et al., 2020a).

V. officinalis, blooming overground parts of the plant, contains flavonoids, terpenoids, phenolic acids, phenylpropanoids and iridoids (Khan et al., 2016, Kubica et al., 2020a, Kubica, Szopa et al., 2017, Shu, Chou et al., 2014). Verbenalin and hastatoside, belonging to iridoids, are characteristic constituents of *V. officinalis*, and they exhibit various biological activities including sleep-promoting, antioxidant, and hepatoprotective activity (Shu et al., 2014). Moreover, the relative content of Verbenalin and hastatoside are higher compared to that of other characteristic iridoids in *V. officinalis*, serving as promising target constituents for quality control of *V. officinalis*.

Kaempferol and apigenin represent the most encountered aglycone flavonoids isolated from *V. officinalis* Kaempferol has been shown to be cardioprotective, neuroprotective, anti-inflammatory, anti-diabetic, anti-oxidant, anti-microbial, and have anticancer activities (Imran, Salehi et al., 2019). Acteoside, belonging to phenylpropanoid glycosides, is water-soluble and exhibits a wide range of biological activities, such as anti-tumor, anti-microbial, anti-inflammatory, anti-thrombotic and wound healing properties (Funes, Laporta et al., 2010).

Natural killer (NK) cells are specialized immune killer cells belonging to innate immune system, which play a key role in eliminating tumorigenic and pathogen-infected cells. To successfully execute their killing function, NK cells need to infiltrate into the target tissue to search for their target cells. Once the target cells are identified, a tight junction termed the immunological synapse (IS) will be formed between the NK cell and the target cells. Consequently, lytic granules (LG) in NK cells, which contain cytotoxic protein such as pore-forming protein perforin and serine proteases granzymes, are enriched and released at the IS to induce destruction of target cells.

NK cells express activating receptors and inhibitory receptors to govern NK activation. Inhibitory receptors engage with major histocompatibility complex (MHC) Class I molecules, which are expressed on all healthy self-cells. In order to bypass immunosurveillance of cytotoxic T lymphocytes, which recognize antigens presented by MHC-I molecules, some tumorigenic cells or pathogen-infected cells down-regulate the expression of their surface MHC-I molecules. Loss or down-regulation of MHC-I molecules results in activation of NK cells to initiate the corresponding killing

processes.

In this work, we aimed to investigate regulation of NK killing efficiency by active constituents of *V. officinalis* and the underlying mechanisms. We were focused on five compounds (Acteoside, Apigenin, Kaempferol, Verbenalin and Hastatoside) from *V. officinalis.* We employed network pharmacology approaches to predict possible targets and various functional assays to determine the effects of the compounds on NK killing functions.

Materials and Methods

Network pharmacological analysis

Prediction of targets of five representative components of *V. officinalis* was performed by TCMSP, TCM-MESH, Symmap, and TCMID database. The Kyoto Encyclopedia of Genes and Genomics (KEGG) pathway analysis and gene ontology (GO) analysis of biological processes (BP), molecular function (MF) and cellular component (CC) using the DAVID online database were used for exploring gene function. We constructed a PPI (protein–protein interaction) network to elucidate the molecular mechanisms of effects of *V. officinalis* by using the Cytoscape software (version 3.7.2; http://www.cytoscape.org) and the STRING database (version 11.0, http://www.string-db.org/) with a required confidence >0.4. Next, the degree of connectivity in the PPI network was analyzed by Cytoscape software (version 3.7.2) and the top 20 hub genes were obtained. We used online tools such as Gene Cards (http://www.genecards.org/) to find potential targets for NK cell-mediated cytotoxicity. Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to find overlapping genes between potential targets of *V. officinalis* and NK cell-mediated cytotoxicity related targets. The further analyses of theses overlapping genes for functional prediction were using CluGO in Cytoscape software (version 3.7.2).

Molecular Docking

To investigate the interactions between small molecules and receptor proteins, the CDOCKER module in Discovery Studio software was used for molecular docking. The three-dimensional structures of five small molecules were download from TCMSP

（https://tcmsp-e.com/）, and then perform hydrogenation through the Prepare Ligand module, optimize the energy with CHARMM force field. The three dimensional structure of target protein was downloaded from the PDB database (https://www.rcsb.org/). Then run the Prepare Protein module to optimize the protein structure: delete redundant protein conformations, delete water molecules, complete incomplete residues, hydrogenation and distribution of related charges. The prepared target proteins and small molecules were introduced into Discovery Studio and docked using CDOCKER module. The semi flexible docking method and simulated annealing algorithm are used to find the optimal conformation of ligand and receptor. And according to the level of CDOCKER Interaction Energy to evaluate the degree of docking, the lower the scoring function value, the stronger the affinity of the small molecule with the receptor.

NK Cell preparation and cell culture

Human primary NK cells were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors using Human NK Cell Isolation Kit (Miltenyi). The isolated NK cells were cultured in AIM V medium (ThermoFischer Scientific) with 10% FCS and 100 U/ml of recombinant human IL-2 (Miltenyi). K562 and K562-pCasper cells were cultured in RPMI-1640 medium (ThermoFischer Scientific) with 10% FCS. For K562-pCasper cells, 1.25mg/ml G418 was added. All cells were kept at 37 °C with 5% CO_2.

Real-time killing assay

Real-time killing assay was conducted as reported previously (Kummerow, Schwarz et

al., 2014). Briefly, target cells (K562 cells) were loaded with Calcein-AM (500 nM, ThermoFisher Scientific) and settled into a 96-well plate (2.5×10^4 target cells per well). NK cells were subsequently added with an effector to target (E:T) ratio of 2.5:1 if not otherwise mentioned. Fluorescence intensity was determined by GENios Pro micro-plate reader (TECAN) using the bottom-reading mode at 37°C every 10 min for 4 hours. Target lysis (t) % = $100 \times (F_{live}(t)-F_{exp}(t))/(F_{live}(t)-F_{lysed}(t))$. (F: fluorescence intensity)

3D killing assay and live cell imaging

Briefly, target cells (K562-pCasper cells) were embedded into 2 mg/ml of pre-chilled neutralized Bovine type I collagen solution (Advanced Biomatrix) in a 96 well plate. The collagen was solidified at 37°C with 5% CO_2 for 40 min. NK cells were subsequently put on top of collagen as effector cells. The cells were visualized using ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices) at 37 °C with 5% CO_2. For 3D killing assay, as described previously (Zhao, Zhou et al., 2021), the killing events were visualized every 20 min for 36 hours, and live target cell numbers were normalized to hour 0 based on area. For live cell imaging to determine time required for killing and the average kills per NK cell, the cells were visualized every 70 sec for 14 hours and tracked manually. Image J software was used to process and analyze the images.

Proliferation assay

To examine proliferation, freshly isolated primary human NK cells were labelled with CFSE (1 μM, ThermoFischer Scientific) and then stimulated with recombinant human IL-2 in presence of Verbenalin at indicated concentrations for 3 days. Fluorescence was

determined with a FACSVerse™ flow cytometer (BD Biosciences) and analyzed with FlowJo v10 (FLOWJO, LLC).*4.7 Determination of cytotoxic protein expression*

To test perforin and granzyme B expression, NK cells were fixed in pre-chilled 4% paraformaldehyde. Permeabilization was carried out using 0.1% saponin in PBS containing 0.5% BSA and 5% FCS. FACSVerse™ flow cytometer (BD Biosciences) was used to acquire data. FlowJo v10 (FLOWJO, LLC) was used for analysis.

CD107a degranulation assay

For degranulation assay, K562 cells were settled with vehicle-treated or Verbenalin-treated NK cells in the presence of Brilliant Violet 421™ anti-human CD107a (LAMP1) antibody (Biolegend) and GolgiStop™ (BD Biosciences). The incubation were carried out at 37°C with 5% CO_2 for 4 hours. The cells were then stained with PerCP anti-human CD16 antibody (Biolegend) and APC mouse anti-human CD56 antibody (BD Biosciences) to define NK cells. Data was obtained with a FACSVerse™ flow cytometer (BD Biosciences) and was analyzed with FlowJo v10 (FLOWJO, LLC).

Results

Analysis of the Ingredient–Target Network

To explore the pharmacological mechanism of *V. officinalis* on immune regulation, five compounds (Acteoside, Apigenin, Kaempferol, Verbenalin and Hastatoside) were selected as bioactive ingredients based on the TCMSP, TCM-MESH, Symmap, and TCMID database analyses (Figure 1A). Then, potential targets of these five ingredients were predicted via the SwissTargetPrediction database based on their structure, and a total of 117 targets were obtained. The corresponding ingredient-target network is shown in Figure 1B. Since the protein-protein interaction (PPI) networks are relevant to visualize the role of various key proteins in disease, a visual PPI network of the 117 potential targets was subsequently constructed using the Cytoscape software. And the 20 potential therapeutic targets with the highest degree of nodes were considered as hub genes (Figure 1C). Among those genes, several targets with the highest degree of nodes are directly involved in killing functions of NK cells, for example AKT1 for activation (Ali, Nandagopal et al., 2015), TP53 for NK maturation (Collin, St-Pierre et al., 2017), JUN for NK cell-mediated target cell killing (Nausch, Florin et al., 2006), and TNF for NK cell interferon-γ production (Almishri, Santodomingo-Garzon et al., 2016). These data indicate that bioactive constituents of *V. officinalis* can regulate NK functions, especially the killing-relevant processes.

Potential Synergistic Mechanism of the five bioactive ingredients from V. officinalis

The 117 targets of the 5 bioactive ingredients from *V. officinalis* were further analyzed for functional prediction by DAVID. Kyoto Encyclopedia of Genes and

Genomes (KEGG) pathway enrichment analysis show that the 117 target genes mainly enriched in tumor, virus infection and immune pathways, including TNF signaling pathway, PI3K-Akt signaling pathway, p53 signaling pathway, HIF-1 signaling pathway, T cell receptor signaling pathway, NF-kappa B signaling pathway (Figure 2A). Several of those most enriched pathways including TNF signaling pathway, PI3K-Akt signaling pathway and NK-kappa B signaling pathway are directly related to NK activation and killing function (Ali et al., 2015). The results of gene ontology (GO) analysis of biological processes (BP), molecular function (MF) and cellular component (CC) were shown in Figure 2B. In the BP category, complex biological processes are described, such as response to drug, negative regulation of apoptotic processes and response to hypoxia. In the MF category, molecular-level activities performed by target proteins include enzyme binding, Heme binding, and identical protein binding. In the CC category, locations on cellular structures in which a target protein performs a function are described, such as the cytosol, Cyclin-dependent protein kinase holoenzyme complex and GABA-A receptor complex. These findings indicate that the five bioactive ingredients in *V. officinalis* may act on multiple pathways to dampen inflammation and on the biological processes, which are closely related to NK killing functions.

Identification candidate targets for five bioactive ingredients from V. officinalis on NK cell-mediated cytotoxicity

To further identify the genes regulated by *V. officinalis* for NK cell-mediated cytotoxicity, we retrieved a total of 2489 targets from the Gene Cards databases in this

aspect. We used Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) to find overlapping genes between potential targets of *V. officinalis* and NK related targets. A total of 71 potential targets of *V. officinalis* on NK mediated cytotoxicity were obtained (Figure 3A). The corresponding PPI network of *V. officinalis* ingredient–NK target is shown in Figure 3B, where the size of the nodes positively correlates with their degree. We noticed that the largest nodes such as AKT1, TP53, JUN, and TNF are the same as identified in Figure 1C, strongly suggesting that these five bioactive ingredients of *V. officinalis* have a great potential to directly regulate NK killing functions. Furthermore, the common target genes between *Verbena officinalis L* and NK cells were further analyzed for functional prediction using CluGO (Figure 3C). The primary enriched GO biological process (BP) terms were mainly related to apoptosis, cell cycle and reactive oxygen species metabolism, suggesting that *V. officinalis* may influence viability of NK cells, especially under conditions with excessive reactive oxygen species.

Bioactive components of V. officinalis bind to NK cell receptors

In addition, we analyzed whether active compounds of *V. officinalis* could bind to key proteins of NK cells. To this end, we tested the interaction between the five active compounds of *V. officinalis* (Acteoside, Apigenin, Kaempferol, Verbenalin and Hastatoside) with inhibitory receptors of NK cells including NKG2A and KIR2DL1. Interestingly, we found that NKG2A can engage with three bioactive compounds (Apigenin, Kaempferol, and Verbenalin) whereas KIR2DL1 can interact with all five (Figure 4). Of note, among these bioactive compounds, with NKG2A Kaempferol

showed the strongest interaction, to comparable extent of which, three compounds (Acteonid, Verbenalin, and Hastaboside) can engage with KIR2DL1 (Table 1).

Bioactive components of V. officinalis enhance NK killing efficiency

In order to verify the impact of bioactive constituents of *V. officinalis* on NK killing efficiency, we cultured primary human NK cells with the corresponding compound (10 μM and 30 μM) for three days in presence with IL-2. Killing kinetics of NK cells was determined using the plate-reader based real-time killing assay (Kummerow et al., 2014). We found that Acteoside, Apigenin, and Kaempferol slightly reduced NK killing efficiency, whereas Verbenalin and Hastatoside enhanced NK killing efficiency (Figure 5A). Verbenalin exhibited the highest potency for elevation of NK killing efficiency (Figure 5A). We further verified the impact of Verbenalin on NK killing efficiency in 3D. Target cells expressing FRET-based apoptosis reporter pCasper were embedded in collagen matrix and NK cells were added from the top. Target cells were yellow when alive and turned green when undergoing apoptosis (Backes, Friedmann et al., 2018). Results show that Verbenalin-treated NK cells exhibited significantly faster killing kinetics compared to their counterparts treated with vehicle. Thus, we conclude that among the bioactive constituents of *V. officinalis*, Verbenalin is able to increase NK killing efficiency under a physiologically relevant condition.

Verbenalin accelerates NK killing processes

Next, we sought for the underlying mechanisms of increase in NK killing efficiency by Verbenalin. We examined proliferation, expression of cytotoxic proteins (perforin and granzyme B), and degranulation of lytic granules. None of those processes were

affected by Verbenalin (Figure 6A-C). Since our molecular docking data suggest that Verbenalin can bind to inhibitory receptors of NK cells (Figure 4), we analyzed the times required for killing. Killing events were visualized with high-content imaging setup every 70 sec for 12 hours (Figure 7A, Movie 1), and the time between the contact and the induced target cell apoptosis for randomly chose NK cells was analyzed. The quantification shows that the time required for killing was reduced by Verbenalin-treatment (Figure 7B). Concomitantly, on average, the numbers of target cells killed per NK cell was almost doubled for Verbenalin-treated NK cells relative to their counterparts (Figure 7C). Notably, it is reported that a portion of NK cells can kill several target cells in a row (Bhat & Watzl, 2007, Prager, Liesche et al., 2019). We thus also analyzed fractions of serial killers (one NK killed more than one target cell), single killers (one NK killed only one target cell) and non-killers (NK cells did not kill any target cells). We found that Verbenalin-treatment substantially enhanced the portion of serial killers and concomitantly reduced the portion of non-killers (Figure 7D). Taken together, our results suggest that Verbenalin potentiates NK cell activation upon target recognition, shortening the time required to initiate destruction of target cells, therefore enhancing killing efficiency of NK cells.

Discussion

In our work, molecular docking predicted interaction between the bioactive components of *V. officinalis* with two prominent inhibitory receptors of NK cells: NKG2A and KIR2DL1. NKG2A (natural killer group 2A) form heterodimers with CD94 (Lazetic, Chang et al., 1996), serving as one of the most prominent inhibitory receptors in NK cells to avoid killing healthy self-cells (Khan, Arooj et al., 2020). NKG2A contains a cytoplasmic immunoreceptor tyrosine-based inhibition motif, which recruits tyrosine phosphatases (SHP1 and SHP2) to initiate inhibition processes involving PI3K, MAPK, and NF-kappaB signaling (Khan et al., 2020). One major ligand of NKG2A is human leukocyte antigen (HLA)-E, non-classical MHC class I molecules in humans (Lee, Llano et al., 1998). Notably, blockage of NKG2A functionality substantially enhances NK killing capacity against HLA-E-expressing tumor cells (Kamiya, Seow et al., 2019). In the context of COVID-19, elevation of NKG2A expression on NK cells from patients is positively correlated with functional exhaustion (Antonioli, Fornai et al., 2020). Thus, blocking interaction between NKG2A with its ligand by bioactive components of *V. officinalis* could dampen the inhibitory signaling, thus leading to enhancement in NK-mediated cytotoxicity.

Killer cell immunoglobulin-like receptors (KIRs) are transmembrane receptors expressing on NK cells and also on a minority of T cells (Pende, Falco et al., 2019). At least 15 different KIRs have been identified in humans (Wende, Colonna et al., 1999). Concerning the nomenclature for KIRs, the first digit following KIR corresponds to the number of the extracellular domains (2 or 3), L or S at the third digit indicates the length

of the cytoplasmic domain (L for long and S for short), P at the third digit refers to pseudogenes (Khan et al., 2016, Pende et al., 2019). Similar as the other inhibitory KIRs, KIR2DL1 is expressed on the plasma membrane and recognize HLA class I ligands (Pende et al., 2019). Remarkably, several interactive residues in KIR2DL1 with bioactive compounds identified by docking methods (HIS 138, TRP188, and TYR186) are very close to the interaction sites with HLA (ASP135, SER184, and GLU187) (Fan, Long et al., 2001). These results implies that these bioactive compounds of *V. officinalis*, especially Verbenalin, can potentially inhibit interaction between KIR2DL1 and its ligand, leading to dampened inhibitory signals, therefore activate NK cells. We postulate that our observation that Verbenalin-treatment shortened required time to initiate apoptosis and enhances average kills per NK cells is very likely due to blockage of inhibitory receptors (including NKG2A and KIR2DL1) by Verbenalin, which eventually enhances NK killing efficiency.

In this work, we identified several promising target genes that are regulated by the five bioactive components of *V. officinalis*, including TP53, JUN, HIF1A, and VEGFA. TP53, as one of the most intensively investigated tumor suppressor genes, plays an essential role in initiation and progression of malignant tumors (Zhu, Pan et al., 2020). A recent study has reported that pharmacological activation of wild-type p53 increases granzyme B-dependent NK killing capacity against tumor cells via elevation of autophagy (Chollat-Namy, Ben Safta-Saadoun et al., 2019). JUN is a key component of the transcription factor AP-1 (activator protein-1), which are involved in killing-related processes in NK cells such as mobilization of lytic granules and production of

cytokines (Abel, Yang et al., 2018). Interestingly, on the target cell side, activation of AP-1 upregulates RAE-1, ligands of the activating receptor NKG2D on NK cells, leading to increased target cell killing mediated by NK cells (Nausch et al., 2006). HIF1A (hypoxia inducible factor 1 subunit alpha) is a key player to regulate cellular functions in response to hypoxia, which often takes place in tumor microenvironments (Lee, Ko et al., 2019). Activity of HIF1A regulates killing activity in tumor-infiltrating NK cells (Ni, Wang et al., 2020). In addition, VEGF (vascular endothelial growth factor) can be induced under hypoxia condition to improve oxygen supply (Lee et al., 2019). In combination with our findings, it suggests that bioactive constituents of *V. officinalis* can regulate NK killing efficiency from the NK side as well as the tumor cell side.

V. officinalis has a long tradition being used as a medical herb throughout the world, e.g. in Europe, Asia, American and Australia (Kubica et al., 2020a, Vogl, Picker et al., 2013). Anti-tumor activity of *V. officinalis* has been reported (Kou, Yang et al., 2013). Recently, a newly developed formula XuanFeiBaiDu composed of thirteen medical herbs including *V. officinalis* has shown very positive clinical outcome treating COVID-19 patients (Xiong, Wang et al., 2020). However, the underlying mechanism is not known. In this work, we have established a direct link between Verbenalin, a bioactive constituent of *V. officinalis*, and killing efficiency of NK cells. It indicates that as a compound, Verbenalin has a promising potential for therapeutical applications fighting against cancer and/or infection.

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Ethical considerations

Research carried out for this study with material from healthy donors (leukocyte reduction system chambers from human blood donors) is authorized by the local ethic committee (declaration from 16.4.2015 (84/15, Prof. Dr. Rettig-Stürmer).

Disclosures

The authors have no financial conflicts of interest.

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Figure legend

Figure 1. The 2-dimensional (2D) molecular structures of the main bioactive ingredients from *V. officinalis* and the ingredients-targets network. (A) The five bioactive ingredients from *V. officinalis*, (B) ingredient–target network, (C) and top 20 hub genes with the highest degree of connectivity from the protein–protein interaction analysis.

Figure 2. Functional enrichment analysis. (A) KEGG pathway enrichment analysis and (B) GO enrichment analysis.

Figure 3. Target genes of *V. officinalis* and NK and their network. (A) The 71 overlapping genes between *V. officinalis* and NK, (B) PPI network of the genes and (C) GO analysis of the target genes of *V. officinalis* on NK cells by CluGO. Different color of the node represents the different cluster of GO terms.

Figure 4. Molecular docking between the representative components of *V. officinalis* and inhibitory receptors of NK cells. Schematic 3D represented the molecular docking model and active sites of the representative components of *V. officinalis* in the protein NKG2A (PDB ID: 3BDW) (A) and KIR2DL1 (PDB ID: 1IM9) (B), respectively.

Figure 5. Bioactive constituents of *V. officinalis* differentially regulate NK killing efficiency. (A) Kinetics of NK killing affected by bioactive constituents of *V. officinalis*. were determined with real-time killing assay. (B, C) Verbenalin accelerates NK killing kinetics in 3D. Selected time points are shown in B and the change in live target cells is shown in C. Scale bars are 40 μm. One representative donor out of four is shown.

Figure 6. Proliferation and lytic granule pathway of NK cells are not affected by Verbenalin. Primary human NK cells were stimulated with IL-2 in presence of Verbenalin with indicated concentrations for 3 days prior to experiments. (A) Proliferation of NK cells. One representative donor out of four is shown. (B, C) Expression of cytotoxic proteins. Expression of perforin (B) and granzyme B (C) was determined by flow cytometry. Results are from four donors. (D) Release of lytic granules was determined with CD107a degranulation assay. Results are from four donors. ns: not significant ($p > 0.5$). Paired Student's t-test was used for statistical analysis.

Figure 7. Verbenalin shortens the time required for killing and increases average kills per NK. Primary human NK cells were stimulated with IL-2 in presence of Verbenalin with indicated concentrations for 3 days prior to experiments. Target cells (K562-pCaspar) were embedded in collagen and NK cells were added from top. Killing events were visualized at 37°C every 70 sec. (A) NK cells make multiple contacts with target cells. One representative NK cell from each condition is shown. NK cells (marked in red) were not fluorescently labeled. The target cells in contact with the corresponding NK cell are numbered. Scale bars are 20 μm. (B) Verbenalin shortens the time required for killing. The period from NK/target contact to target apoptosis was quantified. (C) Verbenalin-treatment enhances number of target cells killed per NK cell. (D) Fraction of serial killers is elevated by Verbenalin treatment. Fraction of serial killer (one NK killed more than one target cell), single killer (one NK killed only one target cell) and non-killer (NK cells did not kill any target cells) for each donor was analyzed.

Results are from two donors. 21 NK cells were randomly chosen from each condition. Mann-Whitney test was used for statistical analysis.

Table 1. Scores of docking and the interactive residues. Lower binding energies correspond to stronger interaction between the compounds and the target proteins.

Movie 1. Verbenalin-treatment reduces time required for killing and enhances average kills per NK. Primary human NK cells were stimulated with IL-2 in presence of Verbenalin with indicated concentrations for 3 days prior to experiments. Target cells (K562-pCaspar) were embedded in collagen and NK cells were added from top. Killing events were visualized at 37°C every 70 sec. One representative NK cell from each condition (0 μM vs 30 μM) is shown. NK cells were not fluorescently labeled and marked with blue tracks. The target cells in contact with the corresponding NK cell are numbered.

Figure 1. The 2-dimensional (2D) molecular structures of the main bioactive ingredients from *V. officinalis* and the ingredients-targets network.

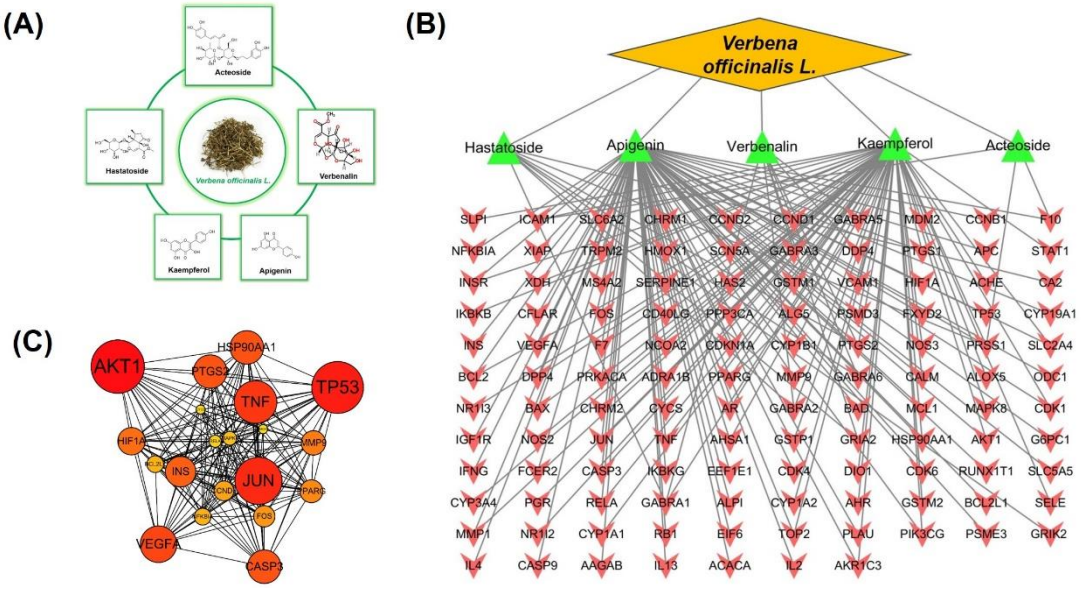
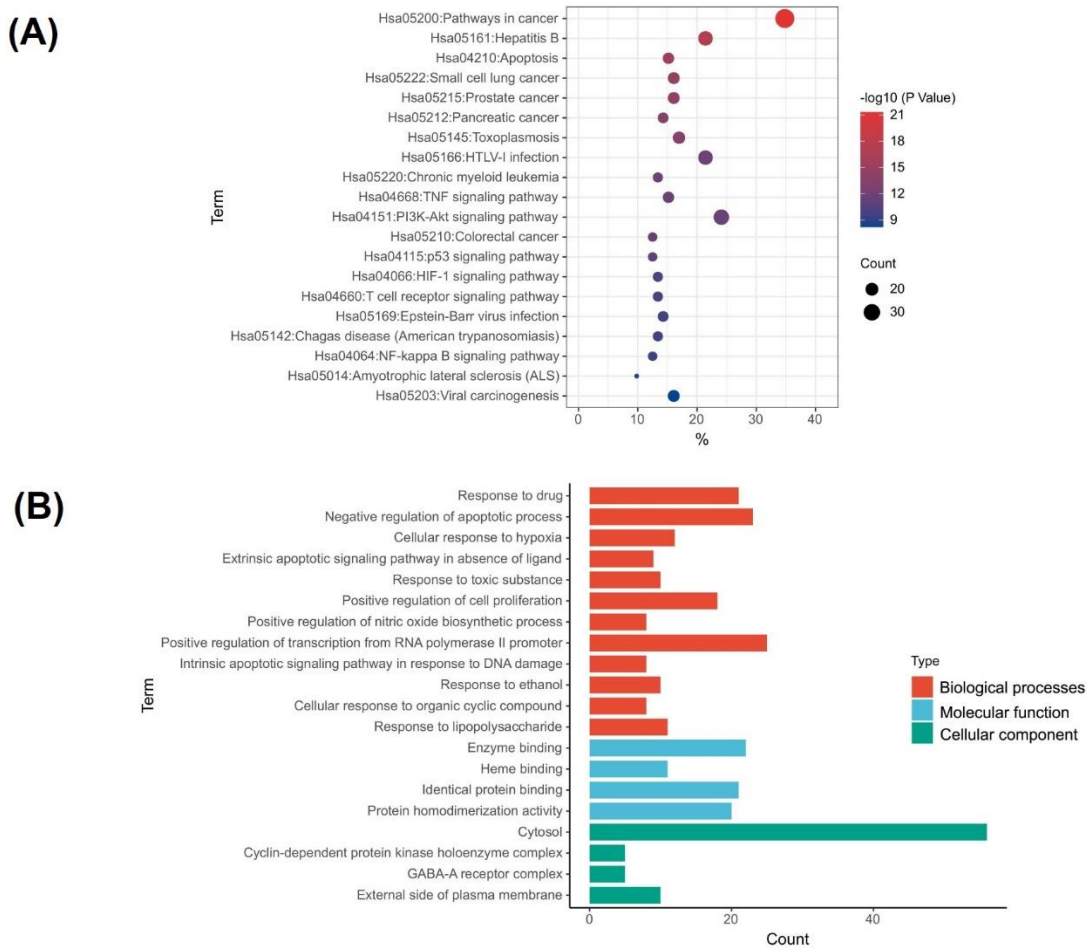


Figure 2. Functional enrichment analysis.



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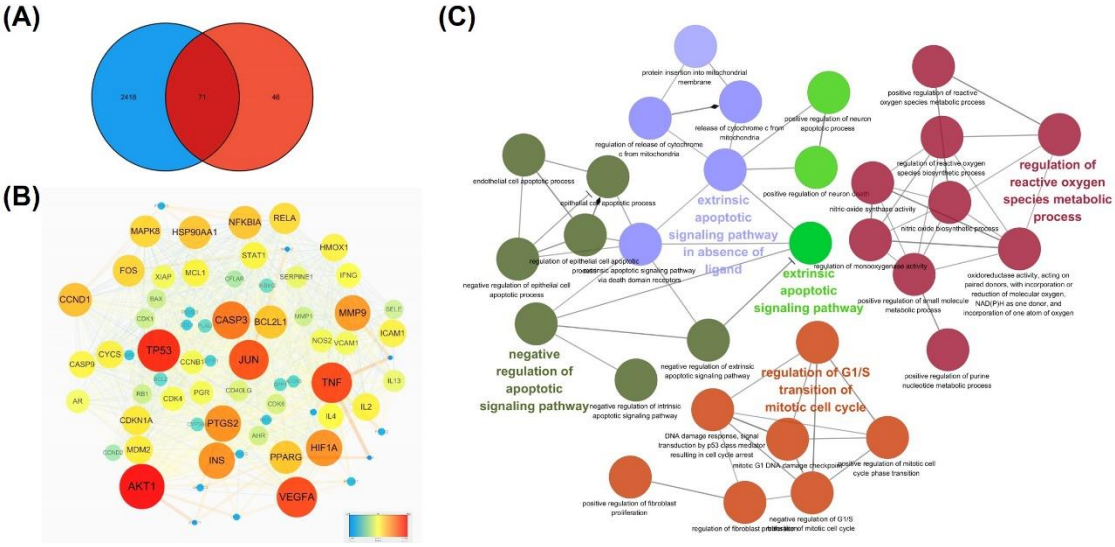

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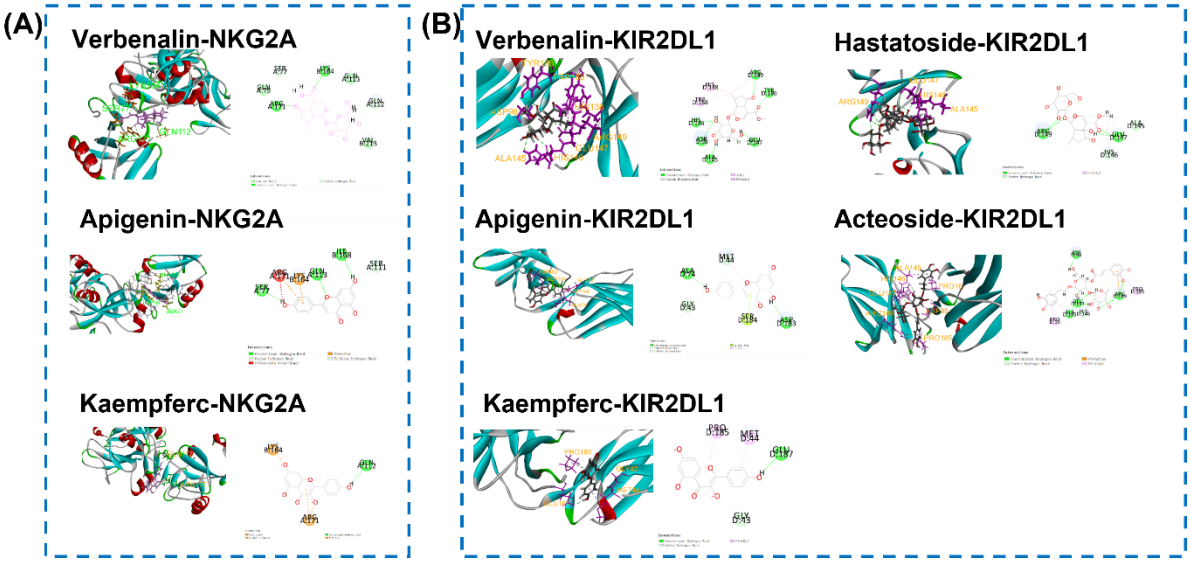
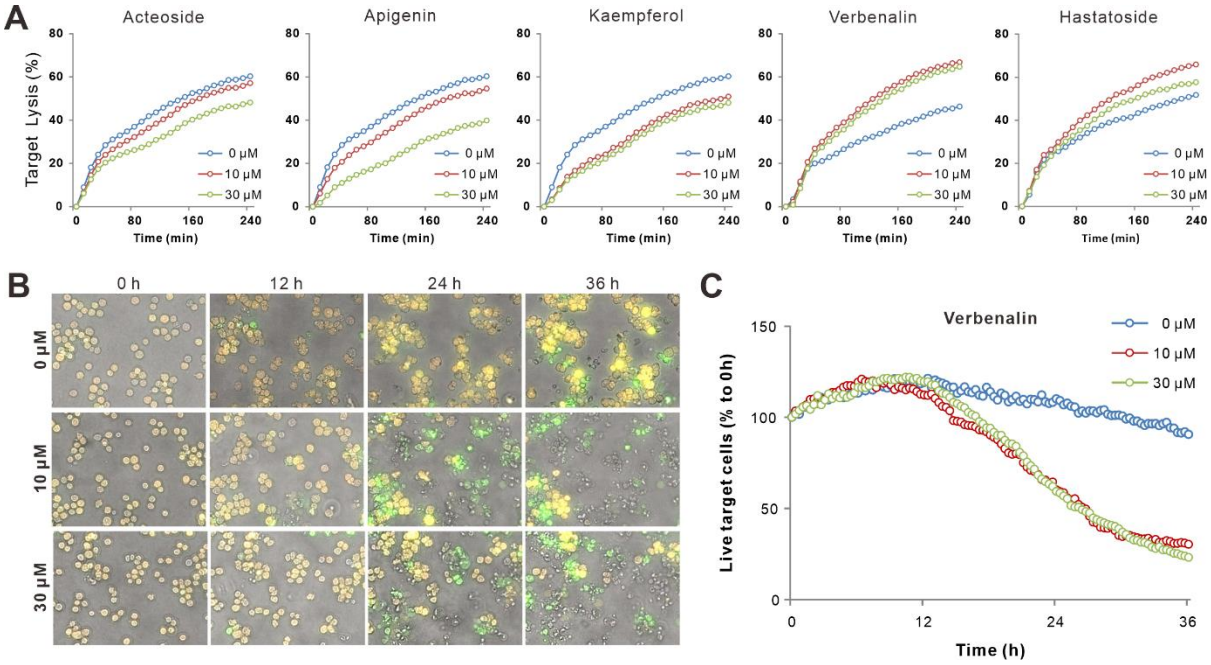


Figure 5. Bioactive constituents of *V. officinalis* differentially regulate NK killing efficiency.



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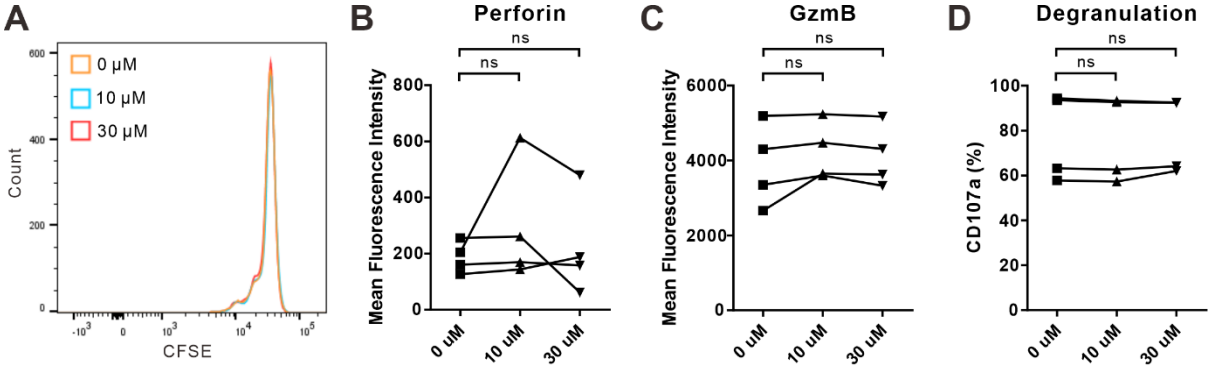

Figure 7. Verbenalin shortens the time required for killing and increases average kills per NK.

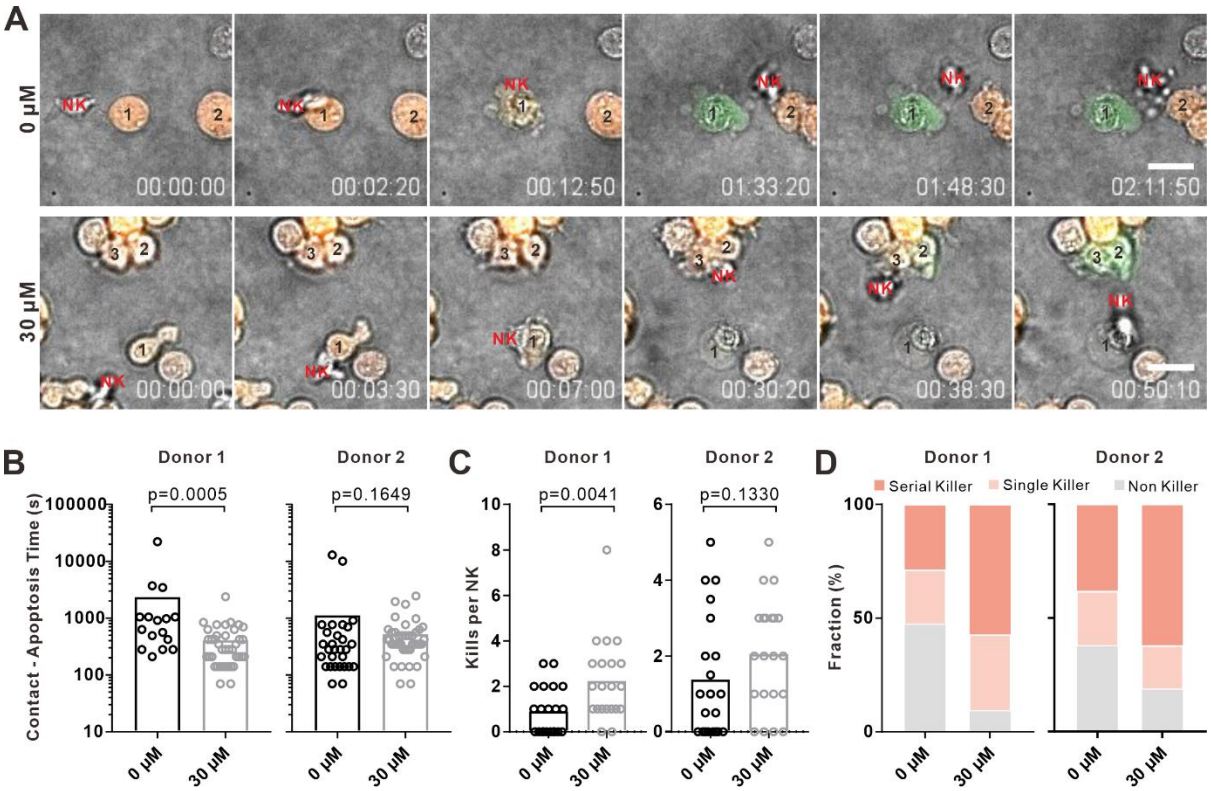

Table 1. Scores of docking and the interactive residues

Receptor protein	Ligand name	CDOCKER Interaction Energy	Interactivate residues
NKG2A	Acteoside	N/A	
	Apigenin	-28.5588	GLN113、ILE168、SER77
	Kaempferol	-54.9669	GLN112、ARG171
	Verbenalin	-29.2176	GLN112
	Hastatoside	N/A	
KIR2DL1	Acteonid	-52,7557	ASP98, ALA145, ARG149, GLU147, HIS146, PRO16, PRO185
	Apigenin	-29,0074	ALA74, ASP183, GLY43, MET44, SER184
	Kaempferol	-24,799	GLU187, GLY43, MET44, PRO185
	Verbenalin	-45,1206	ALA145, ARG149, ASP98, GLU147, HIS138, HIS146, TRP188, TYR186
	Hastatoside	-53,0989	ALA145, ARG149, GLU147, HIS146