ARTICLE

Ellagic acid and Urolithin A modulate the immune response in LPS-stimulated U937 monocytic cells and THP-1 differentiated macrophages

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Sissel Beate Rønning *[a], Vibeke Voldvik [a] , Silje Kristine Bergum [a], Kjersti Aaby [a] and Grethe Iren A. Borge [a]

Abstract

Dietary polyphenols are subjected, following ingestion, to an extensive metabolism, and the molecules that act at cellular and tissue level will be, most likely, a metabolite rather than a native polyphenol. The mechanisms behind the positive effects exerted by polyphenols are not yet completely elucidated, since most *in vitro* studies use unmetabolised polyphenols rather than the metabolites present in the body. The aim of this study was to investigate and compare the potential effect of phenolic metabolites on immune response using U937 monocyte and THP-1 macrophage cell cultures. Of the 16 metabolites tested, urolithins (Uro), and Uro A in particular, was the most potent, measured as modest increase in basal NF-κB activity and a reduction in lipopolysaccaride (LPS)-induced NF-κB activity, gene expression and secretion of pro-inflammatory cytokines. Protocatechuic acid and its metabolites reduced LPS-induced NF-κB activity, but not the IL-6 and TNF-α cytokine secretion. Interestingly, both ellagic acid and its metabolite Uro A had immunomodulating effects, although they regulated the immune response differently, and both reduced LPS-induced NF-κB activity in U937 cells. However, while Uro A dramatically reduced IL-6 and IL-10 mRNA expression, no effect could be observed with ellagic acid. In THP-1 cells, treatment with ellagic acid dramatically reduced expression of Toll-like receptor 4, while Uro A had no effect. **The dual role observed for Uro A, showing both a modest increase in basal NF-κB activity, a reduction in LPS-induced NF-κB activity, as well as reduction in LPS-induced pro-inflammatory cytokine secretion, makes this metabolite particularly interesting for further studies in animals and humans.**

[a] *Nofima AS, Osloveien 1, 1433 ÅS*
[b] *Corresponding author: sissel.ronning@nofima.no, phone: + 47 408 56 287*

Introduction

Dietary polyphenols are a group of molecules that have gained interest because of their broad range of biological activities.[1-3] Polyphenols have been implicated in the prevention of cardiovascular disease (CVD), and evidence from both epidemiological and intervention studies strongly support the notion that increased consumption of polyphenol-rich food reduces CVD risk.[4, 5] Although experimental *in vitro* studies seem to confirm the potential influence of polyphenols in CVD, to date, the specific compounds responsible for this protective effect have not been clearly identified. Polyphenols, in particular flavonoids, have been shown to have anti-inflammatory activity, both *in vitro* and *in vivo*.[2, 6-9] Among the biological properties, the anti-inflammatory activity is crucial in managing chronic inflammatory diseases. The bioactivity of polyphenols might include the inhibition of pro-inflammatory enzymes and cytokines during acute inflammation in addition to the inhibition of the transcriptional factor nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB).[3, 10, 11] It is well known that polyphenols present in foods are subjected, following ingestion, to an extensive metabolism, and the molecules that will act at cellular and tissue level will be, most likely, a metabolite rather than a native polyphenol.[12] During absorption, polyphenols are conjugated in the small intestine, and later in the liver, where methylation, sulfatation and glucuronidation take place.[13, 14]

Anthocyanins, occurring in berries and several fruits and vegetables, have been reported to have low bioavailability, typically urinary recoveries <1-2 % of the intake.[15] However, recent studies show that anthocyanins undergo extensive metabolism and catabolism, including colonic microflora degradation.[16-19] The major human metabolite of anthocyanins, protocatechuic acid, is reported to have longer retention in plasma than other phenolic compounds.[17, 20-22] In addition, a range of phenolic acids, one of them ferulic acid, have been identified in serum after ingestion of anthocyanin-rich berry extracts.[17] Hydroxycinnamic acids, a group of phenolic acids, are common dietary polyphenols found in a broad range of beverages and plant foods, such as coffee, tea, fruit, cereals and potatoes. They are often bound to plant cell constituents, that can be released by microorganisms in the human gut before uptake.[23, 24] Ellagitannins are present in some berries and nuts, including strawberry, raspberry, blackberry, pomegranate, walnuts and muscadine grapes.[25] Ellagitannins, which are hydrolysable tannins, release ellagic acid by hydrolysis. The colonic microbiota further metabolises ellagic acid to urolithins.[26, 27] Recent research of urolithins, mostly based on *in vitro* studies, but also *in vivo* studies, support their promising contribution to the health effects attributed ellagitannin-rich foods including anti-carcinogenic, antioxidant, anti-inflammatory, preservation of the gut-barrier, regulator of skeletal muscle mass, anti-obesity and antimicrobial effects.[28-33] In faeces, urolithin (Uro) A, IsoUro A and Uro B are the most common metabolites.[34] Urolithins are shown to have high bioavailability compared to their precursors, ellagitannins and ellagic acid.[25, 35, 36] Urolithins can be found in plasma and urine, where they can be detected at micromolar concentrations, such as 0.024–35 μM for Uro A glucuronide, see refs in review[36.] Urolithins are absorbed into the circulatory system mainly as glucuronide and sulfate phase II conjugates, of which Uro A glucuronide is the main conjugate found in plasma.[34,36] The conjugated derivatives can exert bioactivity, but show much lower *in vitro* activity than free urolithins.[37-43] Interestingly, upon *in vivo* inflammatory stimuli, Uro A glucuronide was recently demonstrated to be tissue deconjugated to free Uro A in rats.[44]

The mechanisms responsible for the positive effects exerted by polyphenols are not yet completely elucidated, since most *in vitro* studies use unmetabolized polyphenols rather than the metabolites present in the body. The aim of this study was to investigate and compare the potential anti-inflammatory properties of physiologically relevant plasma phenolic metabolites using two *in vitro* cell model systems mimicking biological situations commonly used for screening of bioactivity of a given metabolite; the U937 monocytic cell line and THP-1 macrophages, and to investigate the mechanism of action of the most bioactive metabolites.

Results

NF-κB activity in U937 cells

Basal and lipopolysaccharide (LPS)-induced NF-κB activity was determined in U937 monocytic cells after metabolite treatment at two concentrations (1.5 and 30 μM). The tested metabolites affected NF-κB activity in U937 cells differently (Table 1, Fig. S1). Among the urolithins tested, only Uro A significantly increased the basal NF-κB activity. Hydroxycinnamic acids and protocatechuic acids had no significant effect on the basal NF-κB activity. After LPS-stimulation, Uro A, B, C and ellagic acid significantly reduced the LPS-induced NF-κB activity at both 1.5 and 30 μM. The protocatechuic acids had a significant effect on the LPS-induced NF-κB activity at 1.5 μM, but not 30 μM. Of the tested hydroxycinnamic acids, only dihydrocaffeic acid 4-sulfate at 1.5 μM reduced the NF-κB activity in LPS-stimulated U937 cells. The amount of adenosine triphosphate (ATP) was unchanged when the phenolic metabolites were added to the cells, indicating that the U937 cells were metabolically active and that treatment with the metabolites did not modify cell viability (Fig. S2).

Cytokine secretion in THP-1 cells

Cytokine secretion of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) in LPS-stimulated THP-1 macrophages, treated with the metabolites, are shown in Table 2 and Fig. S3. Uro A and Uro B (30 μM) had significant effect on LPS-induced IL-6 secretion, with 74 and 66 % reduction, respectively. Also, Uro C treatment reduced the secretion, but due to large variation between the replicate experiments, no significant effects were found. Uro A, B and C and ellagic acid showed significant reduction in TNF-α secretion, 57, 34, 45 and 28 % reduction, respectively (Table 2, Fig. S3). Interestingly, treatment with 30 μM Uro D increased the secretion of TNF-α in THP-1 cells with

31 %. None of the hydroxycinnamic acids had significant effect on either of the pro-inflammatory cytokines, except for the low concentration of ferulic acid, which reduced the TNF-α secretion with 43 % (Table 2, Fig. S3). None of the protocatechuic acids had any significant effect on secretion of either of the pro-inflammatory cytokines.

Gene expression of pro- and anti-inflammatory cytokines

Of the tested metabolites, the urolithins, and the deconjugated urolithins in particular, were able to reduce LPS-induced inflammatory responses in both the human cell culture systems (Tables 1 and 2). Uro A was the only metabolite that also increased the basal NF-κB activity. In order to elucidate their mode of action, we chose Uro A, in addition to ellagic acid, ferulic acid and protocatechuic acid, for further experiments. Increased gene expression was observed for both the pro-inflammatory cytokines IL-6 and TNF-α and the anti-inflammatory cytokine IL-10, in LPS-stimulated cells compared with cells without LPS (Fig. 1, supplementary tables 1 and 2). Interestingly, LPS-stimulation of the cells did not induce any change in the gene expression of Toll-like receptor 4 (TLR-4) compared with untreated cells in any of the cell types. Neither ferulic acid nor protocatechuic acid treatment led to any significant change in gene expression of any of the cytokines or TLR-4 receptor in either of the cell types. 30 μM ellagic acid increased the gene expression of TNF-α more than two-fold and reduced the gene expression of TLR-4 in LPS stimulated THP-1 cells compared with control cells with LPS only (Fig. 1A). However, this effect was not observed in U937 cells (Fig. 1B). Interestingly, the pattern observed after treatment with ellagic acid differed from what was observed after treatment with its metabolite Uro A. For the pro-inflammatory factor IL-6, there was significant reduction of gene expression when incubating with Uro A in U937 cells (Fig. 1B). The gene expression of the anti-inflammatory cytokine IL-10 was more than three-fold increased in THP-1 cells, and more than five-fold decreased in U937 monocytes. No change in TLR-4 expression during Uro A treatment was detected. This clearly demonstrate that although both ellagic acid and Uro A display immunomodulating effects, their mode of action seems to be different. Also, the observed effects differed between the cell types investigated.

Intracellular signalling pathways

Translocation of phosphorylated NF-κB subunits is a critical step in the coupling of extracellular stimuli to the transcriptional activation of specific target genes. To access activation of intracellular signalling pathways, localisation and expression of phosphorylated p50 and p65 subunits of the nuclear factor NF-κB in LPS-induced THP-1 cells was evaluated visually by immunofluorescence (Fig. 2). Ellagic acid seemed to increase the expression of phosphorylated p50 (Fig. 2A) which was relocated to the nucleus. However, this activation by ellagic acid was not observed for the p65 subunit. In fact, visual inspection indicated a modest reduction in fluorescence intensity compared with control cells. In contrast to what was observed with ellagic acid, Uro A seemed to reduce the expression of both phosphorylated p50 and p65 subunits (Fig. 2A and B). Protocatechuic acid and ferulic acid had no effect on the expression and translocation of the NF-κB subunit p50 (Fig. 2A), and a modest reduction of p65 (Fig. 2B), similar to the effect observed for ellagic acid.

Discussion

Human cell lines, originating from cancerous cells, are important *in vitro* tools to study cellular functions, mechanisms and responses, as well as signalling pathways. Both monocytes and macrophages detect and respond to pathogens via Toll-like receptors (TLR) and mediate inflammation, and when investigating immune response of a compound both cell types are commonly used. The THP-1 cell line is isolated from the peripheral blood of a 1-year old male patient suffering from acute monocytic leukaemia.[45] This cell line has been widely used to investigate the function and regulation of monocytes and macrophages in the cardiovascular system, with cells not only in the monocyte state, but also in the macrophage-like state.[46] Monocytes are precursor immune cells typically circulating in the blood, before they migrate into a tissue and becomes macrophages. In addition to THP-1 cells, U937 cells are widely used to investigate the function and regulation of responses related to inflammation in monocytes. U937 is a pro-monocytic cell line isolated from the histiocytic lymphoma of a 37-year old male, and these cells exhibit many typical characteristics of monocytes.[47] The basic difference between U937 and THP-1 cells is the origin and maturation stage. U937 cells are of tissue origin, thus at more mature stage, whereas THP-1 cells are from the blood of a young patient with acute leukaemia and are of a less mature stage. The U937 cell line used in this study is stably transfected with a luciferase reporter that contains three NF-κB binding sites and allows rapid detection of NF-κB activity.[48] LPS is the most abundant component within the cell wall of gram-negative bacteria and can stimulate secretion of various inflammatory cytokines in immune cells, leading to an acute inflammatory response.[49] Addition of LPS to immune cells leads to a high increase in IL-6 and TNF-α secretion, and therefore it is possible to test the ability of a given compound to modulate a cellular response to infection by measuring change in secretion. IL-6 and TNF-α are pro-inflammatory cytokines that are secreted to stimulate an immune response. Subsequently, the cells start producing anti-inflammatory cytokines, like IL-10. Receptors on the cell surface, like TLR-4, interact with interleukins produced by immune cells, and induce the systemic inflammation response.[50] NF-κB is a family of transcription factors that play a critical role in cellular stress, and inflammatory responses. Activation of NF-κB lead to transcription of genes that code for inflammatory cytokines and cell-cycle modulators, which are factors that promotes cell survival, while suppression of activity is important for apoptosis. Previous reports have shown that NF-κB regulates the expression of certain genes also under basal conditions.[51] In our study, Uro A was the only metabolite which significantly increased the basal level of NF-κB in the U937 cells. Dihydrocaffeic acid sulfate, the four protocatechuic acids,

ellagic acid and Uro A, B, C and D, significantly inhibited the LPS-induced NF-κB activity in U937 cells. In immune cells, NF-κB is most abundant either as a heteromeric complex of the two subunits p50 and p65, or as a p65/p65 homodimer, that are translocated to the nucleus where it binds to the DNA consensus sequence of various target genes.[52] Generally accepted, the classical NF-κB heterodimer, composed of p50 and p65 subunits, is a transcriptional activator of the pro-inflammatory cytokine IL-6. NF-κB is activated by pro-inflammatory cytokines such as IL-1α, IL-1β and TNF-α. Our data show decreased expression of both p50 and p65 upon treatment with Uro A. This is in contrast with the increased expression and translocation of phosphorylated p50 to the nucleus upon ellagic acid stimulation. In a study performed by Piwowarski et al, Uro A blunted translocation of p65 sub-unit and inhibited DNA binding of p50 in RAW264.7 macrophages.[53] Another study demonstrated that Uro A decreased both protein level and phosphorylate p65 in hepatocellular carcinomas HepG2 cells.[54] In fact, our experiments clearly show that Uro A modulated the immune response in a different way compared with ellagic acid. This is in line with the results performed in a study using human colonic fibroblasts, in which both Uro A and Uro B were able to inhibit activation of NF-κB through receptor-mediated uptake. Interestingly, ellagic acid did not display any anti-inflammatory response in these cells.[55] Also, *in vivo* experiments in damaged kidney tissue in rats demonstrated that Uro A treatment reduced expression of inflammatory cytokines, while ellagic acid failed to reduce these levels. [56] Uro A glucuronide, a common conjugate found in plasma after intake of ellagitannin-containing foods, did not demonstrate strong immunomodulating effects in the cell systems tested in this study, nor did Uro B glucuronide. This is in line with a recent study with human colon cancer cells, where only Uro A, and not its conjugated metabolites, Uro A glucuronide and Uro A sulphate, were able to induce p53-dependent cellular senescence.[57] In an earlier study, using human colon cancer cells, it was suggested that the different mode of action was related to transport and detoxifying of urolithins, particularly in relation to ATP-binding cassette transporters.[38] Also, a study demonstrated that Uro A and Uro B glucuronides had lower effect on TNF-α induced inflammation using human aortic endothelia cells.[58] One explanation to the observed differences in this study between Uro A and its conjugate could be related to migration of immune cells. Uro A glucuronide reduced monocyte adhesion, but did not have any effect on IL-8 secretion, while Uro A affected cell migration but not adhesion. [58] Another reason for dissimilar cellular responses to urolithins and their conjugated metabolites could be due to the metabolic fate of metabolites observed in *in vitro* cell models. The structure of the phenolic molecules might be altered *in vitro* by the cells, and this might affect the bioactivity of the metabolites, thoroughly discussed in a recent review.[59] Combining metabolites could be a mean to overcome this limitation, also tracking compound stability and cell metabolism, could be useful strategies in future studies assessing bioactivity.

Of the phenolic metabolites tested in our study, Uro A, B, and C were the only metabolites that reduced both IL-6 and TNF-α secretion in THP-1 cells upon LPS stimulation. This is in line with a recent study demonstrating that both Uro A and Uro B were able to attenuate LPS-induced inflammation in neural murine cells, J774.1 macrophages and in RAW264.7 macrophages, the latter through supressing of the PI13-K/Akt/NF-κB and JNK/AP-1 signalling pathways.[60] Urolithins are also shown to inhibit IL-6 expression in Caco2 cells.[61] A study by di Gesso et al.[62] reported the effect of several flavonoids or metabolites and in combinations (0.1, 1, 10 or 100 μM) on LPS-induced TNF-α, IL-10 and IL-1β secretion from THP-1 cells. One of the metabolites they found to significantly reduce TNF-α secretion was protocatechuic acid-3-sulfate. In our study, however, we did not observe a similar reduction of TNF-α secretion treating cells with this metabolite. One explanation could be that the pre-treatment times with metabolites were different, since we pre-treated the cells for 21 h with the metabolites and di Gesso et al. used only 30 min prior to addition of LPS.[62] A challenge when using THP-1 cells is to measure the presence of secreted cytokines with ELISA, where long incubation times often are necessary. [63] The challenge of longer incubation times is, however, that this may turn on other signalling pathways. When investigating metabolites for protective capabilities during immune response, it is common to pre-treat the cells followed by disease induction. We did not perform a time-effect response and cannot rule out that the different pre-treatment times could influence the response. Also, kinetic studies in THP-1 macrophages demonstrated that while the gene expression of cytokines peaked at 6 h LPS-stimulation, cytokine secretion did not peak until 18 h LPS-incubation.[64, 65] TNF-α, for example, showed a decline after 24 h LPS stimulation, while IL-10 accumulation increased after 18 h.[65] TNF-α accumulation is regulated by the anti-inflammatory cytokine IL-10, and IL-10 has been shown to suppress TNF-α production upon prolonged stimulation.[66] IL-6 is mostly regarded as a pro-inflammatory cytokine, but it has also anti-inflammatory activities.[67] Stimulation of myelin-reactive T-cells with TGF-α plus IL-6 completely abrogated pathogenic function, and these cells failed to upregulate the pro-inflammatory chemokines crucial for central nervous system inflammation. In addition, these cells produced IL-10, which has potent anti-inflammatory activities.[68] Since cytokine production influences both forward and backward responses, it is feasible to include additional analyses, such as gene expression analyses.[63] Our gene expression studies clearly showed a difference in expression of IL-10 between the two cell types used when treated with Uro A, with increased gene expression in THP1-cells and reduced gene expression in U937 cells. Likewise, we did see a modest up-regulation of TNF-α and a dramatic reduction of TLR-4 gene expression in THP-1 cells when treated with ellagic acid, while the gene expression of TNF-α and TLR-4 was un-changed in U937 cells. This difference could be explained by the fact that THP-1 cells we used are macrophages and U937 cells are monocytes, representing different stages during immune response. In addition, these two different cell lines are originating from different tissue and maturation and might respond differently

to the metabolites. Expression and secretion of IL-10, for example, is dependent on many factors including type of stimulus, type of tissue, in addition to the time point in the process, and molecular mechanisms regulating IL-10 might differ in these two cell types.[69, 70] Likewise, there are numerous TNF-α mediated signalling pathways, depending on cell type.[71] TNF-α is a strong pro-inflammatory agent that regulate macrophage function and is a driver of inflammation. However, this cytokine displays an anti-inflammatory role as well, and is shown to directly limit T cell-mediated immune response.[72, 73] TLR-4 receptor is crucial during inflammatory response, and the expression level often reflect the sensitivity of immune cells to infection.[74] Down-regulation of TLR-4 has been shown to reduce the pro-inflammatory responses.[75, 76] Interestingly, in our study, the cytokine secretion in THP-1 cells was more affected using higher metabolite concentrations (30 µM), while inhibition of NF-κB activity in U937 cells was observed using lower concentrations (1.5 µM). This could be due to different sensitivity to immune stimulants in the two cell systems, and we cannot rule out that also hydroxycinnamic acids and protocatechuic acids could have inhibitory effects on cytokine secretion in THP-1 cells using even higher, non-physiologically relevant concentrations. An earlier study showed that several anthocyanin metabolites, including protocatechuic acids and ferulic acid (0.1-10 µM), reduced IL-6 mRNA levels, but not the protein levels of IL-6.[77] In contrast, Warner et al. [78] demonstrated that a high concentration (100 µM) of protocatechuic acid was necessary to reduce gene expression of VCAM-1, while low concentrations (1 and 10 µM) inhibited the VCAM-1 protein level. The authors suggested that protocatechuic acid was acting post-translationally and not directly on mRNA transcription, interacting with TNF-α converting enzyme ADAM17. This could explain the discrepancies observed for protocatechuic acids in our study, showing a mild increase in secretion of pro-inflammatory cytokines using high concentrations, and reduction of NF-κB activity using low concentrations.

Bioavailability is a key step important when studying bioactive compounds, and the bioactive compound needs to reach the target tissue to have an effect. A recent study shows that the bioavailability of ellagic acid is not as low as previously considered, and concentrations in blood plasma could reach around 0.1 µM, however there were huge inter-individual variability among volunteers.[79]. A tracer study using a multistable-isotope labelled anthocyanin identified a higher bioavailability and greater diversity of circulating metabolites than previously reported [22]. A standard process for evaluating the health-promoting potential of food compounds comprises several stages of analysis involving both *in vitro* and *in vivo* experiments. Ideally, to validate the bioactivity of a given metabolite, human clinical studies are necessary. However, such studies are extremely complicated, expensive, and are typically conducted after selecting a potent molecule through a rigorous screening process. The majority of screening experiments are therefore performed using *in vitro* assays including enzymatic assays, cell cultures, genomic tests and *in vitro* digestion stimulation. Although both the U937 and the THP-1 cell lines are widely used screening models to study effects of food compounds, both come from cells with malignant background, thus the results might not replicate the *in vivo* situation, Also, as these cells are grown outside their natural environment, their response and sensitivity to metabolites might differ. Nevertheless, these cell model systems are valuable tools for mimicking biological situations, they are fast and relative effective, and can be used for assessment of molecular effects. The information provided in our study reflects a first phase screening of phenolic metabolites at the molecular level, using two different cell model systems. Promising molecules should then be evaluated using animal models and eventually used in human intervention studies.

Experimental

Chemicals

Ellagic acid, ferulic acid and protocatechuic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Metabolites of the hydroxycinnamic acids, i.e. ferulic acid 4-sulfate, dihydroferulic acid 4-sulfate, and dihydrocaffeic acid 4-sulfate, were synthesised and provided by the University of Leeds (UL), UK (Prof. Gary Williamson). Anthocyanin metabolites, i.e. protocatechuic acid 3-sulfate, protocatechuic acid 4-sulfate and protocatechuic acid 4-glucuronide, were synthesised and provided by the Quadram Institute, Norwich, UK (Dr. Paul Needs), and protocatechuic acid 3-glucuronide was commercially synthesised and provided from Toronto Research Chemicals (North York, ON, Canada). Metabolites of ellagic acid, i.e. Uro A, B, C and D and glucuronides of Uro A and B, were synthesised and provided by CEBAS-CSIC, Murcia, Spain (Prof. Francisco A. Tomas-Barberan). The concentrations of the metabolites chosen in this study reflect the varying levels used in similar work (i.e. the effect of 20-50 µM UroA in J774.1 murine macrophages, 5, 10 and 40 µM UroA in RAW macrophages, 10 µM Uro A in HepG2 cells, and 0.1-10 µM anthocyanin metabolites in vascular endothelial cells). [53, 54, 80-82]

Cell cultures

The U937-3xκB-LUC cell line, a human monocytic cell line stably transfected with a luciferase reporter that contains three NF-κB binding sites,[48] was maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10 % fetal bovine serum (FBS), 50 U/mL penicillin, 50 µg/mL streptomycin. 75 µg/mL hygromycin was added cell suspension when cells where sub-cultured. All solutions were obtained from Invitrogen (Carlsbad, CA, USA). The cell line was routinely maintained at 37 °C in a humidified atmosphere of 5 % CO_2 and sub-cultivated three times per week. After 20 passages, the cells were replaced by frozen stock. The human monocytic leukaemia cell line THP-1 (TIB-202™, ATCC®) was grown in RPMI-1640 culture medium supplemented with 10 % FBS, 2 mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and

0.05 mM 2-mercaptoethanol. The cell line was routinely maintained at 37 °C in a humidified atmosphere of 5 % CO_2 and sub-cultivated three times per week. After 25 passages, the cells were replaced by frozen stock. To induce mature macrophage-like state of the THP-1 cells, 10^6 cells/ml were seeded out in 12-well tissue culture plates and incubated with phorbol 12-myristate 13-acetate (PMA, SIGMA-Aldrich) for 72 h. Differentiated, plastic adherent cells were then washed twice with sterile Dulbecco's phosphate-buffered saline (Sigma-Aldrich) before incubation with fresh medium without PMA. Experiments were performed on day 2 after PMA removal.

NF-κB activity in U937 cells

To measure NF-κB activity, cells were transferred to medium with 2 % FBS. To measure basal NF-κB activity, cells were treated with ferulic acid, protocatechuic acid and ellagic acid or phenolic metabolites (1.5 and 30 µM in 0.1 % DMSO) or vehicle control (0.1 % DMSO) for 6.5 h. To measure LPS-induced NF-κB activity, cells were incubated with metabolites or vehicle control for 30 min, and then 1 µg/mL LPS (bought as a purified material isolated from E. coli 055:B5 from Sigma-Aldrich, St. Louis, MO, USA; catalogue no. L6529) was added to the cells and the LPS-stimulation continued for 6 h. Previous work in the U937 cell model demonstrated that a plateau of NF-κB activity was reached after 6h incubation using 1 ug/ml LPS, with more than 19-fold increase in activity compared with basal level. [83] The NF-κB activity was determined by measuring the luciferase activity after addition of Bright-Glo Reagent (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. Cell viability was determined using CellTiter-Glo® Luminescent Cell Viability Assay® (Promega, Madison, WI, USA), with a cut-off value of 10 % nonviable cells. Luminescence was detected for 1 sec using a Glomax©96 Microplate Luminometer (Promega, Madison, WI, USA). The cell experiments were performed three independent times seeded out in triplicates.

Cytokine (IL-6 and TNF-α) secretion in THP-1 cells

Twenty-four hours before experiments, differentiated THP-1 cells were plated out in 12-well tissue culture plates at the concentration 1×10^6 cells/mL. To measure LPS-induced cytokine secretion, cells were pre-treated with metabolites (10 and 30 µM in 0.1 % DMSO) or vehicle control (0.1 % DMSO) for 21 h, then 0.5 ng/mL LPS was added to the cells and the LPS-stimulation continued for 6 h.THP1-differentiated macrophages are more sensible to LPS stimulation compared with U937 monocytic cells and requires lower concentrations. The dose of LPS used in this model was selected on the basis of dose-finding experiments in our lab assessing the optimal induction of endotoxin tolerance.[84] The supernatants were then harvested and stored at −20 °C until cytokine assay. The IL-6 and TNF-α concentrations in cell culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA). Purified human recombinant Rat Anti-Human IL-6 or Purified Mouse Anti-Human TNF-α standards (BD Biosciences Pharmingen, San Diego, CA, USA) suspended in coating buffer (0.1 M carbonate/bicarbonate buffer, pH 9.6) were used to generate standard curves for the measurement of secreted cytokines in the samples. The samples and standards were added to MaxiSorp™ 96 MicroWell™, Nunc-Immuno™ plates (Nunc, Roskilde, Denmark) and incubated over night at 4 °C. Plates were washed three times with Dulbecco's Phosphate Buffered Saline (PBS, Merck KGaA, Darmstadt, Germany) containing 0.01 % Tween-20 (PBS-Tween), and unspecific binding-sites were blocked by incubating with 5 % bovine serum albumin in PBS for 1 h at room temperature. After washing five times with PBS-Tween, the samples and the standards were diluted in working-strength, high-performance ELISA (HPE) buffer from Sanquin (Amsterdam, Netherlands). The samples and cytokine standards were added to the plates, incubated for 1.5 h at room temperature, followed by washing five times with PBS-Tween. 1 µg/ml Biotin Rat Anti-Human IL-6 monoclonal antibody or 0.5 µg/ml Biotin Mouse Anti-Human TNF-α (BD Biosciences Pharmingen) in HPE buffer was added to the wells and incubated for 1 h. After another washing step, streptavidin-horseradish peroxidase conjugate (BD Bioscience Pharmingen) (0.1 µl/ml HPE buffer) was added and incubated for 30 min at room temperature. Plates were then washed five times with 30 s between each wash. Addition of 50 µl/well of Invitrogen™ TMB (3,3', 5,5;-tetramethylbenzidine) Chromogen Solution (for ELISA) (Thermo Fisher Scientific, Catalog number: 002023) made blue colour to develop, and after 10 min the reaction was stopped by addition of H_2SO_4 to a final concentration of 0.5 M. Absorbance was measured at 450 nm using SPECTROstar Nano Plate Reader (Ortenberg, Germany). The detection limit of the IL-6 and TNF-α ELISA were 4 pg/mL. The experiments were performed three independent times, seeded out in duplicates.

Immunofluorescence

Differentiated THP-1 cells, seeded out on Nunc™ 8-well chamber slides (Thermo Fisher Scientific, Waltham, MA, US), and treated with metabolites as described for cytokine secretion were fixed in 4 % paraformaldehyde (Honeywell Riedel-De Haën AG, Seelze, Germany) for 15 min, washed 3 times in PBS before the cell were permeabilized with 0.5 % TritonX-100 in PBS for 10 min. The cells were washed in PBS-Tween, and then blocked using 5 % dry-milk in PBS-Tween for 1 h. The primary antibody was incubated in 2 % milk in PBS-Tween for 1 h. Subsequent incubation with secondary antibody was performed for 30 min before mounting using Dako fluorescent mounting medium (Dako Denmark A/S, Glostrup, Denmark). The cells were examined by fluorescence microscopy analysis (Zeiss Axio Observer Z1 microscope, using LCI Plan-Neofluar 63x/1.30 Imm Korr DIC M27 objective), and images were processed using Adobe Photoshop CS3. Primary antibodies used were rabbit phospho-p65 (Ser 311, sc-33039) and phospho-p50 (Ser 337, sc-101744), both from Santa Cruz Biotechnology (Dallas, Texas, USA). Secondary antibody used was Alexa Fluor® 546-conjugated goat anti-rabbit (Thermo Fisher Scientific, Waltham, MA, USA). Hoechst 33342 Fluorescent Stain from Molecular Probes (Thermo Fisher Scientific, Waltham, MA, USA) was used to counterstain the nuclei. The pictures presented were chosen randomly from two seeding experiments, and

expression and localisation of NF-κB in relation to the nuclei was scored by visual inspection.

RT-qPCR

RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to isolate and purify total RNA from THP-1 macrophages and U937 cells, treated with metabolites as described previously, with a DNase step (Invitrogen) to increase purity. A total of 200 ng of RNA was subjected to reverse transcriptase (RT)-reaction by TaqMan Gold RTPCR kit (Applied Biosystems, Foster City, CA, USA). The samples were diluted 4 times before the application of 10 ng samples (in triplicates) to real-time PCR analysis in an ABI prism 7900HT Sequence detection system (Applied Biosystems), using TaqMan Gene expression assay (Applied Biosystems). Amplification of cDNA by 40 two-step cycles (15 s at 95°C for denaturation of DNA, 1 min at 60°C for primer annealing and extension) was performed, and cycle threshold (Ct) values were obtained graphically (Applied Biosystems, Sequence Detection System, Software version 2.2). Gene expression was normalized to the average value of ribosomal protein L32 (RPL32) and ΔCt values were calculated. Comparison of gene expression between samples (control and metabolite treated) was derived from subtraction of ΔCt values between the two samples to give a ΔΔCt value, and relative gene expression (fold change) was calculated as $2^{-\Delta\Delta Ct}$ normalized to control. Applied Biosystems primer/probe assays were used in this study: TLR4 (Hs01060206_m1), RPL32 (Hs00851655_g1), IL-10 (Hs00961622_m1), IL-6 (Hs00985639_m1) and TNF-α (Hs01113624_g1).

Statistical analysis

Significance of metabolite in comparison to control sample was determined using one-way analysis of variance (ANOVA) using Dunnett's multiple comparisons test with a control. Differences were considered significant at $p<0.05$. Results are expressed as means ± SEM. The statistical analysis was performed in GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, USA).

Conclusions

Of the metabolites tested on the human monocytic leukaemia cell line THP-1 and the human monocytic cell line U937-3xκB-LUC, the urolithins, which are metabolites of ellagitannins, and Uro A in particular, was the most active to reduce LPS-induced NF-κB activity. Interestingly, although both ellagic acid and Uro A had immunomodulating effects, the immune response was regulated differently. Both metabolites reduced LPS-induced NF-κB activity in U937 cells. However, while Uro A reduced IL-6 and IL-10 mRNA expression, this effect could not be observed with ellagic acid. In THP-1 cells on the other hand, treatment with ellagic acid dramatically reduced expression of Toll-like receptor 4, while Uro A had no effect. The molecular mechanisms on how the metabolites exerts their effect remains to be elucidates in more details. Different mechanisms of the metabolite groups, as well as the fact that U937 monocytes and the THP-1 macrophages represent different stages during immune response, could explain the different responses observed in the two cell culture systems.

Abbreviations

EA, ellagic acid; FA, ferulic acid; PA, protocatechuic acid; Uro, urolithin; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TNF-α, tumour necrosis factor alpha; Il, interleukin; TLR-4, Toll-like receptor 4; PMA, phorbol 12-myristate 13-acetate.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The research has received funding from the European Union Seventh Framework Program (FP7) under Grant Agreement ID: 312090 (project BACCHUS, Beneficial effects of dietary bioactive peptides and polyphenols on cardiovascular health in humans, 2012-2016), coordinated by Dr. Paul Kroon, Quadram Institute Bioscience, Norwich, UK. Funding from The Norwegian Fund for Research Fees for Agricultural Products (project number 262300) is also acknowledged. Metabolites were provided by partners in the European FP7-KBBE project BACCHUS. Dr. Stine Grimmer is acknowledged for being involved in the experimental planning.

References

1. A. Durazzo, M. Lucarini, E. B. Souto, C. Cicala, E. Caiazzo, A. A. Izzo, E. Novellino and A. Santini, Polyphenols: A concise overview on the chemistry, occurrence, and human health, *Phytotherapy Research*, 2019, **33**, 2221-2243.
2. N. A. Khatkar and K. K. Sharma, Phenylpropanoids and its derivatives: biological activities and its role in food, pharmaceutical and cosmetic industries, *Critical reviews in food science and nutrition*, 2019, DOI: 10.1080/10408398.2019.1653822, 1-21.
3. N. Yahfoufi, N. Alsadi, M. Jambi and C. Matar, The Immunomodulatory and Anti-Inflammatory Role of Polyphenols, *Nutrients*, 2018, **10**.
4. L. Hooper, P. A. Kroon, E. B. Rimm, J. S. Cohn, I. Harvey, K. A. Le Cornu, J. J. Ryder, W. L. Hall and A. Cassidy, Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials, *American Journal of Clinical Nutrition*, 2008, **88**, 38-50.
5. M. L. McCullough, J. J. Peterson, R. Patel, P. F. Jacques, R. Shah and J. T. Dwyer, Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults, *American Journal of Clinical Nutrition*, 2012, **95**, 454-464.
6. M. H. Pan, C. S. Lai and C. T. Ho, Anti-inflammatory activity of natural dietary flavonoids, *Food and Function*, 2010, **1**, 15-31.
7. J. I. Dower, J. M. Geleijnse, L. Gijsbers, C. Schalkwijk, D. Kromhout and P. C. Hollman, Supplementation of the Pure Flavonoids Epicatechin and Quercetin Affects Some Biomarkers of Endothelial Dysfunction and Inflammation in (Pre)Hypertensive Adults: A Randomized Double-Blind, Placebo-Controlled, Crossover Trial, *Journal of Nutrition*, 2015, **145**, 1459-1463.
8. P. Rathee, H. Chaudhary, S. Rathee, D. Rathee, V. Kumar and K. Kohli, Mechanism of action of flavonoids as anti-inflammatory agents: a review, *Inflammmation and Allergy- Drug Targets*, 2009, **8**, 229-235.
9. F. J. Pérez-Cano and M. Castell, Flavonoids, Inflammation and Immune System, *Nutrients*, 2016, **8**, 659.
10. N. Leyva-López, E. Gutierrez-Grijalva, D. Ambriz-Perez and J. Heredia, Flavonoids as Cytokine Modulators: A Possible Therapy for Inflammation-Related Diseases, *International Journal of Molecular Sciences*, 2016, **17**, 921.
11. X. Fu, L.-F. Gong, Y.-F. Wu, Z. Lin, B.-J. Jiang, L. Wu and K.-H. Yu, Urolithin A targets the PI3K/Akt/NF-κB pathways and prevents IL-1β-induced inflammatory response in human osteoarthritis: in vitro and in vivo studies, *Food & function*, 2019, **10**, 6135-6146.
12. D. Del Rio, A. Rodriguez-Mateos, J. P. Spencer, M. Tognolini, G. Borges and A. Crozier, Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases, *Antioxididant Redox Signal*, 2013, **18**, 1818-1892.
13. C. Manach, A. Scalbert, C. Morand, C. Rémésy and L. Jiménez, Polyphenols: food sources and bioavailability, *The American Journal of Clinical Nutrition*, 2004, **79**, 727-747.
14. A. Rodriguez-Mateos, D. Vauzour, C. G. Krueger, D. Shanmuganayagam, J. Reed, L. Calani, P. Mena, D. Del Rio and A. Crozier, Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update, *Arch Toxicol*, 2014, **88**, 1803-1853.
15. B. N. Manolescu, E. Oprea, M. Mititelu, L. L. Ruta and I. C. Farcasanu, Dietary Anthocyanins and Stroke: A Review of Pharmacokinetic and Pharmacodynamic Studies, *Nutrients*, 2019, **11**, 1479.
16. C. Czank, A. Cassidy, Q. Zhang, D. J. Morrison, T. Preston, P. A. Kroon, N. P. Botting and C. D. Kay, Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a (13)C-tracer study, *American Journal of Clinical Nutrition*, 2013, **97**, 995-1003.
17. R. M. de Ferrars, A. Cassidy, P. Curtis and C. D. Kay, Phenolic metabolites of anthocyanins following a dietary intervention study in post-menopausal women, *Molecular Nutrition and Food Research*, 2014, **58**, 490-502.
18. I. A. Ludwig, P. Mena, L. Calani, G. Borges, G. Pereira-Caro, L. Bresciani, D. Del Rio, M. E. Lean and A. Crozier, New insights into the bioavailability of red raspberry anthocyanins and ellagitannins, *Free Radical Biolology and Medicine*, 2015, **89**, 758-769.
19. C. D. Kay, G. Pereira-Caro, I. A. Ludwig, M. N. Clifford and A. Crozier, Anthocyanins and Flavanones Are More Bioavailable than Previously Perceived: A Review of Recent Evidence, *Annual review of food science and technology*, 2017, **8**, 155-180.
20. P. Vitaglione, G. Donnarumma, A. Napolitano, F. Galvano, A. Gallo, L. Scalfi and V. Fogliano, Protocatechuic acid is the major human metabolite of cyanidin-glucosides, *The Journal of nutrition*, 2007, **137**, 2043-2048.
21. K. Krzysztoforska, D. Mirowska-Guzel and E. Widy-Tyszkiewicz, Pharmacological effects of protocatechuic acid and its therapeutic potential in neurodegenerative diseases: Review on the basis of in vitro and in vivo studies in rodents and humans, *Nutritional neuroscience*, 2017, DOI: 10.1080/1028415x.2017.1354543, 1-11.
22. C. Czank, A. Cassidy, Q. Zhang, D. J. Morrison, T. Preston, P. A. Kroon, N. P. Botting and C. D. Kay,

Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a (13)C-tracer study, *Am J Clin Nutr*, 2013, **97**, 995-1003.
23. H. R. El-Seedi, A. M. El-Said, S. A. Khalifa, U. Goransson, L. Bohlin, A. K. Borg-Karlson and R. Verpoorte, Biosynthesis, natural sources, dietary intake, pharmacokinetic properties, and biological activities of hydroxycinnamic acids, *Journal of agricultural and food chemistry*, 2012, **60**, 10877-10895.
24. V. Coman and D. C. Vodnar, Hydroxycinnamic acids and human health: recent advances, *Journal of the Science of Food and Agriculture*, 2019, **n/a**.
25. J. M. Landete, Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health, *Food Res Int*, 2011, **44**, 1150-1160.
26. B. Cerda, F. A. Tomas-Barberan and J. C. Espin, Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability, *Journal of agricultural and food chemistry*, 2005, **53**, 227-235.
27. R. González-Barrio, C. A. Edwards and A. Crozier, Colonic Catabolism of Ellagitannins, Ellagic Acid, and Raspberry Anthocyanins: In Vivo and In Vitro Studies, *Drug Metabolism and Disposition*, 2011, **39**, 1680-1688.
28. M. Larrosa, A. González-Sarrías, M. J. Yáñez-Gascón, M. V. Selma, M. Azorín-Ortuño, S. Toti, F. Tomás-Barberán, P. Dolara and J. C. Espín, Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism, *The Journal of nutritional biochemistry*, 2010, **21**, 717-725.
29. J. C. Espin, M. Larrosa, M. T. Garcia-Conesa and F. Tomas-Barberan, Biological significance of urolithins, the gut microbial ellagic Acid-derived metabolites: the evidence so far, *Evid Based Complement Alternat Med*, 2013, **2013**, 270418.
30. J. Rodriguez, N. Pierre, D. Naslain, F. Bontemps, D. Ferreira, F. Priem, L. Deldicque and M. Francaux, Urolithin B, a newly identified regulator of skeletal muscle mass, *J Cachexia Sarcopenia Muscle*, 2017, **8**, 583-597.
31. S. Alfei, F. Turrini, S. Catena, P. Zunin, M. Grilli, A. M. Pittaluga and R. Boggia, Ellagic acid a multi-target bioactive compound for drug discovery in CNS? A narrative review, *Eur J Med Chem*, 2019, **183**, 111724.
32. R. Singh, S. Chandrashekharappa, S. R. Bodduluri, B. V. Baby, B. Hegde, N. G. Kotla, A. A. Hiwale, T. Saiyed, P. Patel, M. Vijay-Kumar, M. G. I. Langille, G. M. Douglas, X. Cheng, E. C. Rouchka, S. J. Waigel, G. W. Dryden, H. Alatassi, H.-G. Zhang, B. Haribabu, P. K. Vemula and V. R. Jala, Enhancement of the gut barrier integrity by a microbial metabolite through the Nrf2 pathway, *Nature Communications*, 2019, **10**, 89.
33. B. Xia, X. C. Shi, B. C. Xie, M. Q. Zhu, Y. Chen, X. Y. Chu, G. H. Cai, M. Liu, S. Z. Yang, G. A. Mitchell, W. J. Pang and J. W. Wu, Urolithin A exerts antiobesity effects through enhancing adipose tissue thermogenesis in mice, *PLOS Biology*, 2020, **18**, e3000688.
34. R. Garcia-Villalba, J. C. Espin and F. A. Tomas-Barberan, Chromatographic and spectroscopic characterization of urolithins for their determination in biological samples after the intake of foods containing ellagitannins and ellagic acid, *J Chromatogr A*, 2016, **1428**, 162-175.
35. M. A. Nunez-Sanchez, R. Garcia-Villalba, T. Monedero-Saiz, N. V. Garcia-Talavera, M. B. Gomez-Sanchez, C. Sanchez-Alvarez, A. M. Garcia-Albert, F. J. Rodriguez-Gil, M. Ruiz-Marin, F. A. Pastor-Quirante, F. Martinez-Diaz, M. J. Yanez-Gascon, A. Gonzalez-Sarrias, F. A. Tomas-Barberan and J. C. Espin, Targeted metabolic profiling of pomegranate polyphenols and urolithins in plasma, urine and colon tissues from colorectal cancer patients, *Molecular Nutrition and Food Research*, 2014, **58**, 1199-1211.
36. F. A. Tomas-Barberan, A. Gonzalez-Sarrias, R. Garcia-Villalba, M. A. Nunez-Sanchez, M. V. Selma, M. T. Garcia-Conesa and J. C. Espin, Urolithins, the rescue of "old" metabolites to understand a "new" concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status, *Molecular Nutrition and Food Research*, 2017, **61**.
37. J. A. Giménez-Bastida, A. González-Sarrías, M. Larrosa, F. Tomás-Barberán, J. C. Espín and M. T. García-Conesa, Ellagitannin metabolites, urolithin A glucuronide and its aglycone urolithin A, ameliorate TNF-α-induced inflammation and associated molecular markers in human aortic endothelial cells, *Mol Nutr Food Res*, 2012, **56**, 784-796.
38. A. González-Sarrías, J. A. Giménez-Bastida, M. Núñez-Sánchez, M. Larrosa, M. T. García-Conesa, F. A. Tomás-Barberán and J. C. Espín, Phase-II metabolism limits the antiproliferative activity of urolithins in human colon cancer cells, *Eur J Nutr*, 2014, **53**, 853-864.
39. M. Ávila-Gálvez, J. C. Espín and A. González-Sarrías, Physiological Relevance of the Antiproliferative and Estrogenic Effects of Dietary Polyphenol Aglycones versus Their Phase-II Metabolites on Breast Cancer Cells: A Call of Caution, *J Agric Food Chem*, 2018, **66**, 8547-8555.

40. M. Ávila-Gálvez, R. García-Villalba, F. Martínez-Díaz, B. Ocaña-Castillo, T. Monedero-Saiz, A. Torrecillas-Sánchez, B. Abellán, A. González-Sarrías and J. C. Espín, Metabolic Profiling of Dietary Polyphenols and Methylxanthines in Normal and Malignant Mammary Tissues from Breast Cancer Patients, *Mol Nutr Food Res*, 2019, **63**, e1801239.
41. F. Lodi, R. Jimenez, L. Moreno, P. A. Kroon, P. W. Needs, D. A. Hughes, C. Santos-Buelga, A. Gonzalez-Paramas, A. Cogolludo, R. Lopez-Sepulveda, J. Duarte and F. Perez-Vizcaino, Glucuronidated and sulfated metabolites of the flavonoid quercetin prevent endothelial dysfunction but lack direct vasorelaxant effects in rat aorta, *Atherosclerosis*, 2009, **204**, 34-39.
42. K. R. Patel, C. Andreadi, R. G. Britton, E. Horner-Glister, A. Karmokar, S. Sale, V. A. Brown, D. E. Brenner, R. Singh, W. P. Steward, A. J. Gescher and K. Brown, Sulfate metabolites provide an intracellular pool for resveratrol generation and induce autophagy with senescence, *Sci Transl Med*, 2013, **5**, 205ra133.
43. E. Van Rymenant, C. Grootaert, K. Beerens, P. W. Needs, P. A. Kroon, A. Kerimi, G. Williamson, R. García-Villalba, A. González-Sarrías, F. Tomas-Barberan, J. Van Camp and J. Van de Voorde, Vasorelaxant activity of twenty-one physiologically relevant (poly)phenolic metabolites on isolated mouse arteries, *Food & function*, 2017, **8**, 4331-4335.
44. M. A. Avila-Galvez, J. A. Gimenez-Bastida, A. Gonzalez-Sarrias and J. C. Espin, Tissue deconjugation of urolithin A glucuronide to free urolithin A in systemic inflammation, *Food & function*, 2019, **10**, 3135-3141.
45. S. Tsuchiya, M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno and K. Tada, Establishment and characterization of a human acute monocytic leukemia cell line (THP-1), *International journal of cancer*, 1980, **26**, 171-176.
46. W. Chanput, J. J. Mes and H. J. Wichers, THP-1 cell line: an in vitro cell model for immune modulation approach, *Int Immunopharmacol*, 2014, **23**, 37-45.
47. C. Sundström and K. Nilsson, Establishment and characterization of a human histiocytic lymphoma cell line (U-937), *International Journal of Cancer*, 1976, **17**, 565-577.
48. H. Carlsen, J. O. Moskaug, S. H. Fromm and R. Blomhoff, In vivo imaging of NF-kappa B activity, *Journal of Immunology*, 2002, **168**, 1441-1446.
49. M. J. Sweet and D. A. Hume, Endotoxin signal transduction in macrophages, *Journal of Leukococyte Biology*, 1996, **60**, 8-26.
50. V. Kumar, Toll-like receptors in the pathogenesis of neuroinflammation, *Journal of Neuroimmunology*, 2019, **332**, 16-30.
51. J. W. Gordon, J. A. Shaw and L. A. Kirshenbaum, Multiple Facets of NF-kB in the Heart, *Circulation Research*, 2011, **108**, 1122-1132.
52. A. Fiordelisi, G. Iaccarino, C. Morisco, E. Coscioni and D. Sorriento, NFkappaB is a Key Player in the Crosstalk between Inflammation and Cardiovascular Diseases, *International Journal of Molecular Sciences*, 2019, **20**, 1599.
53. J. P. Piwowarski, A. K. Kiss, S. Granica and T. Moeslinger, Urolithins, gut microbiota-derived metabolites of ellagitannins, inhibit LPS-induced inflammation in RAW 264.7 murine macrophages, *Mol Nutr Food Res*, 2015, **59**, 2168-2177.
54. Y. Wang, Z. Qiu, B. Zhou, C. Liu, J. Ruan, Q. Yan, J. Liao and F. Zhu, In vitro antiproliferative and antioxidant effects of urolithin A, the colonic metabolite of ellagic acid, on hepatocellular carcinomas HepG2 cells, *Toxicol In Vitro*, 2015, **29**, 1107-1115.
55. A. González-Sarrías, M. Larrosa, F. A. Tomás-Barberán, P. Dolara and J. C. Espín, NF-κB-dependent anti-inflammatory activity of urolithins, gut microbiota ellagic acid-derived metabolites, in human colonic fibroblasts, *British Journal of Nutrition*, 2010, **104**, 503-512.
56. M. Guada, R. Ganugula, M. Vadhanam and M. N. V. Ravi Kumar, Urolithin A Mitigates Cisplatin-Induced Nephrotoxicity by Inhibiting Renal Inflammation and Apoptosis in an Experimental Rat Model, *J Pharmacol Exp Ther*, 2017, **363**, 58-65.
57. J. A. Giménez-Bastida, M. Á. Ávila-Gálvez, J. C. Espín and A. González-Sarrías, The gut microbiota metabolite urolithin A, but not other relevant urolithins, induces p53-dependent cellular senescence in human colon cancer cells, *Food and Chemical Toxicology*, 2020, **139**, 111260.
58. J. A. Giménez-Bastida, A. González-Sarrías, M. Larrosa, F. Tomás-Barberán, J. C. Espín and M.-T. García-Conesa, Ellagitannin metabolites, urolithin A glucuronide and its aglycone urolithin A, ameliorate TNF-α-induced inflammation and associated molecular markers in human aortic endothelial cells, *Molecular Nutrition & Food Research*, 2012, **56**, 784-796.
59. G. Aragonès, F. Danesi, D. Del Rio and P. Mena, The importance of studying cell metabolism when testing the bioactivity of phenolic compounds, *Trends in Food Science & Technology*, 2017, **69**, 230-242.
60. J. Xu, C. Yuan, G. Wang, J. Luo, H. Ma, L. Xu, Y. Mu, Y. Li, N. P. Seeram, X. Huang and L. Li, Urolithins Attenuate LPS-Induced Neuroinflammation in BV2Microglia via MAPK, Akt, and NF-kappaB Signaling Pathways, *Journal of agricultural and food chemistry*, 2018, **66**, 571-580.

61. G. E. Muku, I. A. Murray, J. C. Espin and G. H. Perdew, Urolithin A Is a Dietary Microbiota-Derived Human Aryl Hydrocarbon Receptor Antagonist, *Metabolites*, 2018, **8**.
62. J. L. di Gesso, J. S. Kerr, Q. Zhang, S. Raheem, S. K. Yalamanchili, D. O'Hagan, C. D. Kay and M. A. O'Connell, Flavonoid metabolites reduce tumor necrosis factor-alpha secretion to a greater extent than their precursor compounds in human THP-1 monocytes, *Molecular Nutrition and Food Research*, 2015, **59**, 1143-1154.
63. J. A. Stenken and A. J. Poschenrieder, Bioanalytical chemistry of cytokines--a review, *Analytica chimica acta*, 2015, **853**, 95-115.
64. W. Chanput, PhD, Wageningen University, 2012.
65. W. Chanput, J. Mes, R. A. Vreeburg, H. F. Savelkoul and H. J. Wichers, Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds, *Food & function*, 2010, **1**, 254-261.
66. D. M. Mosser and X. Zhang, Interleukin-10: new perspectives on an old cytokine, *Immunological reviews*, 2008, **226**, 205-218.
67. J. Scheller, A. Chalaris, D. Schmidt-Arras and S. Rose-John, The pro- and anti-inflammatory properties of the cytokine interleukin-6, *Biochimica Biophysica Acta*, 2011, **1813**, 878-888.
68. M. J. McGeachy, K. S. Bak-Jensen, Y. Chen, C. M. Tato, W. Blumenschein, T. McClanahan and D. J. Cua, TGF-[beta] and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology, *Nature Immunology*, 2007, **8**, 1390-1397.
69. R. Sabat, G. Grütz, K. Warszawska, S. Kirsch, E. Witte, K. Wolk and J. Geginat, Biology of interleukin-10, *Cytokine & Growth Factor Reviews*, 2010, **21**, 331-344.
70. M. Saraiva and A. O'Garra, The regulation of IL-10 production by immune cells, *Nat Rev Immunol*, 2010, **10**, 170-181.
71. N. Parameswaran and S. Patial, Tumor necrosis factor-α signaling in macrophages, *Critical reviews in eukaryotic gene expression*, 2010, **20**, 87-103.
72. M. Zakharova and H. K. Ziegler, Paradoxical Anti-Inflammatory Actions of TNF-α: Inhibition of IL-12 and IL-23 via TNF Receptor 1 in Macrophages and Dendritic Cells, *The Journal of Immunology*, 2005, **175**, 5024-5033.
73. S. Masli and B. Turpie, Anti-inflammatory effects of tumour necrosis factor (TNF)-alpha are mediated via TNF-R2 (p75) in tolerogenic transforming growth factor-beta-treated antigen-presenting cells, *Immunology*, 2009, **127**, 62-72.
74. K. Vijay, Toll-like receptors in immunity and inflammatory diseases: Past, present, and future, *International Immunopharmacology*, 2018, **59**, 391-412.
75. M. T. Abreu, P. Vora, E. Faure, L. S. Thomas, E. T. Arnold and M. Arditi, Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide, *Journal of immunology*, 2001, **167**, 1609-1616.
76. D. Tamandl, M. Bahrami, B. Wessner, G. Weigel, M. Ploder, W. Fürst, E. Roth, G. Boltz-Nitulescu and A. Spittler, Modulation of toll-like receptor 4 expression on human monocytes by tumor necrosis factor and interleukin-6: tumor necrosis factor evokes lipopolysaccharide hyporesponsiveness, whereas interleukin-6 enhances lipopolysaccharide activity, *Shock*, 2003, **20**, 224-229.
77. H. P. Amin, C. Czank, S. Raheem, Q. Zhang, N. P. Botting, A. Cassidy and C. D. Kay, Anthocyanins and their physiologically relevant metabolites alter the expression of IL-6 and VCAM-1 in CD40L and oxidized LDL challenged vascular endothelial cells, *Molecular Nutrition and Food Research*, 2015, **59**, 1095-1106.
78. E. F. Warner, Q. Zhang, K. S. Raheem, D. O'Hagan, M. A. O'Connell and C. D. Kay, Common Phenolic Metabolites of Flavonoids, but Not Their Unmetabolized Precursors, Reduce the Secretion of Vascular Cellular Adhesion Molecules by Human Endothelial Cells, *The Journal of nutrition*, 2016, **146**, 465-473.
79. A. González-Sarrías, R. García-Villalba, M. Á. Núñez-Sánchez, J. Tomé-Carneiro, P. Zafrilla, J. Mulero, F. A. Tomás-Barberán and J. C. Espín, Identifying the limits for ellagic acid bioavailability: A crossover pharmacokinetic study in healthy volunteers after consumption of pomegranate extracts, *Journal of Functional Foods*, 2015, **19**, 225-235.
80. H. P. Amin, C. Czank, S. Raheem, Q. Zhang, N. P. Botting, A. Cassidy and C. D. Kay, Anthocyanins and their physiologically relevant metabolites alter the expression of IL-6 and VCAM-1 in CD40L and oxidized LDL challenged vascular endothelial cells, *Mol Nutr Food Res*, 2015, **59**, 1095-1106.
81. Y. D. Boakye, L. Groyer and E. H. Heiss, An increased autophagic flux contributes to the anti-inflammatory potential of urolithin A in macrophages, *Biochim Biophys Acta Gen Subj*, 2018, **1862**, 61-70.
82. W. Komatsu, H. Kishi, K. Yagasaki and S. Ohhira, Urolithin A attenuates pro-inflammatory mediator production by suppressing PI3-K/Akt/NF-κB and JNK/AP-1 signaling pathways in lipopolysaccharide-stimulated RAW264 macrophages: Possible involvement of NADPH oxidase-derived reactive

oxygen species, *Eur J Pharmacol*, 2018, **833**, 411-424.
83. I. Paur, L. M. Austenaa and R. Blomhoff, Extracts of dietary plants are efficient modulators of nuclear factor kappa B, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 2008, **46**, 1288-1297.
84. A. Rieder, Dietary immune-modulation – carbohydrate specific effects of cereal and yeast beta-glucans, *PhD thesis*, University of Oslo, 2013.

Table 1 Basal and LPS- induced NF-κB activity in U937 cells after metabolite treatment (1.5 and 30 μM concentrations) [a]

Metabolites tested	**Basal NF-κB**				**LPS-induced NF-κB**			
	1.5 μM		30 μM		1.5 μM		30 μM	
	% increase± SEM	Significance	% increase ± SEM	Significance	% inhibition ± SEM	Significance	% inhibition± SEM	Significance
Hydroxycinnamic acids								
Ferulic acid 4-sulfate	15.6 ± 7.9	n.s.	-0.1 ± 2.7	n.s.	9.9 ± 4.5	n.s.	5.0 ± 6.0	n.s.
Dihydroferulic acid 4-sulfate	16.8 ± 8.4	n.s.	-3.7± 3.5	n.s.	13.1 ± 3.3	n.s.	5.1 ± 5.1	n.s.
Dihydrocaffeic acid 4-sulfate	22.1 ± 9	n.s.	-0.7 ± 1.8	n.s.	20.6 ± 5.7	***	12.3 ± 7.5	n.s.
Ferulic acid	-4.5 ± 1.2	n.s.	-1.3 ± 2.1	n.s.	12.7 ± 4.6	n.s.	10.6 ± 3.3	n.s.
Protocatechuic acids								
Protocatechuic acid-3-sulfate	24.3 ±7.7	n.s.	10.8 ± 1.6	n.s.	22.3 ± 4.3	***	6.6 ± 2.9	n.s.
Protocatechuic acid-3-glucuronide	29.8 ± 9.3	n.s.	8.6 ± 1.9	n.s.	16.5 ± 2.8	**	5.5 ± 4.3	n.s.
Protocatechuic acid-4-sulfate	18.2 ± 8.6	n.s.	6.3 ± 2.4	n.s.	19.7 ± 2.6	***	6.6 ± 3.2	n.s.
Protocatechuic acid-4-glucuronide	25.5 ± 8.9	n.s.	5.3 ± 1	n.s.	18.1 ± 2.7	**	6.1 ± 5	n.s.
Protocatechuic acid	22.6 ± 9.1	n.s.	-0.4 ± 1.7	n.s.	18.9 ± 2.2	***	9.9 ± 3.9	n.s.
Urolithins and ellagic acid								
Uro A	35.3 ± 6	*	113.3 ± 39.2	****	28.5 ± 2.1	****	30.4 ± 1.9	****
Uro B	26.2 ± 8.5	n.s.	-1.2 ± 2.3	n.s.	23.1 ± 5.3	****	45.5 ± 5.7	****
Uro C	26.6 ± 9	n.s.	13.0 ± 6.1	n.s.	15.9 ± 2.4	*	24.2 ± 6.5	**
Uro D	24.9 ± 9.2	n.s.	19.3 ± 1.5	n.s.	13.2 ± 2.9	n.s.	9.7 ± 5.6	n.s.
Uro A glucuronide	29.9 ± 6.5	n.s.	8.9 ± 2.8	n.s.	13 ± 2.9	n.s.	11.7 ± 1.9	n.s.
Uro B glucuronide	27.5 ± 7.8	n.s.	5.8 ± 2.4	n.s.	11.8 ± 3.2	n.s.	8.4 ± 3.6	n.s.
Ellagic acid	14.9 ± 6.8	n.s.	-2.8 ± 1.4	n.s.	22.5 ± 2.2	****	19.5 ± 2.2	*

[a]n.s., no significant difference between control and metabolites. Asterisks denote significant differences (**p>0.05, ** p>0.01, *** p>0.001**** p>0.0001) between control cells with or without LPS compared with cells in combination with metabolites assessed by one-way ANOVA.

Table 2 Cytokine secretion of the pro-inflammatory interleukins IL-6 and TNF-α in THP-1 cells after LPS-stimulation of THP-1 cells[a]

Metabolites tested	**IL-6**				**TNF-α**			
	10 μM		30 μM		10 μM		30 μM	
	% inhibition ± SEM	Significance	% inhibition SEM	Significance	% inhibition SEM	Significance	% inhibition SEM	Significance
Hydroxycinnamic acids								
Ferulic acid 4-sulfate	8.5 ±16.3	n.s.	14.4±22.2	n.s.	16.4±10.1	n.s.	22.7 ±10.6	n.s.
Dihydroferulic acid 4-sulfate	5.4 ±12.1	n.s.	2.7 ±9.5	n.s.	20.4 ±7.4	n.s.	7.0 ±6.6	n.s.
Dihydrocaffeic acid 4-sulfate	26.9 ±9.8	n.s.	10.9 ±2.0	n.s.	29.7 ±4.7	n.s.	28.3 ±10.2	n.s.
Ferulic acid	31.6 ±19.1	n.s.	9.8 ±12.9	n.s.	43.2 ±6.5	**	20.8 ±8.9	n.s.
Protocatechuic acids								
Protocatechuic acid-3-sulfate	-1.7 ±5.9	n.s.	1.7 ±4.7	n.s.	24.8 ±49.6	n.s.	20.9 ±43.7	n.s.
Protocatechuicacid-3-glucuronide	-4.6 ±8	n.s.	7.5 ±3.8	n.s.	6.7 ±30.3	n.s.	19.6 ±18.8	n.s.
Protocatechuic acid-4-sulfate	-12.8 ±7.5	n.s.	3.2 ±1.6	n.s.	49.6 ±52.2	n.s.	24.4 ±39.5	n.s.
Protocatechuic acid-4-glucuronide	0.8 ±2	n.s.	12.6 ±6.1	n.s.	35.9 ±50.8	n.s.	23.4 ±25.5	n.s.
Protocatechuic acid	7.6 ± 5.2	n.s.	10.1 ±4.1	n.s.	7.2 ±27.2	n.s.	49.7 ±33.2	n.s.
Urolithins and ellagic acid								
Uro A	14.3 ±2.1	n.s.	73.7 ±7.8	**	-1.90±7.9	n.s.	57.1 ±2.2	****
Uro B	9.0 ±5	n.s.	65.2 ±10.8	**	1.2 ±4.4	n.s.	37.6 ±5.2	****
Uro C	45.6 ±0.7	n.s.	46.7 ±29.2	n.s.	13.6 ±6.4	n.s.	46.2 ±2.1	****
Uro D	n.a.	n.a.	-20.8 ±14.3	n.s.	n.a.	n.a.	-30.8 ±4.8	**
Uro A glucuronide	n.a.	n.a.	-19.6 ±6.1	n.s.	n.a.	n.a.	-10.3 ±3.4	n.s.
Uro B glucuronide	n.a.	n.a.	-5.8 ±8.4	n.s.	n.a.	n.a.	0.1 ±4.3	n.s.
Ellagic acid	n.a.	n.a.	44.6 ±5.9	n.s.	n.a.	n.a.	19.5 ±7.3	**

[a]n.s., no significant difference between control and metabolites. n.a., not analyzed
Asterisks denote significant differences (**p>0.05, ** p>0.01, *** p>0.001**** p>0.0001) between control cells with LPS compared with cells in combination with metabolites assessed by one-way ANOVA.

Figure legends:

Fig. 1 Gene expression in THP-1 macrophages and U937 cells upon treatment with protocatechuic acid (PA), ferulic acid (FA), ellagic acid (EA) and Uro A. Differentiated THP-1 cells were incubated with 0.5 ng/mL LPS alone (control) or in combination with the phenolic metabolites (A). U937-3xΚB-LUC cells were incubated either with 1 µg/mL LPS alone or in combination with phenolic metabolites (B). The cells were harvested, and RNA was isolated before RT-PCR was performed to determine the mRNA expression of IL-6, IL-10, TNF-α and TLR-4. The results are presented as the average of three independent cell culture experiments seeded out in duplicates and presented as mean ± SEM relative to control samples with LPS added. Asterisks denote significant differences (*p>0.05, ** p>0.01, *** p>0.001**** p>0.0001) between cells stimulated with LPS alone compared with LPS in combination with metabolites assessed by one-way ANOVA. n.s. not significant. Statistics of real-time PCR is presented in Supplementary data tables 1 and 2.

Fig. 2 The activation and subcellular location of p50 and p65 subunits of NF-κB upon treatment with protocatechuic acid (PA), ferulic acid (FA), ellagic acid (EA) and Uro A. Differentiated THP-1 cells were incubated with 0.5 ng/mL LPS alone (control) or in combination with 30 µM of the phenolic metabolites. Cells were fixed using 4 % paraformaldehyde, before subjected to immunofluorescence immunostaining against phosphorylated p50 (A) and phosphorylated p65 (B). Alexa 546 conjugated goat-anti rabbit was used as secondary antibody. The nuclei were counterstained using Hoechst (blue). The boxed areas are presented at higher magnification. Arrows show strong nuclear expression assessed by visual inspection of staining intensity and localisation in relation to the nuclei.

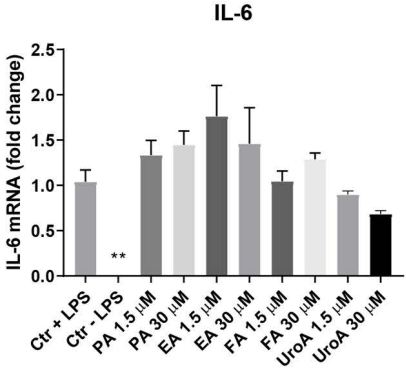
Fig. S1 Effect of the phenolic metabolites on basal (A) or LPS-induced (B) NF-κB activity. U937-3xκB-LIC cells were pre-treated with the phenolic metabolites (30 and 1.5 µM) in cell culture medium for 6.5 h before luciferase activity was measured (A). U937-3xκB-LUC cells were pre-treated with phenolic metabolites (30 and 1.5 µM) in cell culture medium for 30 min before 1 µg/ml LPS was added. The cells were incubated further for 6 h before luciferase activity was measured (B). The results are percentage of control (0.1 % DMSO). Asterisks denote significant differences (*p>0.05, ** p>0.01, *** p>0.001**** p>0.0001) between cells treated with LPS alone compared with LPS in combination with metabolites assessed by one-way ANOVA. n.s. not significant. Each bar represents the mean of three independent experiments ± SEM.

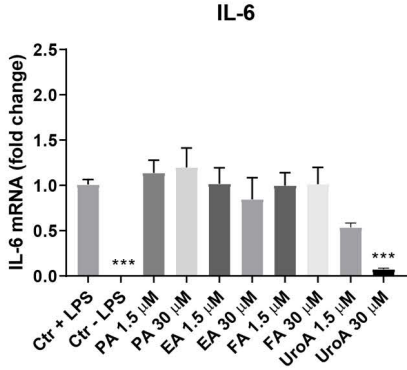
Fig. S2 Effect of phenolic metabolites on cell viability in U937 cells. U937-3xκB-LUC cells were treated with metabolites (30 and 1.5 µM) in cell culture medium for 30 min before 1 µg/mL LPS was added. The cells were incubated further for 6 h before luminescence was measured. The results are percentage of control (0.1 % DMSO). Each bar represents the mean of three independent experiments ± SEM.

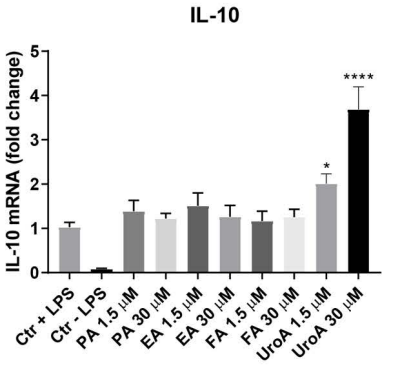
Fig. S3 Effect of phenolic metabolites on LPS-induced cytokine IL-6 and TNF-α secretion. THP1 cells were pre-treated with 30 and 10 µM of phenolic metabolites in cell culture medium for 21 hours before 0.5 ng/ml LPS was added. The cells were incubated further for 6 h before supernatants were harvested. IL-6 and TNF-α secretion was measured by sandwich ELISA. The results are percentage of control (0.1 % DMSO). Asterisks denote significant differences (*p>0.05, ** p>0.01, *** p>0.001**** p>0.0001) between cells treated with LPS alone compared with LPS in combination with metabolites assessed by one-way ANOVA. n.s. not significant. Each bar represents the mean of three independent experiments ± SEM.

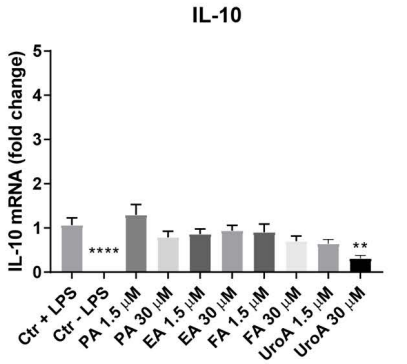
A) mRNA expression in THP-1 cells

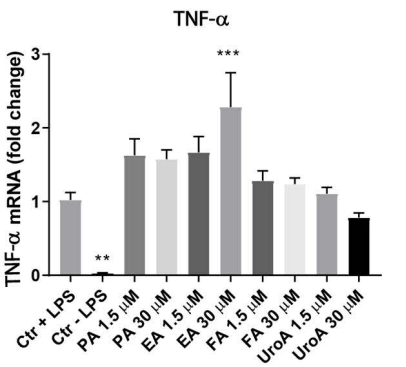
B) mRNA expression in U937 cells

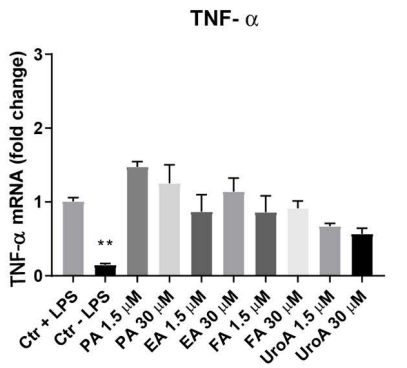


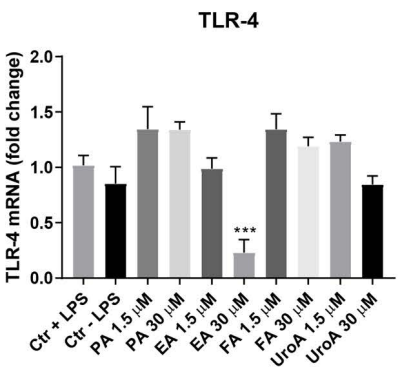


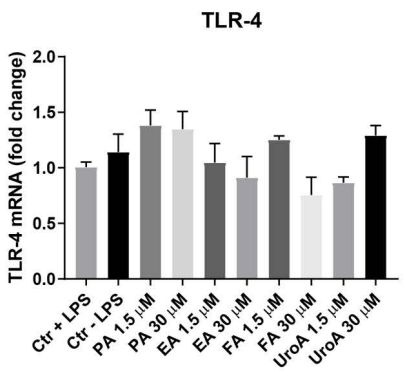


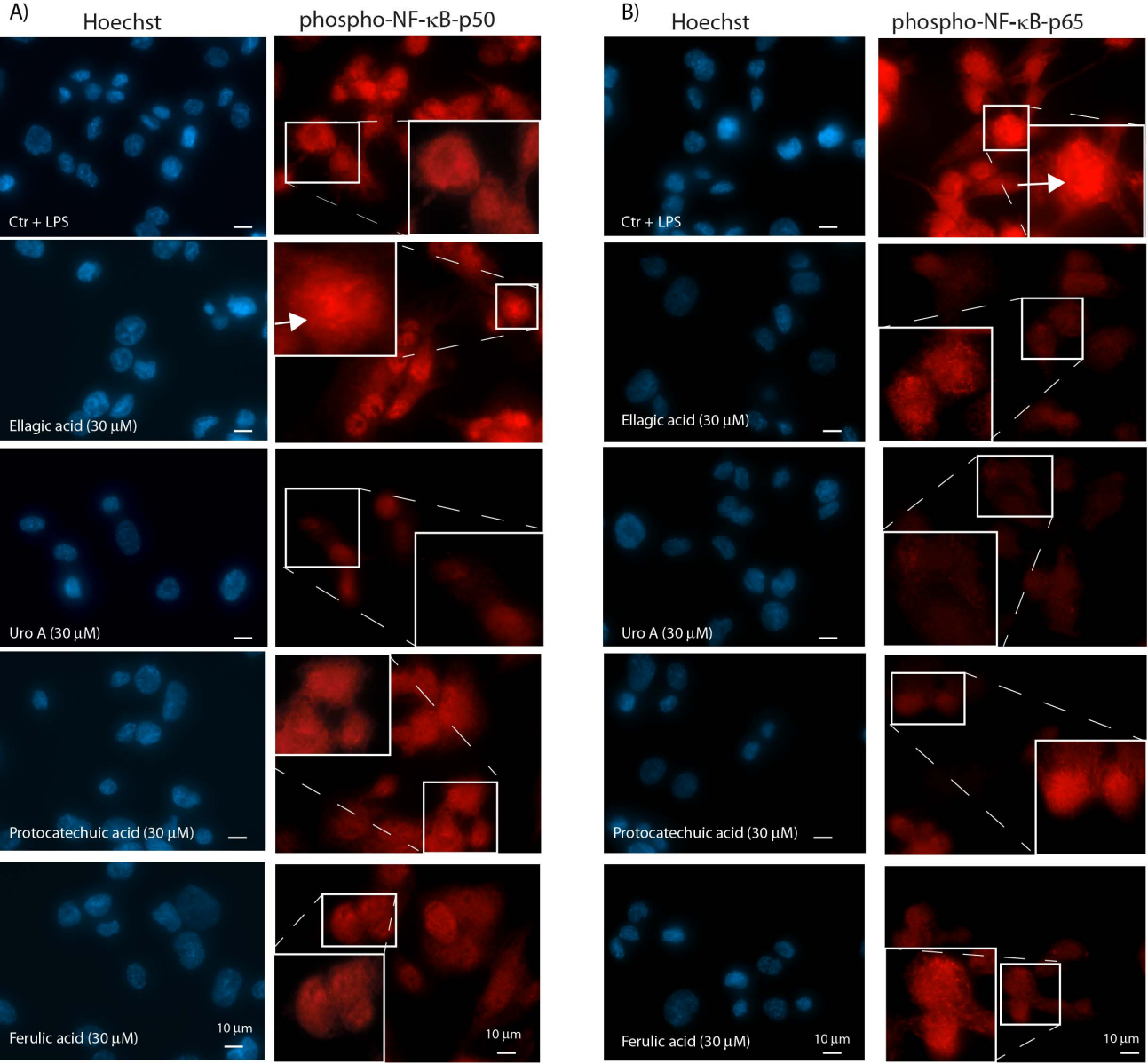




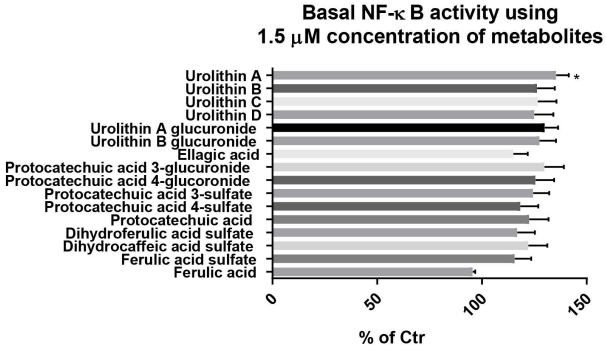


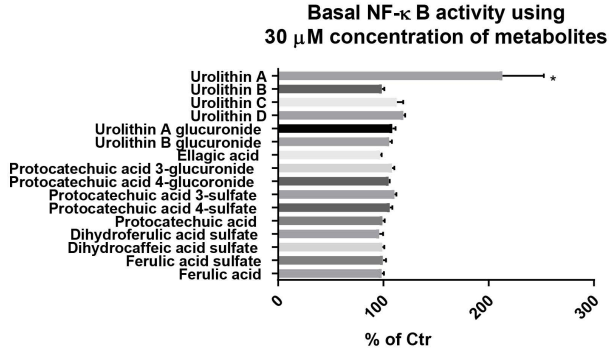




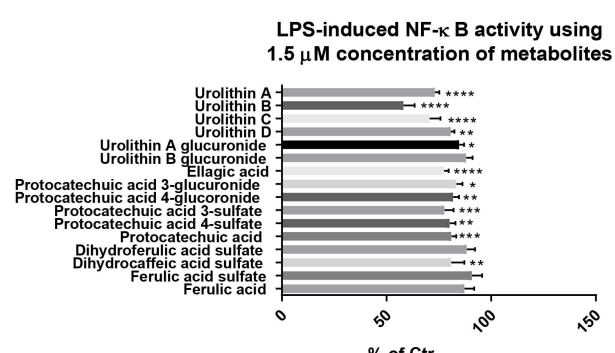

A)
Hoechst
phospho-NF-κB-p50
Ctr + LPS
Ellagic acid (30 μM)
Uro A (30 μM)
Protocatechuic acid (30 μM)
Ferulic acid (30 μM)
10 μm
10 μm
B)
Hoechst
phospho-NF-κB-p65
Ctr + LPS
Ellagic acid (30 μM)
Uro A (30 μM)
Protocatechuic acid (30 μM)
Ferulic acid (30 μM)
10 μm
10 μm

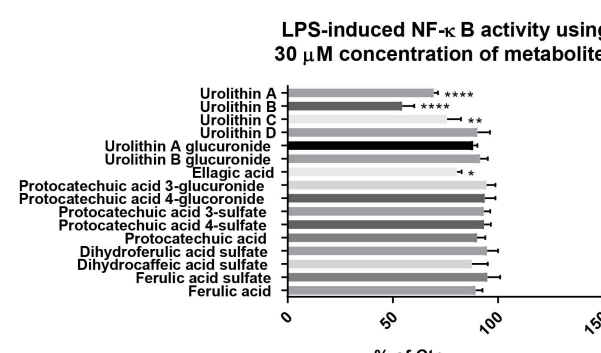
A)


Basal NF-κ B activity using
1.5 μM concentration of metabolites
Urolithin A
Urolithin B
Urolithin C
Urolithin D
Urolithin A glucuronide
Urolithin B glucuronide
Ellagic acid
Protocatechuic acid 3-glucuronide
Protocatechuic acid 4-glucuronide
Protocatechuic acid 3-sulfate
Protocatechuic acid 4-sulfate
Protocatechuic acid
Dihydroferulic acid sulfate
Dihydrocaffeic acid sulfate
Ferulic acid sulfate
Ferulic acid
*
0
50
100
150
% of Ctr

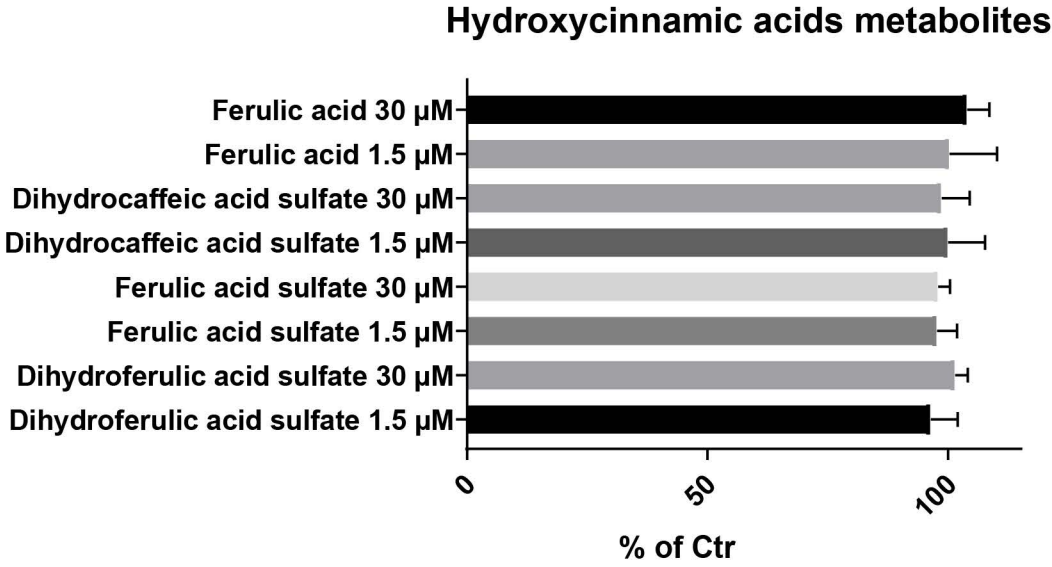

Basal NF-κ B activity using
30 μM concentration of metabolites
Urolithin A
Urolithin B
Urolithin C
Urolithin D
Urolithin A glucuronide
Urolithin B glucuronide
Ellagic acid
Protocatechuic acid 3-glucuronide
Protocatechuic acid 4-glucuronide
Protocatechuic acid 3-sulfate
Protocatechuic acid 4-sulfate
Protocatechuic acid
Dihydroferulic acid sulfate
Dihydrocaffeic acid sulfate
Ferulic acid sulfate
Ferulic acid
*
0
100
200
300
% of Ctr

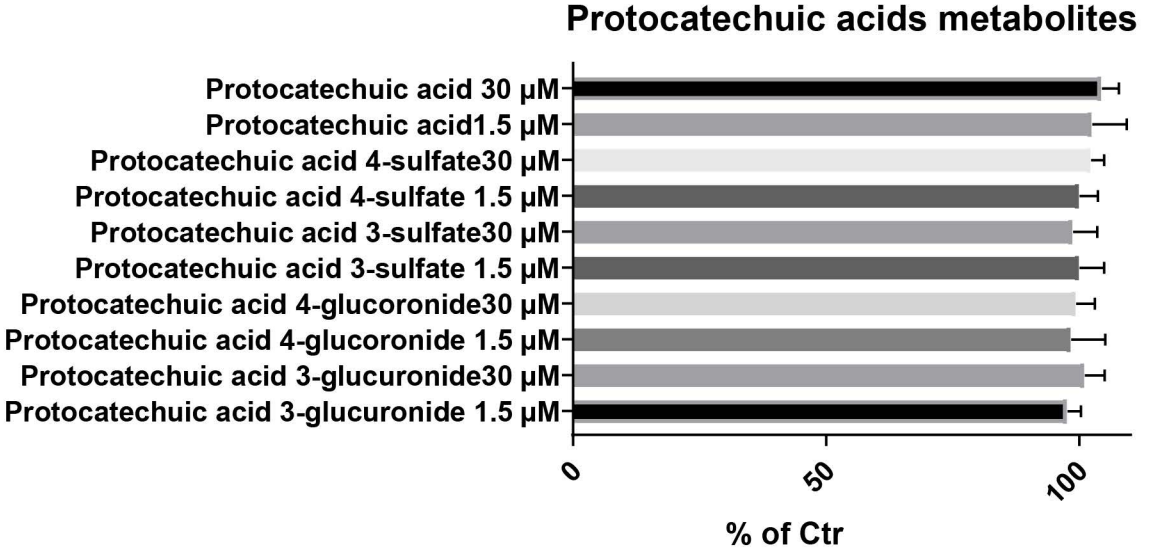
B)

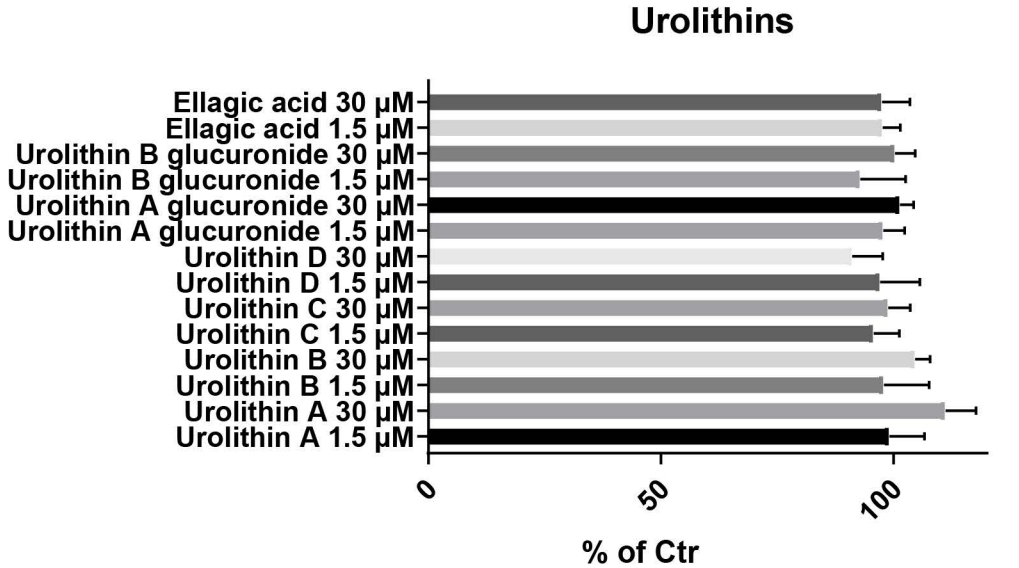

LPS-induced NF-κ B activity using
1.5 μM concentration of metabolites
Urolithin A
Urolithin B
Urolithin C
Urolithin D
Urolithin A glucuronide
Urolithin B glucuronide
Ellagic acid
Protocatechuic acid 3-glucuronide
Protocatechuic acid 4-glucuronide
Protocatechuic acid 3-sulfate
Protocatechuic acid 4-sulfate
Protocatechuic acid
Dihydroferulic acid sulfate
Dihydrocaffeic acid sulfate
Ferulic acid sulfate
Ferulic acid
****
****
****
**
*
****
*
**
***
**
****
**
0
50
100
150
% of Ctr

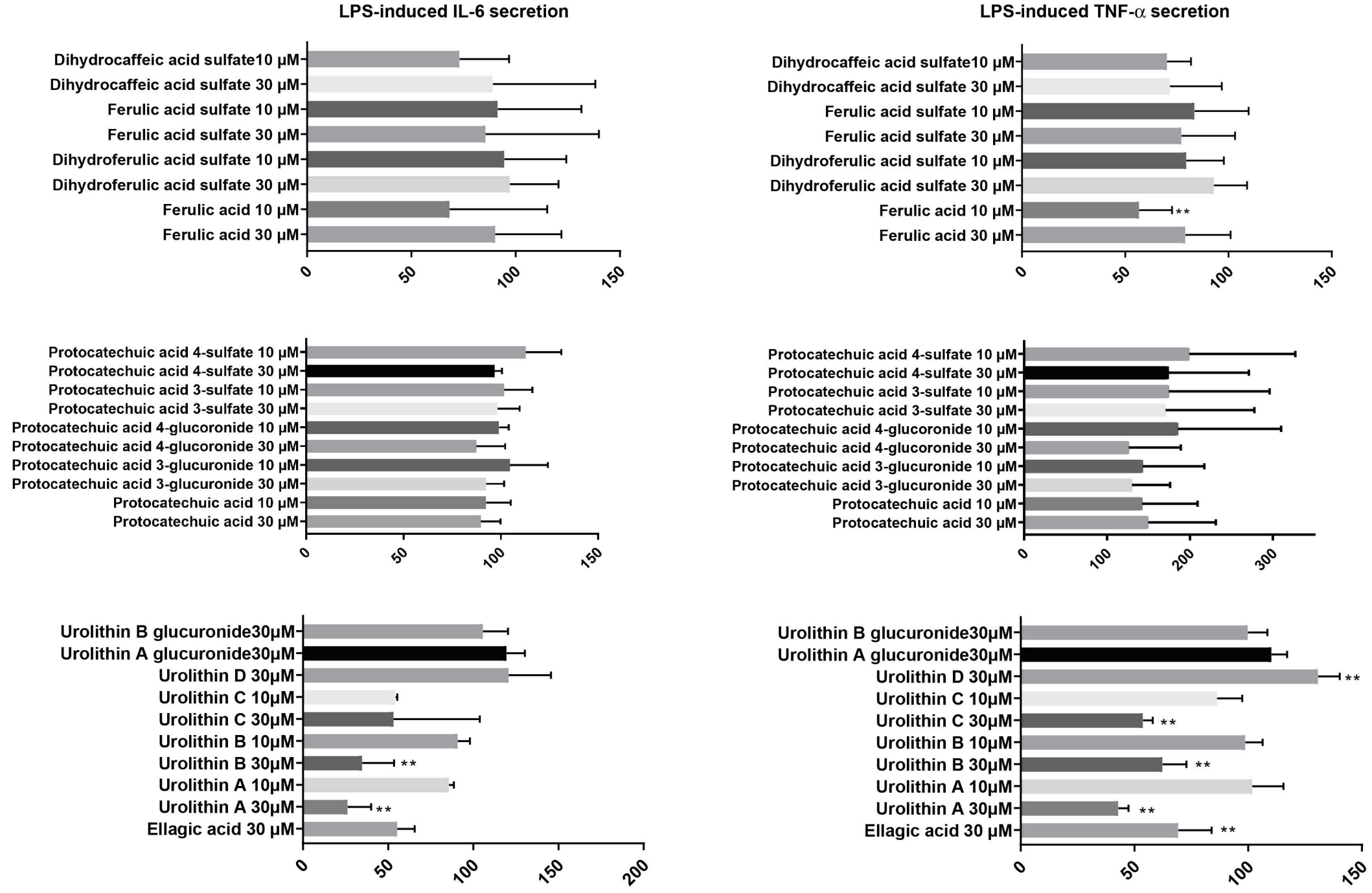

LPS-induced NF-κ B activity using
30 μM concentration of metabolites
Urolithin A
Urolithin B
Urolithin C
Urolithin D
Urolithin A glucuronide
Urolithin B glucuronide
Ellagic acid
Protocatechuic acid 3-glucuronide
Protocatechuic acid 4-glucuronide
Protocatechuic acid 3-sulfate
Protocatechuic acid 4-sulfate
Protocatechuic acid
Dihydroferulic acid sulfate
Dihydrocaffeic acid sulfate
Ferulic acid sulfate
Ferulic acid
****
****
**
*
0
50
100
150
% of Ctr

Amount of ATP (Cell viability)








LPS-induced IL-6 secretion
Dihydrocaffeic acid sulfate10 μM
Dihydrocaffeic acid sulfate 30 μM
Ferulic acid sulfate 10 μM
Ferulic acid sulfate 30 μM
Dihydroferulic acid sulfate 10 μM
Dihydroferulic acid sulfate 30 μM
Ferulic acid 10 μM
Ferulic acid 30 μM
0
50
100
150
LPS-induced TNF-α secretion
Dihydrocaffeic acid sulfate10 μM
Dihydrocaffeic acid sulfate 30 μM
Ferulic acid sulfate 10 μM
Ferulic acid sulfate 30 μM
Dihydroferulic acid sulfate 10 μM
Dihydroferulic acid sulfate 30 μM
Ferulic acid 10 μM
**
Ferulic acid 30 μM
0
50
100
150
Protocatechuic acid 4-sulfate 10 μM
Protocatechuic acid 4-sulfate 30 μM
Protocatechuic acid 3-sulfate 10 μM
Protocatechuic acid 3-sulfate 30 μM
Protocatechuic acid 4-glucoronide 10 μM
Protocatechuic acid 4-glucoronide 30 μM
Protocatechuic acid 3-glucuronide 10 μM
Protocatechuic acid 3-glucuronide 30 μM
Protocatechuic acid 10 μM
Protocatechuic acid 30 μM
0
50
100
150
Protocatechuic acid 4-sulfate 10 μM
Protocatechuic acid 4-sulfate 30 μM
Protocatechuic acid 3-sulfate 10 μM
Protocatechuic acid 3-sulfate 30 μM
Protocatechuic acid 4-glucoronide 10 μM
Protocatechuic acid 4-glucoronide 30 μM
Protocatechuic acid 3-glucuronide 10 μM
Protocatechuic acid 3-glucuronide 30 μM
Protocatechuic acid 10 μM
Protocatechuic acid 30 μM
0
100
200
300
Urolithin B glucuronide30μM
Urolithin A glucuronide30μM
Urolithin D 30μM
Urolithin C 10μM
Urolithin C 30μM
Urolithin B 10μM
Urolithin B 30μM
**
Urolithin A 10μM
Urolithin A 30μM
**
Ellagic acid 30 μM
0
50
100
150
200
Urolithin B glucuronide30μM
Urolithin A glucuronide30μM
Urolithin D 30μM
**
Urolithin C 10μM
Urolithin C 30μM
**
Urolithin B 10μM
Urolithin B 30μM
**
Urolithin A 10μM
Urolithin A 30μM
**
Ellagic acid 30 μM
**
0
50
100
150

Supplementary Table

Table 1: Real-time PCR statistics for all metabolites tested in THP-1 cells. Significance of metabolite in comparison to control sample was determined using one-way analysis of variance (ANOVA) using Dunnett's multiple comparisons test with a control (control cells + LPS stimulation) Asterisks indicate significant differences, *<0.05. **<0.001. *** <0.001. ****p<0.0001. Abbreviations: Ctr, control cells; ns, no significant difference

	Sample sets	Mean Diff.	95% CI of Diff.	Significant?	Summary	Adjusted P Value
IL-6	Ctr + LPS vs. Ctr - LPS	1.040	0.2950 to 1.785	Yes	**	0.0024
	Ctr + LPS vs. PA 1.5 μM	-0.2934	-1.038 to 0.4516	No	ns	0.8552
	Ctr + LPS vs. PA 30 μM	-0.4088	-1.154 to 0.3362	No	ns	0.5566
	Ctr + LPS vs. EA 1.5 μM	-0.7241	-1.469 to 0.02091	No	ns	0.0603
	Ctr + LPS vs. EA 30 μM	-0.4214	-1.166 to 0.3236	No	ns	0.5229
	Ctr + LPS vs. FA 1.5 μM	-0.006090	-0.7511 to 0.7389	No	ns	>0.9999
	Ctr + LPS vs. FA 30 μM	-0.2486	-0.9937 to 0.4964	No	ns	0.9336
	Ctr + LPS vs. UroA 1.5 μM	0.1416	-0.6034 to 0.8866	No	ns	0.9969
	Ctr + LPS vs. UroA 30 μM	0.3562	-0.3888 to 1.101	No	ns	0.7003
IL-10	Ctr + LPS vs. Ctr - LPS	-0.9446	-1.921 to 0.0314	No	ns	0.0620
	Ctr + LPS vs. PA 1.5 μM	0.3595	-0.6165 to 1.336	No	ns	0.8924
	Ctr + LPS vs. PA 30 μM	0.1931	-0.7829 to 1.169	No	ns	0.9967
	Ctr + LPS vs. EA 1.5 μM	0.4824	-0.4937 to 1.458	No	ns	0.6677
	Ctr + LPS vs. EA 30 μM	0.2361	-0.7399 to 1.212	No	ns	0.9904
	Ctr + LPS vs. FA 1.5 μM	0.1410	-0.8350 to 1.117	No	ns	0.9994
	Ctr + LPS vs. FA 30 μM	0.2268	-0.7492 to 1.203	No	ns	0.9921
	Ctr + LPS vs. UroA 1.5 μM	0.9817	0.005699 to 1.958	Yes	*	0.0481
	Ctr + LPS vs. UroA 30 μM	2.663	1.687 to 3.639	Yes	****	<0.0001
TNF-α	Ctr + LPS vs. Ctr - LPS	0.9966	0.2363 to 1.757	Yes	**	0.0048
	Ctr + LPS vs. PA 1.5 μM	-0.6047	-1.365 to 0.1556	No	ns	0.1772
	Ctr + LPS vs. PA 30 μM	-0.5517	-1.312 to 0.2086	No	ns	0.2565
	Ctr + LPS vs. EA 1.5 μM	-0.6435	-1.404 to 0.1168	No	ns	0.1324
	Ctr + LPS vs. EA 30 μM	-1.260	-2.020 to -0.4997	Yes	***	0.0002

	Ctr + LPS vs. FA 1.5 µM	-0.2587	-1.019 to 0.5016	No	ns	0.9267
	Ctr + LPS vs. FA 30 µM	-0.2138	-0.9741 to 0.5465	No	ns	0.9745
	Ctr + LPS vs. UroA 1.5 µM	-0.08388	-0.8442 to 0.6764	No	ns	0.9996
	Ctr + LPS vs. UroA 30 µM	0.2403	-0.5200 to 1.0010	No	ns	0.9503
TLR-4	Ctr + LPS vs. Ctr - LPS	0.1639	-0.2913 to 0.6190	No	ns	0.9034
	Ctr + LPS vs. PA 1.5 µM	-0.3275	-0.7827 to 0.1276	No	ns	0.2645
	Ctr + LPS vs. PA 30 µM	-0.3218	-0.7769 to 0.1333	No	ns	0.2815
	Ctr + LPS vs. EA 1.5 µM	0.02946	-0.4257 to 0.4846	No	ns	0.9997
	Ctr + LPS vs. EA 30 µM	0.7874	0.3322 to 1.2420	Yes	***	0.0001
	Ctr + LPS vs. FA 1.5 µM	-0.3248	-0.7799 to 0.1304	No	ns	0.2728
	Ctr + LPS vs. FA 30 µM	-0.1727	-0.6278 to 0.2824	No	ns	0.8768
	Ctr + LPS vs. Uro A 1.5 µM	-0.2133	-0.6684 to 0.2418	No	ns	0.7192
	Ctr + LPS vs. Uro A 30 µM	0.1727	-0.2825 to 0.6278	No	ns	0.8769

Table 2: Real-time PCR statistics for all metabolites tested in U937 cells. Significance of metabolite in comparison to control sample was determined using one-way analysis of variance (ANOVA) using Dunnett's multiple comparisons test with a control (control cells + LPS stimulation) Asterisks indicate significant differences, *<0.05. **<0.001. *** <0.001. ****p<0.0001. Abbreviations: Ctr, control cells; ns, no significant difference.

	Sample sets	Mean Diff.	95% CI of Diff.	Significant?	Summary	Adjusted P Value
IL-6	Ctr + LPS vs. Ctr - LPS	1.008	0.4329 to 1.584	Yes	***	0.0001
	Ctr + LPS vs. PA 1.5 µM	-0.1309	-0.7063 to 0.4446	No	ns	0.9926
	Ctr + LPS vs. PA 30 µM	-0.1912	-0.7667 to 0.3843	No	ns	0.9352
	Ctr + LPS vs. EA 1.5 µM	-0.008652	-0.5841 to 0.5668	No	ns	>0.9999
	Ctr + LPS vs. EA 30 µM	0.1605	-0.4150 to 0.7360	No	ns	0.9758
	Ctr + LPS vs. FA 1.5 µM	0.01031	-0.5652 to 0.5858	No	ns	>0.9999
	Ctr + LPS vs. FA 30 µM	-0.006020	-0.5815 to 0.5695	No	ns	>0.9999
	Ctr + LPS vs. Uro A 1.5 µM	0.4723	-0.1032 to 1.048	No	ns	0.1536
	Ctr + LPS vs. Uro A 30 µM	0.9339	0.3584 to 1.509	Yes	***	0.0003
IL-10	Ctr + LPS vs. Ctr - LPS	1.063	0.5160 to 1.610	Yes	****	<0.0001
	Ctr + LPS vs. PA 1.5 µM	-0.2316	-0.7789 to 0.3156	No	ns	0.8055
	Ctr + LPS vs. PA 30 µM	0.2720	-0.2753 to 0.8192	No	ns	0.6621
	Ctr + LPS vs. EA 1.5 µM	0.2038	-0.3435 to 0.7510	No	ns	0.8868
	Ctr + LPS vs. EA 30 µM	0.1252	-0.4221 to 0.6725	No	ns	0.9924
	Ctr + LPS vs. FA 1.5 µM	0.1576	-0.3896 to 0.7049	No	ns	0.9706
	Ctr + LPS vs. FA 30 µM	0.3609	-0.1863 to 0.9082	No	ns	0.3529
	Ctr + LPS vs. Uro A 1.5 µM	0.4187	-0.1286 to 0.9659	No	ns	0.2090
	Ctr + LPS vs. Uro A 30 µM	0.7524	0.2051 to 1.300	Yes	**	0.0028

TNF-α	Ctr + LPS vs. Ctr - LPS	0.8594	0.2751 to 1.444	Yes	**	0.0011
	Ctr + LPS vs. PA 1.5 μM	-0.4695	-1.054 to 0.1147	No	ns	0.1710
	Ctr + LPS vs. PA 30 μM	-0.2466	-0.8308 to 0.3377	No	ns	0.8127
	Ctr + LPS vs. EA 1.5 μM	0.1388	-0.4454 to 0.7230	No	ns	0.9916
	Ctr + LPS vs. EA 30 μM	-0.1323	-0.7165 to 0.4520	No	ns	0.9929
	Ctr + LPS vs. FA 1.5 μM	0.1461	-0.4382 to 0.7303	No	ns	0.9891
	Ctr + LPS vs. FA 30 μM	0.09293	-0.4913 to 0.6772	No	ns	0.9993
	Ctr + LPS vs. Uro A 1.5 μM	0.3357	-0.2486 to 0.9199	No	ns	0.5105
	Ctr + LPS vs. Uro A 30 μM	0.4382	-0.1460 to 1.022	No	ns	0.2288
TLR-4	Ctr + LPS vs. Ctr - LPS	-0.1354	-0.6169 to 0.3461	No	ns	0.9790
	Ctr + LPS vs. PA 1.5 μM	-0.3735	-0.8760 to 0.1289	No	ns	0.2425
	Ctr + LPS vs. PA 30 μM	-0.3421	-0.8446 to 0.1603	No	ns	0.3318
	Ctr + LPS vs. EA 1.5 μM	-0.04077	-0.5432 to 0.4617	No	ns	0.9997
	Ctr + LPS vs. EA 30 μM	0.09554	-0.4069 to 0.5980	No	ns	0.9971
	Ctr + LPS vs. FA 1.5 μM	-0.2456	-0.7480 to 0.2569	No	ns	0.7004
	Ctr + LPS vs. FA 30 μM	0.2511	-0.2141 to 0.7163	No	ns	0.5954
	Ctr + LPS vs. Uro A 1.5 μM	0.1411	-0.3241 to 0.6063	No	ns	0.9668
	Ctr + LPS vs. Uro A 30 μM	-0.2860	-0.7512 to 0.1791	No	ns	0.4463