Biochemical and Biophysical Research Communications 708 (2024) 149763

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

DT-13 attenuates inflammation by inhibiting NLRP3-inflammasome related genes in RAW264.7 macrophages

Shikha Raina [a], Emely Hübner [a,b,c], Esther Samuel [a], Gregor Nagel [a], Hendrik Fuchs [a,*]

[a] Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Institute of Diagnostic Laboratory Medicine, Clinical Chemistry and Pathobiochemistry, Augustenburger Platz 1, D-13353, Berlin, Germany
[b] Hochschule Bonn-Rhein Sieg, 53359, Rheinbach, Germany
[c] HAN University of Applied Sciences, Groenewoudseweg, 6524, Nijmegen, Netherlands

ARTICLE INFO

Keywords:
Steroidal saponin
Anti-inflammatory effects
NLRP3 inflammasome
NFκB pathway

ABSTRACT

Plant derived saponins or other glycosides are widely used for their anti-inflammatory, antioxidant, and anti-viral properties in therapeutic medicine. In this study, we focus on understanding the function of the less known steroidal saponin from the roots of *Liriope muscari* L. H. Bailey – saponin C (also known as DT-13) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages in comparison to the well-known saponin ginsenoside Rk1 and anti-inflammatory drug dexamethasone. We proved that DT-13 reduces LPS-induced inflammation by inhibiting nitric oxide (NO) production, interleukin-6 (IL-6) release, cycloxygenase-2 (COX-2), tumour necrosis factor-alpha (TNF-α) gene expression, and nuclear factor kappa-B (NFκB) translocation into the nucleus. It also inhibits the inflammasome component NOD-like receptor family pyrin domain containing protein 3 (NLRP3) regulating the inflammasome activation. This was supported by the significant inhibition of caspase-1 and interleukin-1 beta (IL-1β) expression and release. This study demonstrates the anti-inflammatory effect of saponins on LPS-stimulated macrophages. For the first time, an *in vitro* study shows the attenuating effect of DT-13 on NLRP3-inflammasome activation. In comparison to the existing anti-inflammatory drug, dexamethasone, and triterpenoid saponin Rk1, DT-13 more efficiently inhibits inflammation in the applied cell culture model. Therefore, DT-13 may serve as a lead compound for the development of new more effective anti-inflammatory drugs with minimised side effects.

1. Introduction

Inflammation is a consequence of a series of events occurring in the body as a defence mechanism against impaired cells, pathogens, and other unfavourable external stimuli. Generally, the body has various physiological barriers that form the innate immune system leading to activation of immune cells like macrophages, and further activating the adaptive immune cells like antibodies and T-cells, up against the stimuli. But in case of its ineffectiveness, the inflammation persists and leads to even more detrimental chronic inflammation that underlies various diseases for example, Alzheimer's disease, cancer, cardiovascular disease, and type 2 diabetes [1–4].

Inflammation occurs through various pathways, but one transcription factor is the most common in all pathways, namely, nuclear factor kappa B (p65 or NFκB). Various external factors like tumour necrosis factor-α (TNF-α) or lipopolysaccharide (LPS) lead to the activation of the NFκB pathway. In the LPS-induced inflammatory pathway, LPS binds to toll-like receptor-4 (TLR4) and results in the dimerization of the receptor [5]. This in turn, leads to the activation and phosphorylation of NFκB. The activated NFκB translocates into the nucleus and initiates the expression of various pro-inflammatory genes, e.g., cyclooxygenase 2 (COX-2), TNF-α, interleukin 6 (IL-6), and inducible nitric oxide (iNOS) [6]. This marks the initiation and activation of parallel pathways that in concert cause inflammation. There are already drugs on the market, which have an inhibitory effect on cytokines like COX-2, interleukin-1 beta (IL-1β) or NFκB but the side effects of strong individual inhibition of these molecules are apparently abundant, highlighting the need to develop pan-inhibitors that act broadly but do not completely inhibit required individual pathways [7].

Saponins are glycosides occurring as amphiphilic secondary metabolites in plants and are present in almost all the parts including bark, roots, and fruits protecting them from metabolic stress and damage by

* Corresponding author.
E-mail address: hendrik.fuchs@charite.de (H. Fuchs).

https://doi.org/10.1016/j.bbrc.2024.149763
Received 5 December 2023; Received in revised form 4 March 2024; Accepted 6 March 2024
Available online 13 March 2024

herbivores [8]. The class of saponins is well studied and known for exceptional anti-inflammatory [9–11], antioxidant, and adjuvant properties [12,13]. Some members have already been used in ancient medicinal practices like ashwagandha [14], curcumin [15], Panax Ginseng C. A. Mey [16], among others. Therefore, it is important to have a comparative study with different pathways and key mediators of specific inflammatory diseases to ensure an effective drug molecule design. Structurally, saponins are made of both a hydrophobic aglycone part and a hydrophilic glycosidic part, which upon acid hydrolysis liberates sapogenins [17,18], the otherwise widely studied component of saponins. The sapogenin component can be categorised into two different types, namely, steroid or triterpenoid. It has been observed in various studies that the number of sugar residues and modifications of sugar residues within the same type of saponins affect the functional properties of the whole molecule [19,20]. It is therefore important to explore the structurally different saponins, to have a better understanding of the functioning and efficacy of the molecule.

Ginsenosides, a well-known group of saponins, are known to inhibit the inflammasome pathway and thereby, chronic inflammatory progression to cell death [21,22]. Inflammasome formation and pyroptosis (cell death) are part of the inflammatory response [23] and are thereby interlined with the pathways involved in inflammation. Inflammasomes are categorised into four different types based on the active component, but NLRP3 inflammasome is the one extensively studied in relation to inflammatory disease. NLRP3 inflammasome formation is regulated at two steps, namely priming and activation. The priming step is induced by various pathogen associated molecular patterns (PAMPs) like LPS recognised by pattern recognising receptors (PRRs) such as toll-like receptor, or by cytokines like TNF-α [24], via activation of NFκB gene and in turn regulating the NLRP3 and IL-1β gene expression [25]. The second activation step can occur due to mitochondrial reactive oxygen species (ROS), adenosine triphosphates (ATP), LPS or PAMPs and external irritants released by impaired cells that activate caspase-1 and form activated inflammasomes. Mutations in the NLRP3 gene has been found in many auto-inflammatory diseases [26,27], emphasising the need for and importance of generating NLRP3-targeted drug therapies.

Very few literature studies have shown that DT-13 exhibits anti-inflammatory properties and function via inhibiting NFκB [9,28]. DT-13 is also observed to have anti-cancer [29,30], anti-tumour [31,32], and anti-thrombosis [33] properties in different disease models. In this study, we want to explore the effective mechanism of DT-13 against LPS-stimulated inflammation-derived inflammasomes. Our aim is to have a comparative analysis of two structurally different saponins, DT-13 and Rk1 (steroidal and triterpenoid backbone respectively), and the commercially available synthetic drug dexamethasone with regard to anti-inflammatory efficiency and mode of action. The *in vitro* analysis in LPS-stimulated macrophages is suitable to provide a general inflammation model system to compare drug candidates and aid in an effective drug molecule design.

2. Results

2.1. DT-13 inhibits NO release in a dose dependent manner

The LPS-stimulated macrophages express inducible nitric oxide synthase (iNOS), which in turn generates nitric oxide (NO). The validity of this *in vitro* inflammation model system is checked by determining the iNOS levels with increasing concentration of LPS (Supplemental Fig. S1). We also assessed the cytotoxicity of all investigated anti-inflammatory compounds and found no or solely minimal effects on the metabolic activity of LPS-stimulated RAW264.7 cells in the presence of Rk1, DT-13 or dexamethasone in the tested concentration range (Supplemental Fig. S2).

To check the effect of saponins on NO release, macrophages were pre-treated with either DT-13 or Rk1 (5, 10, 20 μM) for 1 h and stimulated with LPS (10 ng/mL) for a total of 19 h (Fig. 1). In both, Rk1 and DT-13 treated cells, NO production was significantly reduced compared to the cells treated only with LPS (considered as 100%). Moreover, saponins alone did not show any increased NO release at the maximal concentration of 20 μM indicating that saponins do not have influence on NO production or activation of macrophages.

2.2. Inhibitory effect of DT-13 on pro-inflammatory cytokines in comparison to Rk1 and dexamethasone

The LPS-activated macrophages mediate the inflammatory signals by the expression and release of pro-inflammatory cytokines, e.g., TNF-α, IL-6, COX-2. The inhibition of cyclooxygenases has been specifically exploited in screening for efficient non-steroidal anti-inflammatory drugs like acetylsalicylic acid, ibuprofen, dexamethasone, and others. In our experiments, both DT-13 and Rk1 inhibited LPS-induced IL-6 cytokine release from the cells in a dose dependent manner. Considering the 20 μM-concentration as most effective (and non-toxic), we observed significant inhibition of TNF-α, IL-6, and COX-2 expression in LPS-stimulated RAW264.7 cells when pre-treated with Rk1, DT-13, or dexamethasone (Fig. 2). We saw that DT-13 has an inhibitory efficiency of 90% for IL-6 and 85% for TNF-α compared to the acclaimed anti-inflammatory drug dexamethasone with 24% and 15% for IL-6 and TNF-α, respectively, and ginseng saponin Rk1 with 78% for IL-6 (Table 1). This indicates enhanced anti-inflammatory properties of DT-13 in contrast to previously studied molecules.

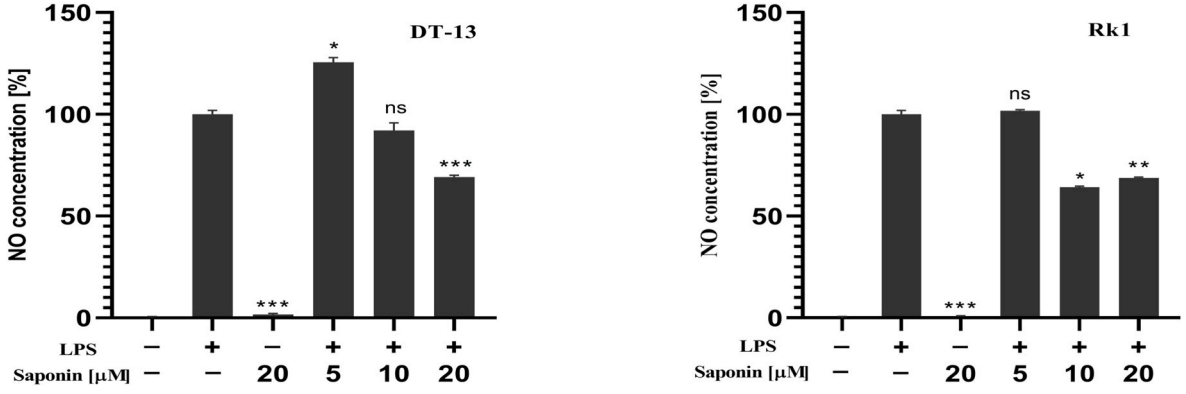

Fig. 1. NO release in LPS-stimulated RAW264.7 macrophages is reduced after pre-treatment with Rk1 and DT-13. The NO concentration was determined by Greiss reagent assay in cell culture supernatants treated with different concentrations of Rk1 or DT-13 in comparison to LPS alone. The concentrations were normalized with a relative concentration of 0% NO for unstimulated cells and 100% for cells treated with LPS alone. Data is represented as mean ± SD of three independent experiments, all of them conducted in triplicates *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 'ns' statistically not significant with respect to LPS alone.

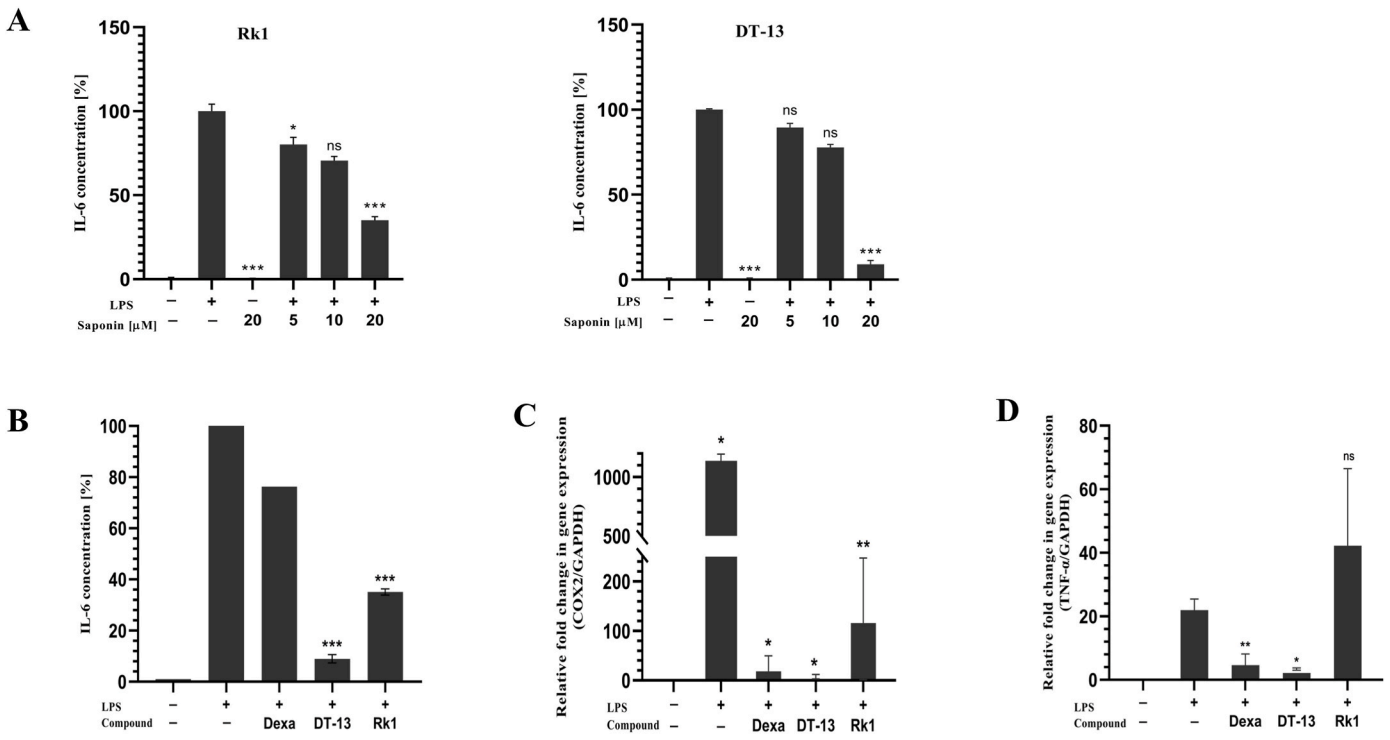

Fig. 2. DT-13 effectively inhibits pro-inflammatory cytokines in LPS-stimulated macrophages. (A) Dose dependent release of IL-6 cytokine after pre-treatment with Rk1 and DT-13 as measured by ELISA and expressed in percentage (normalized with unstimulated cells as 0% and LPS-treated cells as 100%). (B) Comparative analysis of IL-6 cytokine release after pre-treatment with dexamethasone, DT-13, and Rk1 (both 20 μM). Relative mRNA expression of (C) COX-2, and (D) TNFα. Data is represented as mean ± SD of three independent experiments *p < 0.05, **p < 0.01, ***p < 0.001, 'ns' statistically not significant with respect to LPS alone.

Table 1
Percent inhibition efficiency of DT-13, Rk1 and dexamethasone for various cytokines after LPS stimulation. Untreated samples were considered as 100% inhibition and LPS treated cells (without drug) as 0% inhibition. The average values have been derived from the individual experiments presented in this article. ns = no significant inhibition.

Markers	Inhibition Efficiency (%)		
	DT-13	Rk1	Dexamethasone
NO	69.2	68.8	–
IL-6	89.95	77.73	23.72
TNF-α	85.3	ns	14.7
COX-2	99.5	94.5	97.5
p-NFκB	31.8	19.2	26.3
NLRP3	73.5	ns	62.3
IL-1β	92.0	83.9	98.5

2.3. NFκB phosphorylation and translocation is inhibited by DT-13 pre-treatment

For the activation of gene expression, NFκB is phosphorylated (p-NFκB) and translocates to the nucleus where it serves as a transcription factor. To visualise the translocation process, we applied the immunofluorescence technique in RAW264.7 macrophages stimulated with LPS (200 ng). Within 30 min of LPS stimulation, NFκB appears as a ring like structure in the nucleus (green signal in Supplemental Fig. S3). For cells pre-treated with DT-13 for 1 h and then induced with LPS, NFκB localisation to nucleus is inhibited as implicated by the absence of the green ring-like structure inside the nucleolar region (Fig. 3 (A)). This was also quantified considering the nuclear region marked by 4,6-diamidino-2-phenylinodole (DAPI) nuclear staining (Fig. 3 (B)). We also observed that in untreated macrophages, as expected, phosphorylated NFκB as shown in green colour in Fig. 4 (A) is less and mostly visible in the cytoplasmic region whereas in LPS stimulated cells, the green signal of phosphorylated NFκB was comparatively high in the nuclear region (observed as a blue-green signal in merged LPS only image). In cells pre-treated with either dexamethasone, DT-13 or Rk1, NFκB phosphorylation was reduced in contrast to cells treated with LPS alone and the cells appeared more like the untreated cells.

2.4. DT-13 pre-treatment inhibits LPS-induced inflammasome activation

As mentioned earlier, with inefficient translocation and activation of NFκB, various gene expressions are affected. NLRP3-inflammasome related genes are among those. NLRP3-inflammasomes directly activate caspase-1 and indirectly IL-1β that eventually leads to formation of membrane attack complex (MAC) and pyroptosis. We observed that LPS induces an increase in NLRP3 gene expression and the pre-treatment with DT-13 reduces this expression significantly (Fig. 5 (A)). A similar inhibitory trend was observed for IL-1β gene expression (Fig. 5 (B)). This effect was stronger than for Rk1, which is already known to inhibit the inflammasome pathway. Dexamethasone is also known to inhibit NLRP3 inflammasome activation [34]. In comparison to DT-13, we observed similar inhibition for NLRP3 gene expression and slightly more pronounced attenuation of IL-1β gene expression for dexamethasone. The confirmatory test for the inflammasome inhibition was done by analysing the activity of the NLRP3-inflammasome triggered caspase-1 release by Caspase-Glo inflammasome. In this assay, caspase-1, an important marker of inflammasome activation is measured with the help of a specific substrate that detects catalytically active caspase-1 in cell culture mediums. We observed a significant reduction in caspase-1 activity in cells pre-treated with DT-13 and Rk1 (Fig. 5 (D)). To confirm the caspase catalytic activity, we measured the IL-1β release from cells by ELISA. DT-13 inhibited the release of IL-1β from LPS stimulated cells in a dose dependent manner (Fig. 5 (C)). We also conducted an ELISA based assay to understand the role of the two saponins in activating the complement system because it is known that complement activation in addition to inflammasomes leads to the formation of the pore complex

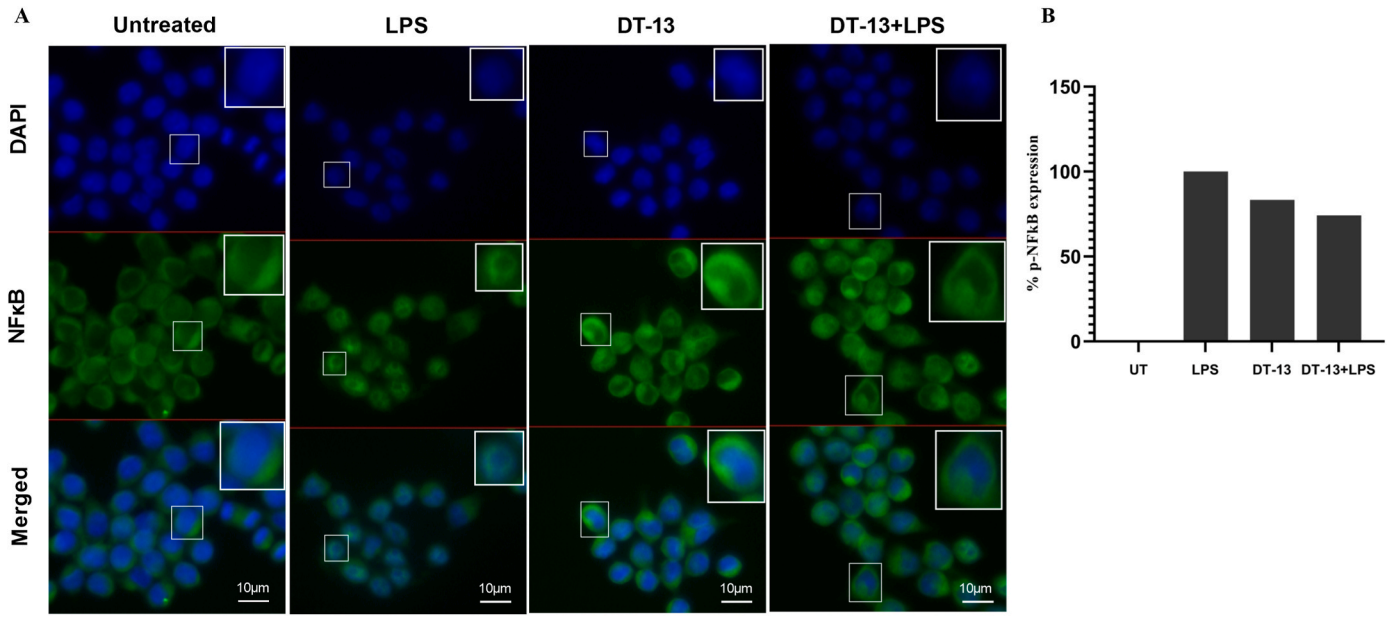

Fig. 3. DT-13 pre-treatment inhibits phosphorylation of NFκB in LPS-stimulated RAW 264.7 cells. (A) Representative images of phosphorylated NFκB in DT-13 pre-treated cells, as observed by fluorescent microscopy. (B) Image analysis using ImageJ software of expression of p-NFκB in comparison to cells treated with LPS alone (LPS alone is taken as 100% and untreated cells were normalized to 0%). Blue colour represents DAPI for nucleus staining and green colour shows FITC-labelled antibody directed against NFκB protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

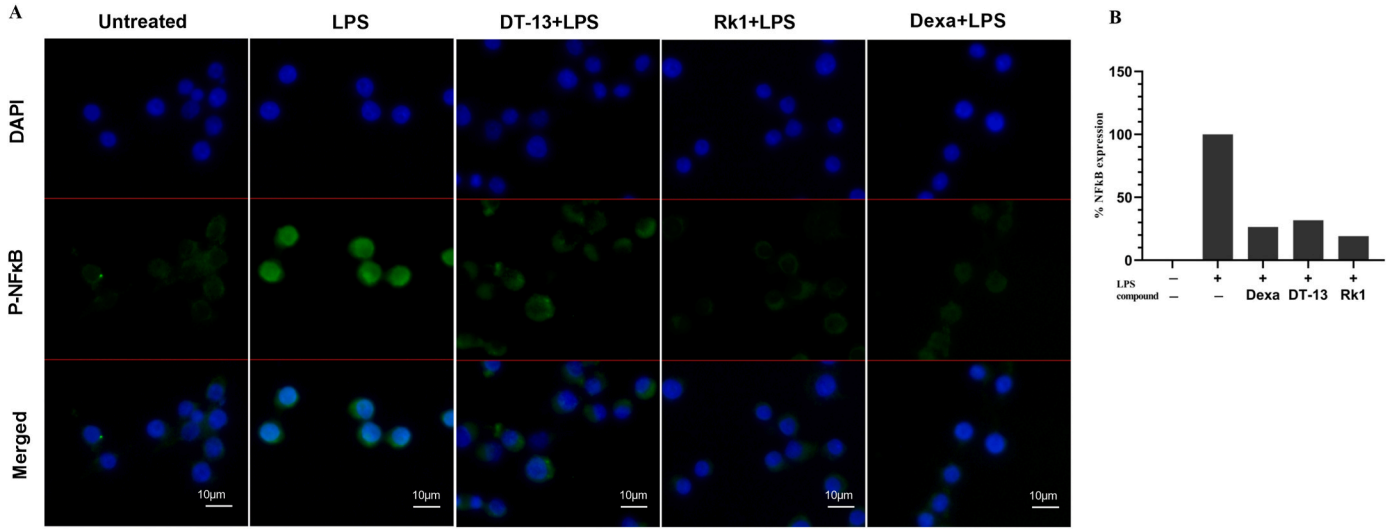

Fig. 4. Pre-treatment of Rk1, DT-13 and dexamethasone inhibits NFκB translocation into the nucleus (A) Representative fluorescent images of NFκB expression in the nucleus after treatment with LPS with or without pre-treatments. (B) Quantitation of the nuclear localisation of the NFκB using ImageJ software (LPS alone is taken as 100% and untreated cells were normalized to 0%). Blue colour represents DAPI for nucleus staining and green colour shows FITC-labelled antibody against NFκB protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

called as membrane attack complex consisting of C5b-9 proteins. The amount of C5b-9 protein measured in the assay corresponds to the complement activation and thereby the terminal pore complex formation. In the assay, the serum containing the positive activator of the complement system was taken as 100% activation. We observed that in sera incubated with the saponins, the activation of the pathway was inhibited in a dose dependent manner for both DT-13 and Rk1 (Fig. 5 (E)).

3. Discussion

On the one hand, non-steroidal anti-inflammatory drugs (NSAIDs), or COX-2 inhibitors, are widely used in various disease treatments but accompanied by several side effects. On the other hand, saponins, used in ancient medicinal practices are well-studied molecules in various disease pathophysiologies [27] and are commercially available as diet supplements for example ginseng. Most of the research regarding the functional mechanism of plant saponins is usually done with high concentrations of crude plant extracts containing various isoforms of the saponins; hence, lacking clear evidence of the functional significance of the individual active compounds [35,36]. Triterpenoid saponins are most widely studied and known to have diverse beneficiary effect on our body whereas steroidal saponins have recently gained attention in the research field of immunomodulatory effects [10,37]. Passo et al. summarised 45 articles published on steroidal saponins and emphasised the necessity of more detailed pre-clinical research on single molecular

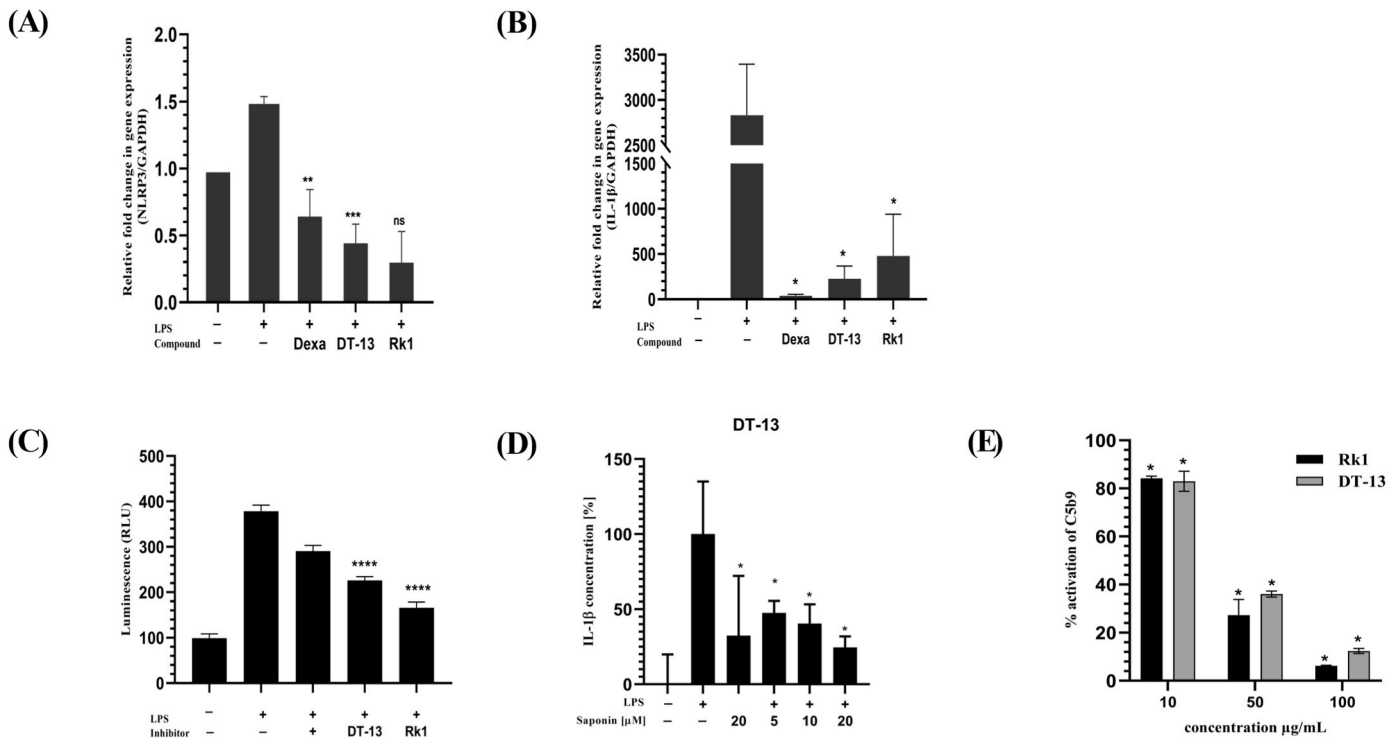

Fig. 5. LPS induced NLRP3-inflammasome priming inhibited by DT-13 pre-treatment. mRNA expression analysis of (A) NLRP3, (B) IL-1β, and (C) caspase-1 activity in response to pre-treatments with DT-13 or Rk1, respectively, (D) IL-1β release with increasing concentration of DT-13 pretreatment as measured by ELISA, (E) Complement Alternate Pathway (AP) assay for the detection of MAC complex specific protein C5b-9 in the presence of Rk1 and DT-13. Data is represented as mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 'ns' statistically not significant with respect to LPS alone).

structures [10]. Therefore, we looked at the functional role of DT-13 in comparison to the already known anti-inflammatory compounds Rk1 ginsenoside and dexamethasone to enhance the existing knowledge about the less known steroidal saponin.

In the present study, we used non-toxic concentrations of compounds as confirmed by MTT assay and pre-treated LPS-activated macrophages to evaluate the release of NO from the cells first. The release of NO by activated macrophages is known to play an important role in innate immunity by regulating the cell signalling [38]. Otherwise, over-production of NO is associated with chronic inflammation and tissue damage [39]. Therefore, it is the first and important pro-inflammatory mediator to be analysed while screening for anti-inflammatory drugs since 1985 [40,41]. Our results confirmed the previous findings that saponin pre-treatment of LPS-stimulated cells lead to inhibition of NO release in a dose dependent manner (Fig. 1). However, we observed an increase in NO production at the initial concentration of 5 μM of DT-13 and Rk1 in LPS-stimulated cells that might be due to the ineffectiveness of the saponins below a certain threshold concentration and an additional increase in stress in presence of LPS.

Once the macrophages are activated by the LPS stimulation, all the pro-inflammatory cytokines are expressed and released out of the cells as a response to the induced inflammation. NFκB plays the key role in mediating the cross talk between those signalling pathways [42,43]. Upon activation and phosphorylation, NFκB translocates into the nucleus and mediates the transcription of pro-inflammatory genes, e.g., TNF-α, IL-6, COX-2, and IL-1β. According to our results, NFκB translocation was inhibited by pre-treatment with DT-13 proved to be more efficient in inhibiting some pro-inflammatory cytokines (IL-6, TNF-α) in comparison to Rk1 and dexamethasone. Additionally, we found that DT-13 has very similar (99.5%) inhibitory effect on COX-2 levels compared to dexamethasone (97.5%) at the same concentration. This is an intriguing result as COX-2 is the main target for dexamethasone. It can be noted that we tested only RAW macrophages as a simple inflammation model system but saponins' effect can be cell- or tissue-specific. For example, Rk1 is reported to have contrasting apoptotic effects on tumour cells, which is in line with its anti-tumour functional role [37,38]. Therefore, further studies on other cell and tissue models are required.

It is known that non-steroidal anti-inflammatory drugs reduce inflammasome formation and thereby exhibit their anti-inflammatory function [44]. Rk1 and dexamethasone are also implicated in inhibiting inflammasome formation [34]. Additionally, PAMPs like LPS primes the macrophages for inflammasome formation and in some cases are also mediating activation due to intrinsic ROS production [45]. This is marked by increased NLRP3 and IL-1β gene expression. Therefore, we checked the role of DT-13 in inflammasome, and it was interesting to observe that DT-13 significantly inhibited NLRP3-inflammasome related genes – NLRP3 and IL-1β. DT-13 and dexamethasone inhibited NLRP3 significantly by 73% and 62% respectively whereas IL-1β was reduced by 92% with DT-13, 84% with Rk1 and 98% with dexamethasone treatment. Caspase-1 is the major protein that upon activation along with NLRP3 forms the activated inflammasome complex that is involved in the release of IL-1β and pyroptosis via formation of a pore complex also called MAC complex. To analyse the levels of active caspase-1 we performed Caspase-Glo inflammasome assay from Promega. DT-13 significantly downregulated the activated caspase-1 release in LPS-stimulated cells indicating the inhibition of inflammasome activation. This was also confirmed by performing an ELISA to analyse the release of IL-1β from the cells. A significant dose dependent decrease in IL-1β levels were seen with DT-13 pretreatment. Our aim was only to investigate the possibility of DT-13 involved in inflammasome activation in comparison to Rk1 and dexamethasone. Nevertheless, more research can be done to confirm the mechanism followed by DT-13 to prevent inflammasome formation. The present study implies that DT-13, via NFκB inhibition, forms the link between inflammation and inflammasome cross talk pathways by regulating the NLRP3 and IL-1β gene expression. Supporting the role of DT-13 for inflammasomes, with the help of an ELISA-based complement assay, it was observed that DT-13

inhibits the protein C5b-9 activation that plays a role in the formation of pore complexes in the cells in response to inflammasome activation and thereby inhibiting the cell death.

Considering the amount of time and expenditure required to perform in vivo studies, we could not analyse and validate our *in vitro* results in mice. However, various studies *in vitro* and *in vivo* have shown that steroidal saponins [46–48] and triterpene saponins [49–51] are involved in the inhibition of NLRP3 inflammasomes. Although not proven, these studies make it appear likely that the effect we observed here also occurs *in vivo*. However, whether the effect is also stronger *in vivo* than with other saponins must be shown experimentally. This study enumerates the comparable data of two structurally different saponins and their inhibition efficiency in an inflammatory *in vitro* model with respect to the already marketed anti-inflammatory drug dexamethasone as summarised in Table 1.

We observed that DT-13 significantly reduces the expression of NLRP3, IL-1β-release, active caspase-1, and MAC complex formation in addition to NFκB and pro-inflammatory cytokines TNF-α, COX-2, and IL-6. We hypothesised that DT-13 inhibits LPS-induced NFκB activation and its translocation to the nucleus. As activated NFκB acts as a transcription factor in the nucleus for the expression of cytokines and NLRP3 genes [52], its attenuation in the cytosol leads to suppression of gene activity related to inflammation and inflammasome formation. Additionally, caspase-1 activity and release of IL-1β from cells is also reduced by DT-13 pretreatments in the experimental model. Therefore, our results concluded that DT-13 suppresses inflammasome formation by inhibiting the NLRP3 gene via regulating LPS-induced NFκB pathway in addition to suppressed release of caspase-1 and IL-1β from RAW264.7 cells. This study therefore elucidated the role of DT-13 as an inhibitor of inflammasome priming via inhibition of NFκB-mediated inflammatory pathway. This is, indeed, significant regarding the efficient mechanism of how DT-13 overcomes the LPS-induced inflammation leading to inflammasome formation and cell death by pyroptosis.

This is the first study to report the effect of DT-13 on NLRP3-inflammasome inhibition in RAW264.7 macrophages and the fact that the saponins, DT-13 and Rk1, are functionally active at otherwise non-toxic concentrations. All the observations signify that DT-13 is involved in multiple pathways and acting as a pan-inhibitor rather than a specific target in the cell. The study can be valuable for introduction of anti-inflammatory NLRP3-target specific compounds for future *in vivo* and pre-clinical studies.

4. Materials and methods

4.1. Chemicals

Ginsenoside Rk1 (Merck, Darmstadt, Germany), *Liriope muscari* L. H. Bailey saponin C (Biosynth-Carbosynth, Staad, Switzerland), dexamethasone (Fisher Scientific, Waltham, US), dimethyl sulfoxide (DMSO) (Sigma, St. Louis, US), lipopolysaccharide from *Escherichia coli* O111:B4 (Sigma, St. Louis, US), Gibco's Dulbecco's modified eagle medium (DMEM), 0.25% trypsin-EDTA, fetal calf serum (FCS) (Thermo Fischer Scientific, Schwerte, Germany), penicillin-streptomycin (Merck, Darmstadt, Germany).

4.2. Cell culture

RAW264.7 mouse macrophages obtained from ATCC (Manassas, VA, USA) were grown at 37 °C in DMEM medium with 10% FCS and 1% penicillin-streptomycin. For treatments, cells were grown overnight at a density of 10^5 cells in complete growth medium in a 6-well plate. Cells were pre-treated for 1 h with saponins at specified concentrations followed by stimulation with LPS (10 ng/ml) for a total of 19 h. The cell pellets were used for protein and RNA isolation and the supernatants were used for ELISA and NO assay.

4.3. MTT assay

RAW264.7 macrophages were seeded in a 96-well plate at a density of 10^5 cells per well in complete growth medium and kept overnight at 37 °C, 5% CO_2. Various concentration of the saponins were added to check the cytotoxicity caused by the saponins alone and in the presence of LPS over a period of 24 h. Post incubation, 50 μL of 5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, US) was added in each well and was incubated for 4 h at 37 °C. The solution was removed later as the purple-coloured crystals of formazan were seen and dissolved in 50 μL formazan solubiliser solution per well followed by absorbance measured at 560 nm using a spectrophotometer – Spectramax 340 PC (SJ, California, US). Percent survivability is calculated by comparing the sample type with untreated and LPS alone.

4.4. NO assay

Cell supernatant was used from the experimental setup mentioned earlier for the analysis post incubation. Modified Greiss Reagent was used to analyse the nitric oxide concentration. 10 g of Greiss reagent modified (Sigma, St. louis, US) was dissolved in 250 mL of deionised water and 50 μL was added to the samples. After addition of Greiss reagent, the samples were incubated in dark for 15 min and then absorbance was measured at 540 nm using the spectrophotometer. Sodium nitrite was used as a standard reference.

4.5. ELISA

The macrophages were treated with the compounds and LPS as described earlier. The release of cytokine IL-6, IL-1β and TNF-α was determined as per manufacturer's protocol using ELISA Max kits (Biolegend, San Diego, California, US).

4.6. RNA isolation and qPCR

RNA was isolated using RNA isolation kit (Macherey- Nagel, Duren, Germany) followed by reverse transcription using MMLV reverse transcriptase (Promega, Mannheim, Germany) into cDNA. Real-time PCR was performed to analyse the expression of iNOS, COX-2, TNF-α, NLRP3, IL-1β, and GAPDH (for primer sequences refer to Supplemental Table 1) using the GoTaq qPCR master mix (Promega, Mannheim, Germany) and a thermal cycler Stratagene, Mx3000P (Agilent technologies, SC, US).

4.7. Immunofluorescence

RAW264.7 macrophages were seeded on a coverslip in a 6-well plate at a density of 10^5 cells per well and kept at 37 °C for overnight. Cells were pre-treated with saponins for 1 h followed by LPS (200 ng/ml) for 30 min. Primary antibody (1:500) incubated overnight at 4 °C. Goat anti-rabbit FITC labelled secondary antibody (1:2000) was added for 1.5 h at room temperature followed by the nucleus staining with DAPI for 10 min in dark. The coverslips were mounted on the glass cover slides using Shandon Immu-Mount (Thermo Scientific, Schwerte, Germany) and observed under fluorescence microscope Zeiss Axio Observer Z1 (Carl Zeiss, Jena, Germany).

4.8. Caspase-1 Glo inflammasome assay

Macrophages were seeded in a 6-well plate at a density of 6×10^6 in complete media and pre-treated with saponins followed by LPS stimulation. The assay was performed using the cell supernatants after 19 h of incubation and following the manufacturer's protocol from the Caspase-Glo 1 Inflammasome Assay (Promega, Mannheim, Germany).

4.9. Complement assay

Complement system alternative pathway was measured using Wieslab® complement system (Weislab, Malmö, Sweden), an ELISA-based assay wherein the pre-coated strips with specific activator are provided. This pathway was used to understand how saponins affect the activation of the complement system. Saponins were diluted in the serum provided with the kit in described concentration and incubated for 1 h at 37 °C onto the coated microtiter strips. The activation of the pathway was measured by the detection of C5b-9, which is formed during MAC formation, with an alkaline phosphatase labelled antibody. The assay was analysed according to the kit's protocol.

4.10. Statistical test

Statistical analysis was done using GraphPad Prism 9. Wilcoxon test was used to analyse the ELISA and NO assay. Non-parametric Student t-test with Welch's correction was used for others. Samples were compared to the LPS-alone samples after normalisation with the unstimulated control.

ANOVA one way analysis was done within the samples and was found to be significant for all experiments. The number of replicates and graphical representation is mentioned separately in figure legends.

Funding

This research was supported by the Deutsche Forschungsgemeinschaft (DFG) (Grant no: FU 408/11-1).

CRediT authorship contribution statement

Shikha Raina: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Emely Hübner:** Investigation, Formal analysis. **Esther Samuel:** Investigation, Formal analysis. **Gregor Nagel:** Writing – review & editing. **Hendrik Fuchs:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hendrik Fuchs reports financial support was provided by German Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors like to acknowledge Dr. Jens Dernedde and Marten Kagelmacher for their assistance and invaluable comments.

List of abbreviations

ATP	Adenosine triphosphate
COX-2	Cyclooxygenases-2
DAPI	4,6-diamidino-2-phenylinodole
ELISA	Enzyme linked immunosorbent assay
IKB	I kappa B
IL-1β	Interleukin beta-1
IL-6	Interleukin-6
iNOS	Inducible nitric oxide
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	Nuclear factor kappa B
NLRP3	Nod, -LRR and Pyrin domain containing protein 3
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
PAMPs	Pathogen associated molecular patterns
ROS	Reactive oxygen species
TLR4	Toll-like receptor-4
TNF-α	Tumour necrosis factor alpha

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2024.149763.

References

[1] D. Foell, H. Wittkowski, J. Roth, Mechanisms of disease: a 'DAMP' view of inflammatory arthritis, Nat. Clin. Pract. Rheumatol. 3 (7) (2007) 382–390.
[2] F.L. Heppner, R.M. Ransohoff, B. Becher, Immune attack: the role of inflammation in Alzheimer disease, Nat. Rev. Neurosci. 16 (6) (2015) 358–372.
[3] N.G. Cruz, et al., The linkage between inflammation and Type 2 diabetes mellitus, Diabetes Res. Clin. Pract. 99 (2) (2013) 85–92.
[4] L.M. Coussens, Z. Werb, Inflammation and cancer, Nature 420 (6917) (2002) 860–867.
[5] B.S. Park, J.O. Lee, Recognition of lipopolysaccharide pattern by TLR4 complexes, Exp. Mol. Med. 45 (2013) e66.
[6] A.V. Bagaev, et al., Elevated pre-activation basal level of nuclear NF-kappaB in native macrophages accelerates LPS-induced translocation of cytosolic NF-kappaB into the cell nucleus, Sci. Rep. 9 (1) (2019) 4563.
[7] J.N. Sharma, N.M. Jawad, Adverse effects of COX-2 inhibitors, Sci. World J. 5 (2005) 629–645.
[8] A.E. Osbourn, Preformed antimicrobial compounds and plant defense against fungal attack, Plant Cell 8 (10) (1996) 1821–1831.
[9] G.J. Khan, et al., Pharmacological effects and potential therapeutic targets of DT-13, Biomed. Pharmacother. 97 (2018) 255–263.
[10] F.R.S. Passos, et al., Anti-inflammatory and modulatory effects of steroidal saponins and sapogenins on cytokines: a review of pre-clinical research, Phytomedicine 96 (2022) 153842.
[11] J. Zhu, et al., Saikosaponin A mediates the inflammatory response by inhibiting the MAPK and NF-kappaB pathways in LPS-stimulated RAW 264.7 cells, Exp. Ther. Med. 5 (5) (2013) 1345–1350.
[12] H.X. Sun, Y. Xie, Y.P. Ye, Advances in saponin-based adjuvants, Vaccine 27 (12) (2009) 1787–1796.
[13] S. Dong, et al., Emerging role of natural products in cancer immunotherapy, Acta Pharm. Sin. B 12 (3) (2022) 1163–1185.
[14] A. Sikandan, T. Shinomiya, Y. Nagahara, Ashwagandha root extract exerts antiinflammatory effects in HaCaT cells by inhibiting the MAPK/NFkappaB pathways and by regulating cytokines, Int. J. Mol. Med. 42 (1) (2018) 425–434.
[15] Y. Peng, et al., Anti-inflammatory effects of curcumin in the inflammatory diseases: status, limitations and countermeasures, Drug Des. Dev. Ther. 15 (2021) 4503–4525.
[16] D.C. Lee, A.S. Lau, Effects of Panax ginseng on tumor necrosis factor-alpha-mediated inflammation: a mini-review, Molecules 16 (4) (2011) 2802–2816.
[17] J. Love, C.R. Simons, Acid hydrolysis of saponins extracted in tincture, PLoS One 15 (12) (2020) e0244654.
[18] T. Herrera, et al., Acid hydrolysis of saponin-rich extracts of quinoa, lentil, fenugreek and soybean to yield sapogenin-rich extracts and other bioactive compounds, J. Sci. Food Agric. 99 (6) (2019) 3157–3167.
[19] K. Mizutani, et al., Sweetness of glycyrrhetic acid 3-O-β-d-Monoglucuronide and the related glycosides, Biosci., Biotechnol., Biochem. 58 (3) (1994) 554–555.
[20] W. Dong, et al., Inhibition of influenza A virus infection by ginsenosides, PLoS One 12 (2) (2017) e0171936.
[21] Y. Shi, et al., Ginsenoside Rg3 suppresses the NLRP3 inflammasome activation through inhibition of its assembly, Faseb. J. 34 (1) (2020) 208–221.
[22] W. Song, et al., Protective effect of ginsenoside metabolite compound K against diabetic nephropathy by inhibiting NLRP3 inflammasome activation and NF-kappaB/p38 signaling pathway in high-fat diet/streptozotocin-induced diabetic mice, Int. Immunopharm. 63 (2018) 227–238.
[23] Z. Wang, et al., NLRP3 inflammasome and inflammatory diseases, Oxid. Med. Cell. Longev. 2020 (2020) 4063562.
[24] L. Franchi, T. Eigenbrod, G. Nunez, Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation, J. Immunol. 183 (2) (2009) 792–796.
[25] F.G. Bauernfeind, et al., Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression, J. Immunol. 183 (2) (2009) 787–791.
[26] H. Nakanishi, et al., NLRP3 mutation and cochlear autoinflammation cause syndromic and nonsyndromic hearing loss DFNA34 responsive to anakinra therapy, Proc. Natl. Acad. Sci. U.S.A. 114 (37) (2017) E7766–E7775.

[27] G. Meng, et al., A mutation in the Nlrp3 gene causing inflammasome hyperactivation potentiates Th17 cell-dominant immune responses, Immunity 30 (6) (2009) 860–874.
[28] R. Fan, et al., DT-13 ameliorates TNF-alpha-induced nitric oxide production in the endothelium in vivo and in vitro, Biochem. Biophys. Res. Commun. 495 (1) (2018) 1175–1181.
[29] H. Li, et al., DT-13, a saponin monomer of dwarf lilyturf tuber, induces autophagy and potentiates anti-cancer effect of nutrient deprivation, Eur. J. Pharmacol. 781 (2016) 164–172.
[30] Y.W. Li, et al., Novel cytotoxic steroidal glycosides from the roots of Liriope muscari, Chin. J. Nat. Med. 13 (6) (2015) 461–466.
[31] C. Wang, et al., DT-13 induced apoptosis and promoted differentiation of acute myeloid leukemia cells by activating AMPK-KLF2 pathway, Pharmacol. Res. 158 (2020) 104864.
[32] X. Wei, et al., DT-13 inhibited the proliferation of colorectal cancer via glycolytic metabolism and AMPK/mTOR signaling pathway, Phytomedicine 54 (2019) 120–131.
[33] Y. Tian, et al., Anti-thrombotic activity of DT-13, a saponin isolated from the root tuber of Liriope muscari, Indian J. Pharmacol. 45 (3) (2013) 283–285.
[34] J.W. Yang, et al., Corticosteroids alleviate lipopolysaccharide-induced inflammation and lung injury via inhibiting NLRP3-inflammasome activation, J. Cell Mol. Med. 24 (21) (2020) 12716–12725.
[35] J.S. Park, et al., Anti-inflammatory mechanism of ginseng saponins in activated microglia, J. Neuroimmunol. 209 (1–2) (2009) 40–49.
[36] J.O. Lee, et al., Korean Red Ginseng saponin fraction exerts anti-inflammatory effects by targeting the NF-kappaB and AP-1 pathways, J. Ginseng. Res. 46 (3) (2022) 489–495.
[37] J. Harmatha, et al., Spirostanol saponins from flowers of allium porrum and related compounds indicating cytotoxic activity and affecting nitric oxide production inhibitory effect in peritoneal macrophages, Molecules 26 (21) (2021).
[38] C. Bogdan, M. Rollinghoff, A. Diefenbach, The role of nitric oxide in innate immunity, Immunol. Rev. 173 (2000) 17–26.
[39] J.N. Sharma, A. Al-Omran, S.S. Parvathy, Role of nitric oxide in inflammatory diseases, Inflammopharmacology 15 (6) (2007) 252–259.
[40] D.J. Stuehr, M.A. Marletta, Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide, Proc. Natl. Acad. Sci. U.S.A. 82 (22) (1985) 7738–7742.
[41] R. Zamora, Y. Vodovotz, T.R. Billiar, Inducible nitric oxide synthase and inflammatory diseases, Mol. Med. 6 (5) (2000) 347–373.
[42] T. Liu, et al., NF-kappaB signaling in inflammation, Signal Transduct. Targeted Ther. 2 (2017).
[43] P.P. Tak, G.S. Firestein, NF-kappaB: a key role in inflammatory diseases, J. Clin. Invest. 107 (1) (2001) 7–11.
[44] M.J. Daniels, et al., Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer's disease in rodent models, Nat. Commun. 7 (2016) 12504.
[45] K.M. Lin, et al., IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation, Proc. Natl. Acad. Sci. U.S.A. 111 (2) (2014) 775–780.
[46] G. Cao, et al., Ruscogenin attenuates cerebral ischemia-induced blood-brain barrier dysfunction by suppressing TXNIP/NLRP3 inflammasome activation and the MAPK pathway, Int. J. Mol. Sci. 17 (9) (2016) 1418.
[47] X. Wu, et al., Dihydronortanshinone, a natural product, alleviates LPS-induced inflammatory response through NF-κB, mitochondrial ROS, and MAPK pathways, Toxicol. Appl. Pharmacol. 355 (2018) 1–8.
[48] X. Jiang, et al., Dioscin alleviates periodontitis by inhibiting NLRP3 inflammasome activation via regulation of K(+) homeostasis and mitochondrial function, Int. J. Biol. Sci. 20 (4) (2024) 1375–1388.
[49] H. Honda, et al., Isoliquiritigenin is a potent inhibitor of NLRP3 inflammasome activation and diet-induced adipose tissue inflammation, J. Leukoc. Biol. 96 (6) (2014) 1087–1100.
[50] Y. Zhu, et al., Protective effect of ginsenoside Rg5 against kidney injury via inhibition of NLRP3 inflammasome activation and the MAPK signaling pathway in high-fat diet/streptozotocin-induced diabetic mice, Pharmacol. Res. 155 (2020) 104746.
[51] H. Honda, et al., Glycyrrhizin and isoliquiritigenin suppress the LPS sensor toll-like receptor 4/MD-2 complex signaling in a different manner, J. Leukoc. Biol. 91 (6) (2012) 967–976.
[52] T. Liu, et al., NF-κB signaling in inflammation, Signal Transduct. Targeted Ther. 2 (2017) 17023.