Agathisflavone suppresses NF-κB-mediated release of inflammatory mediators from BV2 microglia and produces SIRT1 mediated neuroprotection in APPSwe-transfected neuronal cells

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Complete List of Authors:	Velagapudi, Ravikanth; University of Huddersfield, Department of Pharmacy; National Institutes of Health, Neurobiology Laboratory, National Institute of Environmental Health Sciences Ajileye, Olusiji; Obafemi Awolowo University, Department of Chemistry Okorji, Uchechukwu; University of Huddersfield, Department of Pharmacy Jain, Priya; University of Huddersfield, Department of Pharmacy Aderogba, Mutalib; Obafemi Awolowo University, Ile-Ife., chemistry; University of Pretoria, Paraclinical sciences Olajide, Olumayokun; University of Huddersfield, Department of Pharmacy
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Agathisflavone isolated from *Anacardium occidentale* suppresses SIRT1-mediated neuroinflammation in BV2 microglia and neurotoxicity in APPSwe-transfected SH-SY5Y cells

Ravikanth Velagapudi[a,c], Olusiji O Ajileye[b], Uchechukwu Okorji[a], Priya Jain[a], Mutalib A. Aderogba[b], Olumayokun A Olajide[a*]

[a] Department of Pharmacy, School of Applied Sciences, University of Huddersfield, Huddersfield, HD1 3DH, United Kingdom

[b] Department of Chemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria

[c] Present Address: Neurobiology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA

***Author for Correspondence**

Dr Olumayokun A Olajide
Department of Pharmacy
University of Huddersfield
Queensgate
Huddersfield, HD1 3DH
United Kingdom
Email: o.a.olajide@hud.ac.uk
Telephone: +44 (0) 1484 472735

Abstract

Agathisflavone is a bioactive compound in *Anacardium occidentale*. In this study we investigated inhibition neuroinflammation in BV2 microglia by agathisflavone. Neuroprotective activity of the compound was investigated in differentiated SH-SY5Y cells. Experiments in LPS-activated BV2 microglia showed that pre-treatment with agathisflavone (5-20 μM) produced significant reduction in the release of TNFα, IL-6, IL-1β, NO and PGE_2 from the cells. Immunoblotting experiments also revealed that agathisflavone reduced levels of iNOS and COX-2 protein. Further studies revealed that agathisflavone reduced neuroinflammation by targeting critical steps in NF-κB signalling in BV2 microglia. Treatment of SH-SY5Y cells with conditioned medium from LPS-activated BV2 microglia produced a significant reduction in neuronal viability. However, conditioned medium from BV2 cells which were stimulated with LPS in the presence of agathisflavone did not induce neurotoxicity. Agathisflavone also produced neuroprotection in APPSwe plasmid-transfected SH-SY5Y neurons. The compound further attenuated LPS- and APPSwe plasmid-induced reduction in SIRT1 in BV2 microglia and SH-SY5Y, respectively. In the presence of EX527, agathisflavone lost its anti-inflammatory and neuroprotective activities. Our results suggest that agathisflavone inhibits neuroinflammation in BV2 microglia by targeting NF-κB signalling pathway. The compound also reduces neurotoxicity through mechanisms that are possibly linked to SIRT1 in the microglia and neurons.

Keywords

Agathisflavone, *Anacardium occidentale*, Neuroprotection, NF-κB, SIRT1

Introduction

Neuroinflammation is now known to be strongly linked to the progression of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Hou et al., 2014). Neuroinflammation has been particularly linked to the pathogenesis of AD. An important feature of neuroinflammation is excessive microglia activation and the production of large amounts of pro-inflammatory cytokines and other inflammatory mediators such as tumour necrosis factor-α (TNFα), interleukin-1β (IL-1β), nitric oxide, and reactive oxygen species (Ishihara et al., 2015). Consequently, neuroinflammation remains a critical target for novel therapeutics for AD and other neurodegenerative conditions.

Nuclear Factor-kappa B (NF-κB) is a transcription factor which regulates the production of pro-inflammatory mediators during microglia activation. Inactive NF-κB is normally complexed to the inhibitory protein IκB in the cytoplasm. However, following activation of the microglia by inflammatory stimuli such as lipopolysaccharide (LPS) or the pro-inflammatory cytokines, IκB becomes phosphorylated and degraded. This results in nuclear translocation of NF-κB (Verma et al., 1995; Ghosh et al., 1998; Cao et al., 2011), with subsequent activation resulting in pro-inflammatory gene expression (Baldwin Jr, 1996).

Natural products are an important source of new treatments for neurodegenerative disorders like AD. For example, natural polyphenols modulate the functions of a number of therapeutic targets involved in the disease. Polyphenols are known to be effective in investigations into neurodegeneration due to their antioxidant, anti-inflammatory, and antiamyloidogenic effects (Pérez-Hernández et al., 2016).

Anacardium occidentale is a medicinal plant that is commonly employed in Africa for the treatment of arthritis, fever, aches, pains, and as anti-inflammatory (Olajide et al., 2004). Previously, extracts from the plant have been shown to produce anti-inflammatory effect *in vivo* (Olajide et al., 2004; Mota et al., 1985; Pawar et al., 2000; Vilar et al., 2016). Recently, we showed that the stem bark extract of the plant inhibited NF-κB-mediated neuroinflammation in LPS-activated BV2 microglia (Olajide et al., 2013). Agathisflavone is a biflavonoid found in many plants, including *Anacardium occidentale* (Ajileye et al., 2015). This compound has been reported to produce hepatoprotective effect (Anand et al., 1992), cytotoxicity to cancer cells

(Konan et al., 2012; Ndongo et al., 2015; Taiwo et al., 2017) and antioxidant activities (Ajileye et al., 2015). In spite of the pharmacological potentials of agathisflavone, nothing is known about its ability to inhibit neuroinflammation. In this study, we explored inhibition of NF-κB-mediated neuroinflammation by agathisflavone isolated from the leaves of *A. occidentale*. We also report significant inhibition of neuroinflammation- and amyloid beta-induced neurotoxicity by the compound.

Materials and methods

Plant material

Leaves of *Anacardium occidentale* were collected in Ile-Ife, Nigeria and authenticated in the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria. A voucher specimen (IFE Herbarium: 16834) was deposited in the Herbarium. The leaves were air dried at room temperature and ground to a powder.

Extraction, isolation and characterization of agathisflavone

Activity directed isolation procedure for agathisflavone from the ethyl acetate soluble fraction of the crude extract of 80% methanol of *A. occidentale* leaves and subsequent characterisation of the compound using spectroscopic methods were as previously reported (Ajileye et al., 2015).

Cell culture

BV2 mouse microglia cell line ICLC ATL03001 (Interlab Cell Line Collection, Banca Bilogica e Cell Factory, Italy) were cultured in RPMI 1640 medium with 10% foetal bovine serum (Sigma), 2 mM L-glutamine (Sigma), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma) in a 5% CO_2 incubator. Cells were treated with LPS (100 ng/mL; from *Salmonella typhimurium*; Innaxon, UK).

SH-SY5Y human neuroblastoma cells (ECACC 94030304) were cultured in Ham's F12: EMEM (EBSS) with15% FBS, 2mM Glutamine and 1% non-essential amino acids (NEAA) in a 5% CO_2 incubator. SH-SY5Y were differentiated with retinoic acid (10 μM).

BV2 Cell viability experiment

The MTT assay was performed as described previously (Olajide et al., 2013), and was used to determine the viability of BV2 microglia which were pre-treated with agathisflavone (5-20 μM) 30 min prior to stimulation with LPS (100 ng/mL) for 24 h. After stimulation, 200 μL MTT solution (5 mg/mL) was added to cells followed by incubation at 37°C for 4 h. Then, 200 μL of medium was removed from each well, and 150 μL of DMSO solution added to wells. The plate was shaken briefly and absorbance was read at 540 nm with a plate reader (Infinite F50, Tecan).

Production of pro-inflammatory cytokines, nitrite and PGE_2

BV2 cells were treated with agathisflavone (5-20 μM) for 30 min and then stimulated with LPS for 24 h. Culture supernatants were collected and centrifuged. Concentrations of TNFα and IL-6 were measured with commercially available ELISA kits (BioLegend, UK), followed by measurements in a plate reader at a wavelength of 450 nm. Levels of nitrite in culture supernatants were measured using commercially available Griess assay kit (Promega) according to manufacturer's instructions. Absorbance was measured at 540 nm in a microplate reader. Levels of PGE_2 in supernatants were measured using commercially available enzyme immunoassay kit (Arbor Assays, Ann Arbor, Michigan, USA) according to manufacturer's instructions. Absorbance was measured at 450 nm in a microplate reader.

Detection of cellular reactive oxygen species (ROS)

The effect of LPS on intracellular ROS levels in BV2 microglia was carried out using the fluorescent 2', 7'-dichlorofluorescin diacetate (DCFDA)-cellular ROS detection assay kit (Abcam). DCFDA (10 μM) was added to cultured BV2 microglia and incubated for 30 min at 37°C. Following removal of excess DCFDA, cells were washed and then treated with agathisflavone (5-20 μM) for 30 min and then stimulated with LPS (100 ng/mL) for a further 4 h at 37°C. Intracellular generation of ROS was detected by in a microplate reader with a fluorescence excitation wavelength of 485 nm and emission wavelength of 535 nm.

Western blotting

Western blotting was carried out as described elsewhere (Olajide et al., 2013). The following primary antibodies were used: anti-rabbit iNOS (1:500, Santa Cruz), rabbit

anti-COX-2 (1:500; Santa Cruz), rabbit phospho-IκBα (1:250; Santa Cruz), rabbit anti-total IκB (1:250; Santa Cruz) and rabbit anti-actin (1:1000; Sigma). Proteins were detected with horseradish peroxidase (HRP)-coupled secondary antibodies using enhanced chemiluminescence (ECL) western blotting substrate (Licor). All Western blot experiments were carried out at least three times.

ELISA for phospho-p65

Cultured BV2 microglia were treated with agathisflavone (5-20 μM) for 30 min. This was followed by stimulation with LPS (100 ng/mL) for a further 15 min. Following stimulation, cell lysates were analysed for levels of phospho-p65 protein using an ELISA kit (eBioscience, UK), according to the manufacturer's instructions.

Immunofluorescence for NF-κB

This was carried out using a procedure described elsewhere (Velagapudi et al., 2017). BV2 cells were pre-treated with agathisflavone (5-20 μM) for 30 min followed by stimulation with LPS (100 ng/mL) for 60 min. Immunofluorescence detection of rabbit anti-NF-κB p65 antibody (Santa Cruz; 1:100) was carried with Alexa Fluor 488-conjugated donkey anti rabbit IgG secondary antibody (Life Technologies; 1:500) and images obtained using EVOS® FLoid® cell imaging station.

NF-κB reporter gene assay

In order to determine the effect of agathisflavone on NF-κB-mediated gene expression, a luciferase reporter gene assay was carried out as described earlier (Okorji et al., 2014). HEK293 cells were transfected with a Cignal® NF-κB (SABiosciences) reporter, using TransIT®-LT1 transfection reagent (Mirus Bio LLC) and incubated for a further 16 h at 37°C in 5% CO_2. Transfected HEK293 cells treated with agathisflavone (5-20 μM), and followed by stimulation with TNFα (1 ng/mL) for 6 h. NF-κB-mediated gene expression was measured with Dual-Glo luciferase assay kit (Promega, Southampton, UK) according to the manufacturer's instructions using a Polarstar Optima Plate reader.

NF-κB DNA binding assay

NF-κB DNA binding assay was carried out as earlier described (Okorji et al., 2016). DNA binding assay was carried on nuclear extracts using the TransAM NF-κB transcription factor EMSA kit (Activ Motif, Belgium), which employs a 96-well plate to

which an oligonucleotide containing the NF-κB consensus site (5' GGGACTTTCC-3') has been immobilised. Cells were treated with agathisflavone (5-20 μM) followed by LPS (100 ng/ml).

Conditioned media-induced neurotoxicity

BV2 cells were pre-treated with agathisflavone (5-20 μM) for 30 min and stimulated with LPS (1 μg/ml) for 48 h. Stimulation was terminated by removing supernatants (conditioned medium) from the cells. Supernatants were centrifuged at 2500 rpm for 5 min to remove cellular debris, and then stored at -80°C. At confluence, cultured SH-SY5Y neuronal cells were seeded at a density of 2×10^5 cells /ml in 24-well cell culture plates. Cells were allowed to settle for ~24 h in a 5% CO_2 incubator at 37°C. Later, medium was removed from the SH-SY5Y cells and replaced with conditioned medium. Cells were incubated for a further 24 h in 5% CO_2 at 37°C. Neuronal viability was determined using MTT cell viability assay.

APPSwe-transfection of SH-SY5Y neuronal Cells

pCAX APP Swe/Ind (Addgene plasmid # 30145) and pCAX APP 695 (Addgene plasmid # 30137) were gifts from Dennis Selkoe & Tracy Young-Pearse (Young-Pearse et al., 2007). SH-SY5Y cells were transiently transfected with 4 μg of each plasmid using and 10 μl of lipofectamine 2000 (Thermo Fisher Scientific) in Opti-MEM according to the manufacturer's instructions. Six hours after transfection, cells were treated with agathisflavone (5-20 μM). After 48 h, the degree of neurotoxicity was measured using MTT.

SIRT1 ELISA

Following exposure to conditioned medium or transfection with APPSwe plasmid, SH-SY5Y were assayed for SIRT1 activity using SIRT1 ELISA (Abcam).

Statistical analyses

Values of all experiments were represented as a mean ± SEM of at least 3 independent experiments. Values were compared using one-way analysis of variance (ANOVA) followed by a post-hoc Student Newman-Keuls test.

Results and discussion

Agathisflavone did not affect viability of BV2 microglia

A cell viability assay was carried out in this study to assess whether concentrations of agathisflavone used in this study affected the viability of cultured BV2 microglia. Results of the MTT viability assay in Figure 2 show that at concentrations of 5, 10 and 20 μM agathisflavone did not affect the viability of BV2 microglia. This observation suggests that any reduced signals in subsequent experiments were not due to a reduction in cell numbers.

Agathisflavone suppressed the release of pro-inflammatory cytokines in LPS-activated BV2 microglia

Recruited microglia are known to release excessive amounts of pro-inflammatory cytokines. Furthermore, mutations in genes which control the release of IL-1β, IL-6 and TNFα have been associated with increased risk of late onset AD (Papassotiropoulos et al., 1999; Nicoll et al., 2000; McCusker et al., 2001; Minter et al., 2016). In our study, stimulation of BV2 microglia resulted in a characteristic increase in the levels of pro-inflammatory cytokines TNFα, IL-6 and IL-1β (Figures 2A, B and C). However, in the presence of agathisflavone, there was a significant ($p<0.05$) and concentration-dependent reduction in the production of all the cytokines in LPS-activated BV2 microglia. Consequently, inhibition of pro-inflammatory cytokine production by agathisflavone indicates that the compound may have a promising application in AD therapeutics.

Agathisflavone prevented LPS-induced ROS generation in BV2 microglia

During chronic activation of the microglia, neurotoxic ROS (H_2O_2, O_2^-) are released to promote neurotoxicity (Block and Hong, 2005). Furthermore, there is evidence that NADPH oxidase (NOX) is activated in microglia, resulting in the formation of ROS which damages neighbouring neurons in AD (Shimohama et al., 2007). We therefore tested the effect of agathisflavone on LPS-induced ROS generation in BV2 microglia. In Figure 4, LPS is shown to produce a significant ($p<0.001$) elevation of ROS generated in BV2 microglia, when compared with unstimulated cells. Pre-treatment with agathisflavone (5-20 μM) resulted in significant ($p<0.01$) and concentration-dependent reduction in ROS production by LPS. Studies have also shown that (NOX)-mediated increase in intracellular ROS levels in microglia amplifies

pro-inflammatory cytokine release and primes the microglia to be sensitive to further stimuli (Qin et al., 2004; Block et al., 2007; Surace and Block, 2012). These results therefore suggest that reduction in elevated ROS levels in neuroinflammation would contribute to the activity of agathisflavone in neurodegeneration.

Agathisflavone reduced the production of nitric oxide and levels of iNOS protein in BV2 microglia stimulated with LPS

Excessive nitric oxide production has been identified as one of the critical factors in the pathogenesis of several neurodegenerative diseases. Furthermore, excessive release of nitric oxide in neuroinflammation leads to the formation of reactive nitrogen species and subsequent neuronal cell death (Yutse et al., 2015). We were therefore interested in determining whether agathisflavone would attenuate iNOS-mediated NO production in LPS-induced neuroinflammation. Expectedly, stimulation of BV2 microglia resulted in significant ($p<0.001$) increase in nitrite production, which was then significantly ($p<0.05$) reduced by agathisflavone in a concentration-dependent manner (Figure 5A). Similarly, LPS produced a marked increase in protein levels of iNOS (Figure 5B). Pre-treating cells with agathisflavone (5-10 μM) resulted in significant ($p<0.001$) reduction in elevated levels of iNOS protein, suggesting that the compound prevented NO production through inhibition of iNOS protein expression in LPS-activated microglia.

COX-2-mediated PGE_2 production in LPS-activated BV2 microglia was inhibited by agathisflavone

We also investigated effects of increasing concentrations of agathisflavone on PGE_2 production and COX-2 protein in LPS-stimulated BV2 microglia. Our results show that pre-treatment with agathisflavone prior to LPS stimulation produced marked reduction in PGE_2 production in BV2 microglia (~3-fold reduction with 5 μM) (Figure 6A). Also, protein levels of COX-2 were significantly ($p<0.001$) increased with LPS stimulation, compared with control unstimulated cells. When we pre-treated the cells with agathisflavone (5-20 μM), there was significant ($p<0.05$) and concentration-dependent reduction in COX-2 protein expression (Figure 6B).

Agathisflavone targets IκB/NF-κB activation to inhibit neuroinflammation in BV2 microglia

Encouraged by results showing that agathisflavone inhibited neuroinflammation in LPS-activated BV2 microglia, we decided to determine whether the compound acts through activation of IκB/NF-κB signalling. Firstly, we were interested to determine whether agathisflavone would produce a general effect on NF-κB-dependent gene expression. To achieve this we transfected HEK293 cells with a plasmid construct bearing a luciferase reporter gene under the control of NF-κB. Figure 7A shows that stimulation of transfected HEK293 cells with TNFα (1 ng/mL) resulted in significant ($p<0.001$) NF-κB-driven luciferase expression. However, on treating the cells with agathisflavone (5-20 μM), there was a concentration-dependent reduction in luciferase activity.

We next investigated individual steps in the signalling pathway involved in the activation of this transcription factor. One of the early steps in the activation of NF-κB following LPS stimulation is the phosphorylation and degradation of IκB (the inhibitor of NF-κB) (Hayden and Ghosh, 2012). Using immunoblotting, we showed that agathisflavone (10 and 20 μM) produced significant ($p<0.001$) inhibition of LPS-induced IκB phosphorylation in BV2 microglia; at a concentration of 5 μM, the compound did not produce an inhibitory effect. (Figure 7B). Interestingly, significant ($p<0.05$) inhibition of LPS-induced degradation of IκB was observed at all tested concentrations of the compound (Figure 7B). These data suggest that agathisflavone possibly acts to inhibit NF-κB signalling in LPS-activated microglia by preventing events which result in its nuclear translocation. This was confirmed with an ELISA, which shows a significant ($p<0.01$) inhibition of LPS-induced elevation of the levels of phosphorylated p65 subunit by agathisflavone (5-20 μM) (Figure 7C). Also, immunofluorescence experiments revealed that nuclear accumulation of p65 subunit as a result of LPS stimulation was reduced in the presence of agathisflavone (Figure 7D).

Finally, we carried out a DNA binding assay to establish the effect of agathisflavone on DNA binding of NF-κB following stimulation with LPS. Figure 7E shows that that stimulation of BV2 cells with LPS (100 ng/mL) resulted in an increase in DNA binding of NF-κB, when compared with unstimulated control cells. When cells were treated

with agathisflavone (5-20 μM), there was a concentration-dependent and significant ($p<0.001$) reduction of DNA binding. These results confirm that agathisflavone acts on the early stages of NF-κB signalling pathway following LPS stimulation of BV2 microglia. The results also confirm that the compound prevents neuroinflammation by targeting NF-κB.

Several flavonoids have been shown to exert NF-κB inhibitory activity in neuroinflammation. Similar results have been reported for flavonoids such as tiliroside, kolaviron (a biflavonoid in *Garcinia kola*), genistein, luteolin and apigenin (Velagapudi et al., 2014; Onasanwo et al., 2016; Jang et al., 2010; Ha et al., 2008; Park et al., 2007).

Agathisflavone protected SH-SY5Y cells from neuroinflammation-and amyloid-mediated neurotoxicity

A strong link now exists between neuroinflammation and neurodegeneration (Calsolaro and Edison, 2016). Based on our observation that agathisflavone inhibits neuroinflammation in BV2-stimulated microglia, we were encouraged to evaluate neuroprotective effect of the compound. Firstly, we treated BV2 microglia with agathisflavone (5-20 μM) prior to stimulation with a high concentration (1 μg/mL) LPS. The resulting conditioned medium was added to cultured SH-SY5Y to induce characteristic neurotoxicity. Expectedly, exposure of SH-SY5Y to conditioned medium from LPS-stimulated microglia resulted in marked reduction in cell viability (Figure 8A). On exposing the cells to conditioned media from BV2 microglia pre-treated with agathisflavone (5-20 μM) prior to LPS, cell viability was significantly ($p<0.05$) increased in a concentration-related manner. It appears that the ability of agathisflavone to inhibit neuroinflammation in the microglia resulted in reduced levels of neurotoxic mediators in the conditioned media. Activation of the microglia is a critical contributor to neurotoxicity due to the production of the pro-inflammatory cytokines, ROS, NO and PGE2 (Gao et al., 2011). These results therefore suggest that neuroinflammation-mediated toxicity was attenuated by agathisflavone, resulting in a neuroprotective activity.

We also tested the effect of agathisflavone on amyloid-induced neurotoxicity, by transfecting SH-SY5Y with APPSwe plasmid and then treating cells with graded concentrations of the compound. MTT cell viability assay shows that APPSwe

transfection of SH-SY5Y cells resulted in reduced cell viability, which was prevented when cells were pre-treated with agathisflavone (Figure 8B). This observation suggests that agathisflavone may be exerting a direct neuroprotective effect against amyloid beta in neuronal cells.

Neuroprotective activity of agathisflavone is mediated through SIRT1

Several mechanisms are known to mediate neuroinflammation and neurotoxicity as well as their prevention in both microglia and neurons. Emerging evidence suggests that the sirtuins, especially SIRT1 are linked to neuroprotection in both the microglia and neurons (Tang and Chua, 2008; Yeung et al., 2004).

Having observed that agathisflavone produced neuroprotection through anti-inflammatory effect in activated microglia, and through a direct action on neuronal cells, we were interested in determining whether the actions of the compound are attributable to possible increase in SIRT1 activity. Firstly, we investigated effects of agathisflavone on SIRT1 levels in BV2 microglia stimulated with LPS and observed that LPS stimulation resulted in significant ($p<0.01$) reduction in SIRT1 levels when compared to unstimulated cells (Figure 9A). On pre-treating cells with agathisflavone (5-20 μM) prior to LPS, there was a significant increase in the levels of SIRT1 (Figure 9A). It is likely that enhancement of SIRT1 levels by agathisflavone contributes to anti-inflammatory activity (Yeung et al., 2004), and hence attenuation of neuroinflammation-induced neuronal damage.

Similar results were obtained when SH-SY5Y neuronal cells were transfected with APPSwe plasmid. There was a marked reduction in SIRT1 levels resulting from Aβ effect (Figure 9B). When we treated cells with agathisflavone (5-20 μM) following exposure to APPSwe plasmid, we observed a significant ($p<0.05$) restoration in SIRT1 levels. Studies have shown that the expression of SIRT1 was significantly decreased while that of APP was increased in AD patients and in SK-N-SH cells treated with amyloid beta (Hou et al., 2016). This report is consistent with our findings showing lowered levels of SIRT1 following APPSwe transfection of SH-SY5Y neuronal cells. Agathisflavone is therefore likely producing neuroprotection in neurons through epigenetic mechanisms similar to DNA methylation inhibitor 5-aza-2′-deoxycytidine and the histone deacetylase inhibitor trichostatin A.

To confirm the role of SIRT1 in the neuroprotective effect of agathisflavone, we compared viability of SH-SY5Y exposed to conditioned medium from LPS-stimulated BV2 microglia treated with agathisflavone (20 μM) and medium from microglia treated with agathisflavone and SIRT1 inhibitor, EX527 (1 μM). In Figure 10A, exposure to conditioned medium shows a characteristic significant ($p<0.01$) reduction in cell viability, prevented when pre-treated with agathisflavone (20 μM). Neuroprotection by agathisflavone and against APPSwe transfection-induced neurotoxicity was significantly ($p<0.01$) reversed by EX527, suggesting that SIRT1 contributes to the effects of agathisflavone in both LPS-stimulated BV2 microglia ans amyloid beta-induced neuronal death.

Inhibition of NF-κB-mediated neuroinflammation by a stem bark extract of *A. occidentale* has been previously reported (Olajide et al., 2013) but this study shows that the leaf also contains bioactive compounds, one of which, agathisflavone, we have identified as being neuroprotective. This is the first report of its effects in APPSwe-induced neurotoxicity and, to our knowledge, the first evidence of the role played by SIRT1 in the anti-inflammatory and neuroprotective properties of a bioactive isolated from *A. occidentale*.

Conclusions

Taken together, our results suggest that agathisflavone inhibits neuroinflammation in BV2 microglia by targeting NF-κB signalling pathway. The compound also attenuated neuroinflammation- and amyloid beta –mediated neurotoxicity through mechanisms that are possibly linked to epigenetic mechanisms involving SIRT1 in the microglia and neurons.

Conflicts of interest

Authors declare no conflicts of interest.

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Figure Legends

Figure 1

Structure of agathisflavone

Figure 2

Agathisflavone (5-20 μM) did not affect the viability of BV2 microglia stimulated with LPS (100 ng/ml) following incubation for 24 h. Cell viability was determined using MTT assay.

Figure 3

Pre-treatment with agathisflavone (5-20 μM) prior to stimulation with LPS (100 ng/ml) produced significant reduction in the secretion of pro-inflammatory cytokines TNFα (A), IL-6 (B) and IL-1β (C). Cytokine levels were determined in culture supernatants 24 h after stimulation, using mouse ELISA kits for TNFα, IL-6 and IL-1β. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, *p<0.05, **p<0.01, ***p<0.001 in comparison with LPS control.

Figure 4

Agathisflavone reduced generation of cellular ROS in LPS-activated BV2 microglia. BV2 cells were treated with agathisflavone (5-10 μM) for 30 min prior to LPS stimulation for 24 h. ROS generation was measured in live cells by the fluorescence detection of dichlorofluorescein. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, **p<0.01, ***p<0.001 in comparison with LPS control.

Figure 5

Agathisflavone produced iNOS-mediated inhibition of NO production in LPS stimulated BV2 microglia. BV2 cells were treated with agathisflavone (5-20 μM) for 30 min prior to LPS (100 ng/ml) stimulation for 24 h. (A) Culture supernatants were analysed for nitrite production using the Griess assay. (B) Cell lysates were analysed using immunoblotting for iNOS and actin. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for

multiple comparison with post-hoc Student Newman-Keuls test, *p<0.05, **p<0.01, ***p<0.001 in comparison with LPS control.

Figure 6

Agathisflavone produced COX-2-mediated inhibition of PGE_2 release in LPS stimulated BV2 microglia. BV2 cells were treated with agathisflavone (5-20 μM) for 30 min prior to LPS (100 ng/ml) stimulation for 24 h. (A) Culture supernatants were analysed for PGE_2 production using an enzyme immunoassay (EIA) for PGE_2 (B) Cell lysates were analysed using immunoblotting for COX-2 and actin. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, *p<0.05, ***p<0.001 in comparison with LPS control.

Figure 7

Inhibition of neuroinflammation by agathisflavone is mediated by targeting NF-κB signalling. (A) Agathisflavone (5-20 μM) supressed NF-κB luciferase activity in HEK273 cells transfected with pGL4.32 [luc2P/NF-κB RE/Hygro] vector and stimulated with TNFα (1 ng/ml) for 6 h. (B) BV2 cells were treated with agathisflavone (5-20 μM) for 30 min prior to LPS stimulation for 60 min. Cell lysates were analysed using immunoblotting for phospho- and total IκB. (C) Agathisflavone reduced phosphorylation of p65 sub-unit in LPS-activated BV2 microglia. BV2 cells were treated with agathisflavone (5-20 μM) prior to stimulation with LPS for 60 min. Cell lysates were analysed using immunoblotting for phospho-p65 and actin. (D) Agathisflavone prevented nuclear localisation of p65 subunit in LPS-stimulated BV2 microglia. BV2 cells were treated with agathisflavone (5-20 μM) prior to LPS (100 ng/ml) for 60 min. Immunofluorescence experiments were carried out to detect p65 protein localisation using an anti-p65 antibody and Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibody. Cells were counterstained with DAPI and fluorescence images acquired with an EVOS® FLoid® cell imaging station (scale bar: 100 μm). (E) Agathisflavone inhibited DNA binding of NF-κB in LPS-stimulated BV2 microglia. Nuclear extracts from cells were added to 96-well plates to which an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3') has been immobilised, followed by addition of NF-κB and HRP-conjugated antibodies. Absorbance was read in a microplate reader. All quantitative experimental values are

expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, *p<0.05, **p<0.01, ***p<0.001 in comparison with LPS control.

ns: not significant

Figure 8

Agathisflavone is neuroprotective against neuroinflammation- and amyloid beta-induced neuronal toxicity. (A) Cultured SH-SY5Y neurons were exposed to conditioned media from LPS (1 µg/ml)-stimulated BV2 microglia that have been pre-treated with agathisflavone (5-20 µM). Neuronal viability was determined using MTT assay. (B) SH-SY5Y neurons were transfected with APPSwe plasmid and then treated with agathisflavone (5-20 µM). Viability of the neurons was measured using MTT viability assay. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, *p<0.05, **p<0.01, ***p<0.001 in comparison with conditioned medium or APPSwe control.

Figure 9

Agathisflavone increases levels of SIRT1 in LPS-activated microglia and APPSwe-transfected SH-SY5Y neurons. (A) BV2 microglia were stimulated with LPS (100 ng/ml) in the presence or absence of agathisflavone (5-20 µM) for 24 h. Levels of SIRT1 were then measured in nuclear extracts using mouse SIRT1 ELISA. (B) SH-SY5Y neuronal cells were transfected with APPSwe plasmid and then treated with agathisflavone (5-20 µM). Levels of SIRT1 were then measured in nuclear extracts using human SIRT1 ELISA. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, *p<0.05, **p<0.01, in comparison with LPS or APPSwe control.

Figure 10

Neuroprotection by agathislavone is reversed in the presence of SIRT1 inhibitor EX527 (1 µM). (A) Cultured SH-SY5Y neurons were exposed to conditioned media from LPS (1 µg/ml)-stimulated BV2 microglia that have been pre-treated with agathisflavone (20 µM) or EX527 (1 µM) + agathisflavone (20 µM). Neuronal viability was determined using MTT assay. (B) SH-SY5Y neurons were transfected with

APPSwe plasmid and then treated with agathisflavone (20 μM) or EX527 (1 μM) + agathisflavone (20 μM). Neuronal viability was determined using MTT assay. Viability of the neurons was measured using MTT viability assay. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, **$p<0.01$, in comparison with cells with no conditioned medium exposure, ***$p<0.001$ in comparison with non-transfected cells, $^{\#}p<0.05$ in comparison with cells exposed to conditioned medium, $^{\#\#\#}p<0.001$ in comparison with APPSwe transfected cells, $^{\&\&}p<0.01$ agathisflavone (20 μM) treatment versus EX527 (1 μM) + agathisflavone (20 μM) treatment.

Figure 1

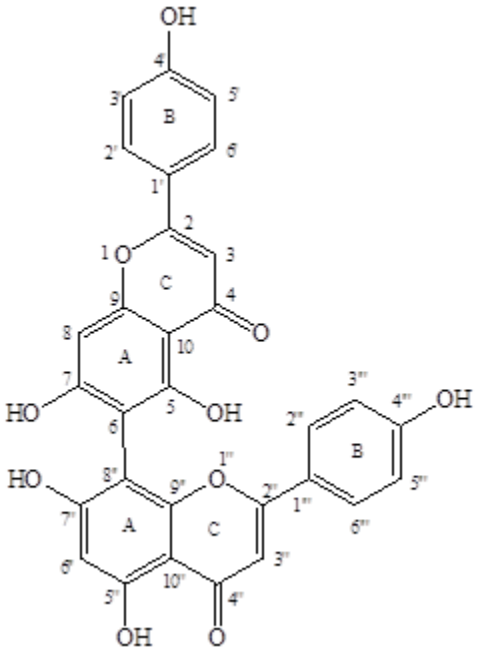

OH
4'
3'
5'
B
2'
6'
1'
2
1
O
3
C
9
4
O
8
A
10
7
3''
4''
OH
HO
5
OH
2''
6
B
1''
HO
8'
9'
O
2'
5''
7'
A
C
1''
6''
6'
3''
10'
5'
4'
OH
O

Figure 2

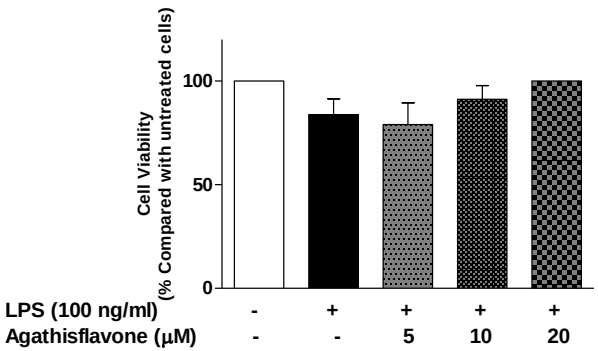

Cell Viability
(% Compared with untreated cells)
100
50
0
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20

Figure 3

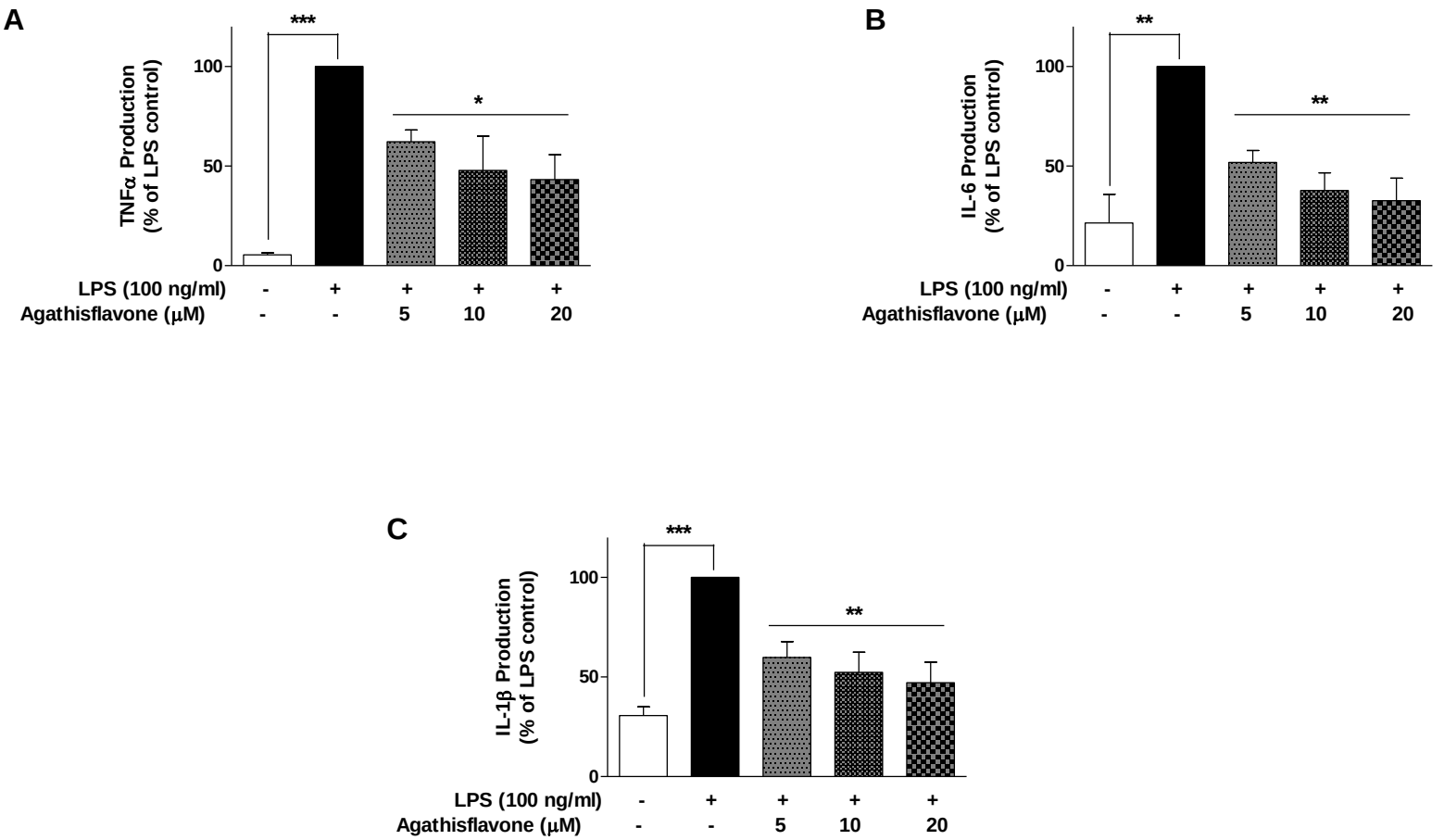

A
***
*
100
50
0
TNFα Production
(% of LPS control)
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20
B
**
**
100
50
0
IL-6 Production
(% of LPS control)
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20
C
***
**
100
50
0
IL-1β Production
(% of LPS control)
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20

Figure 4 **ROS Generation**

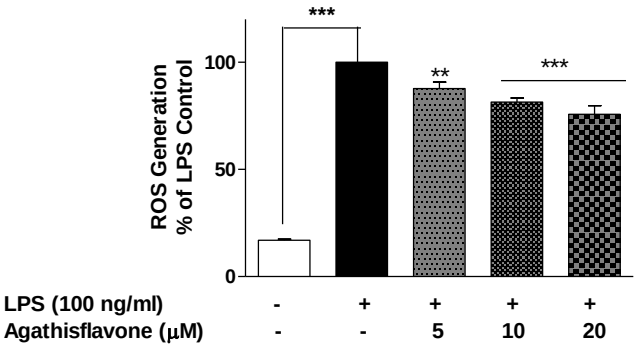


Figure 5

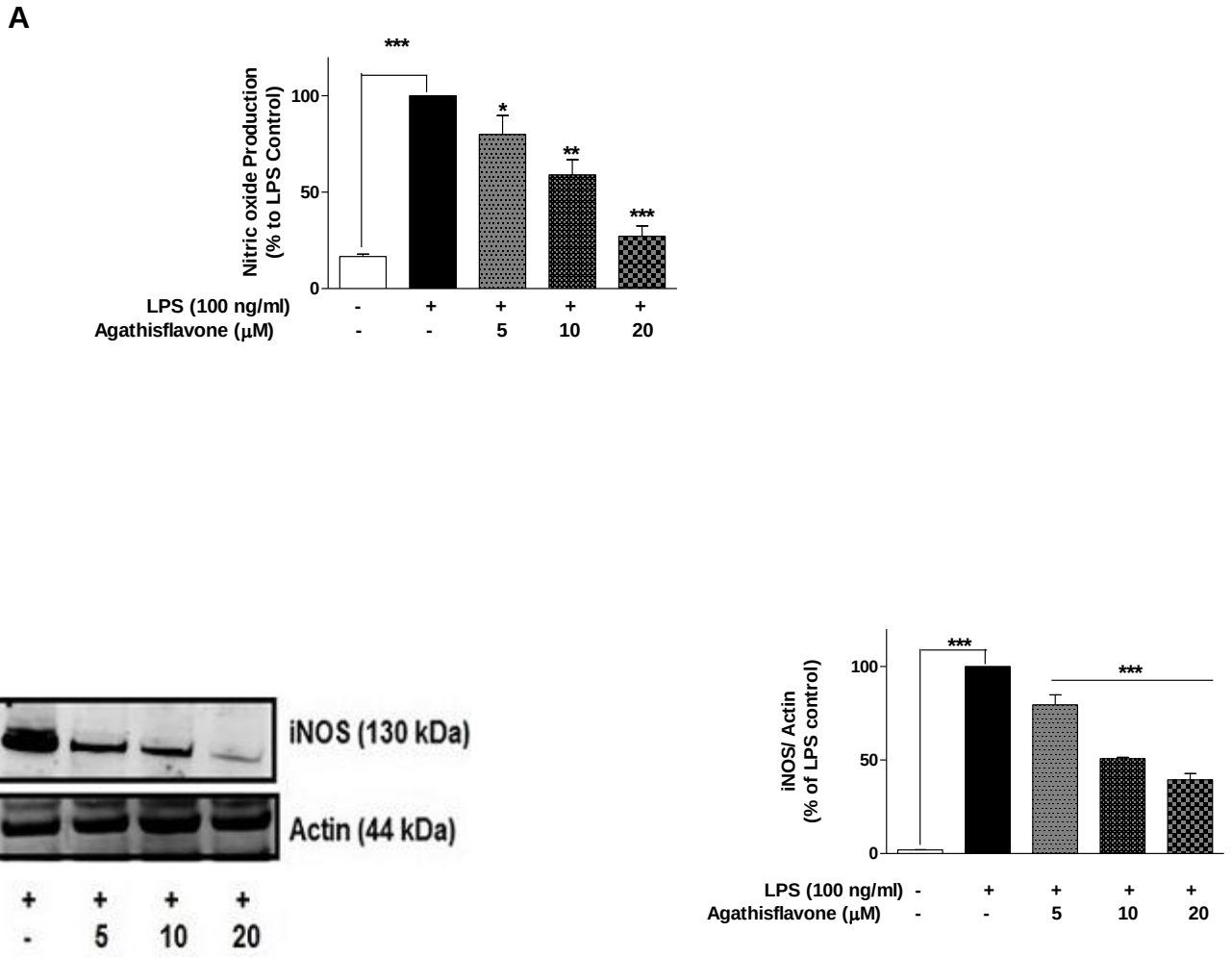

A
Nitric oxide Production (% to LPS Control)
100
50
0
***
*
**
***
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20
B
iNOS (130 kDa)
Actin (44 kDa)
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20
iNOS/ Actin (% of LPS control)
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20

Figure 6

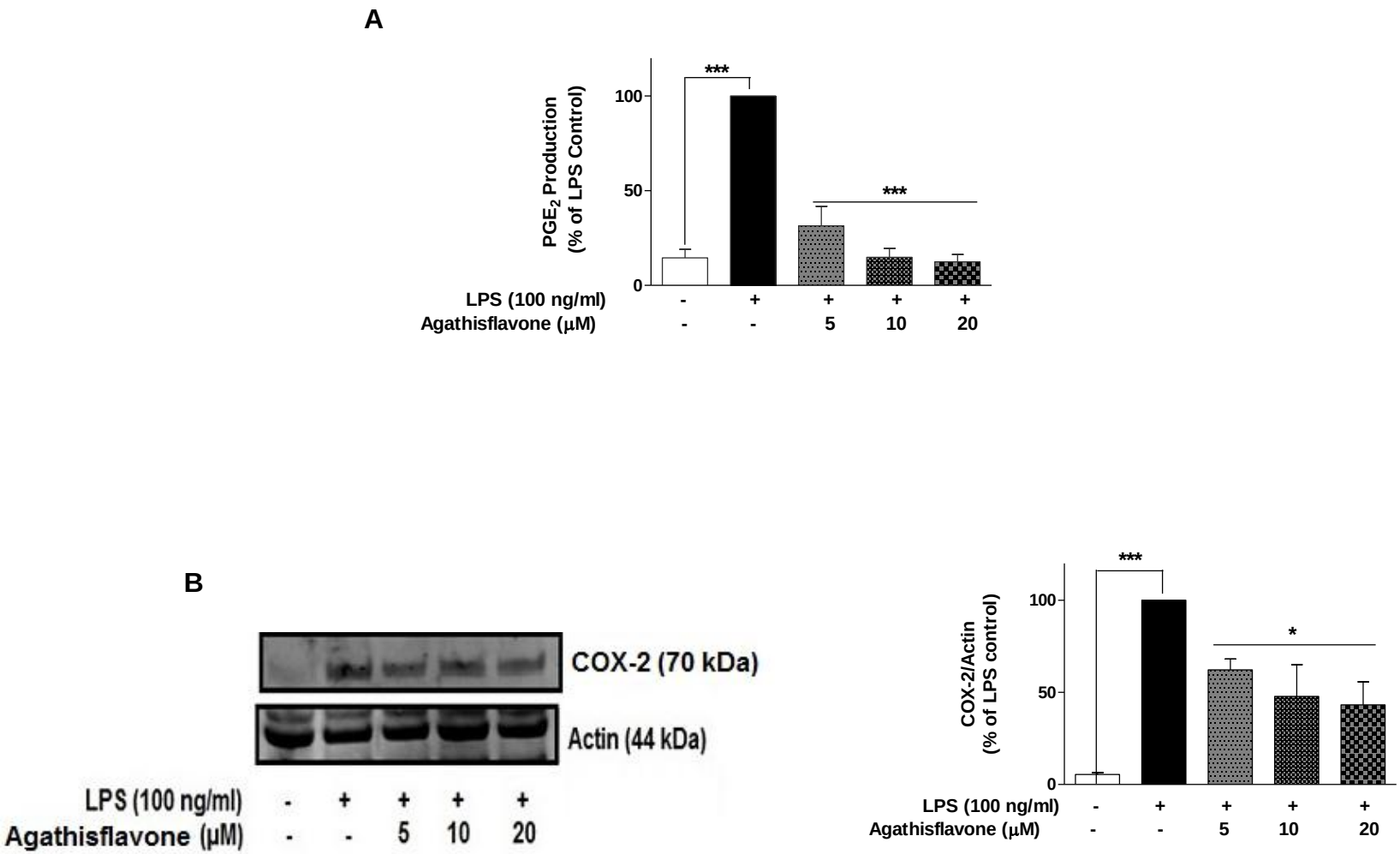

A
PGE2 Production (% of LPS Control)
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20
B
COX-2 (70 kDa)
Actin (44 kDa)
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20
COX-2/Actin (% of LPS control)
100
50
0
***
*
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20

Figure 7

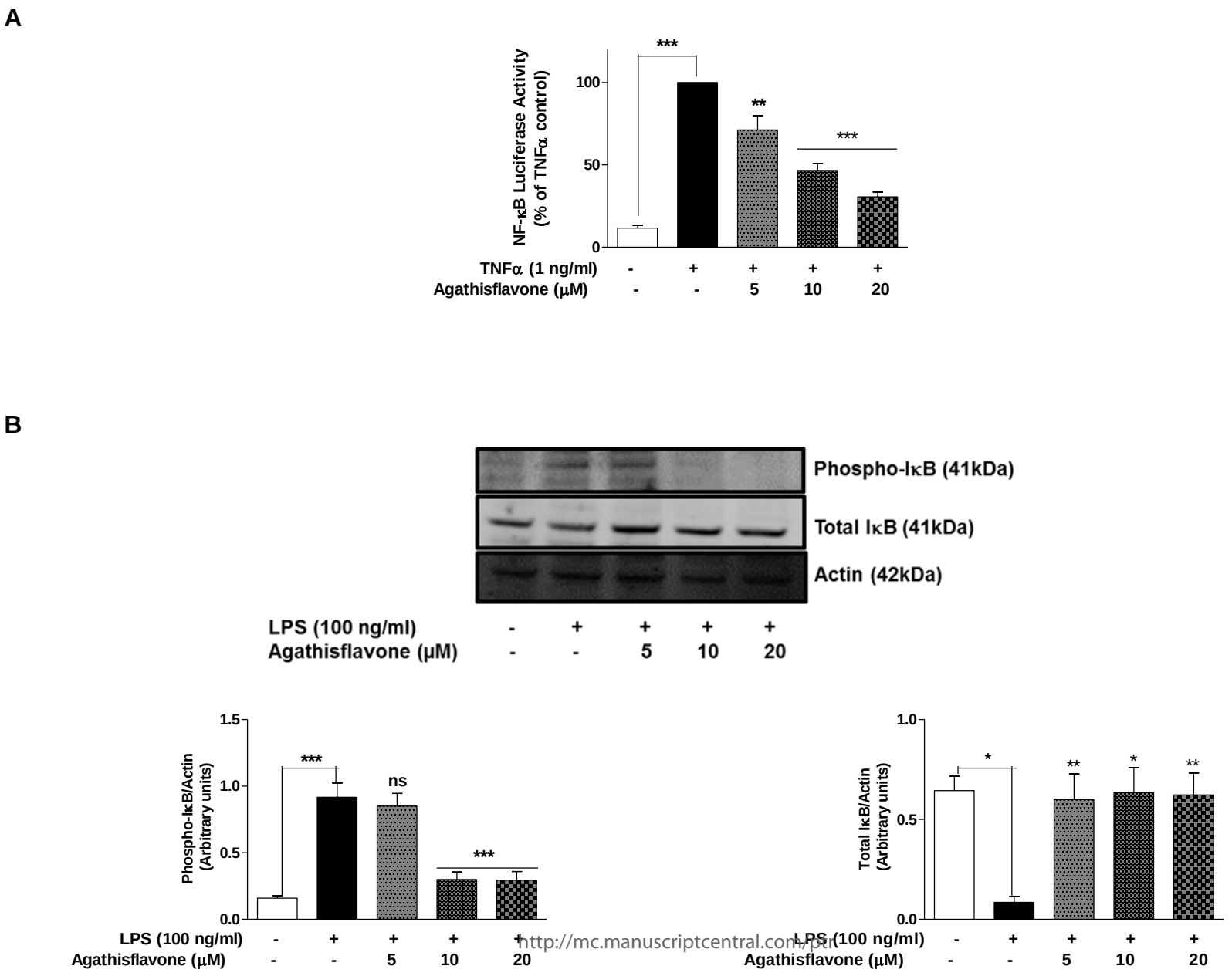
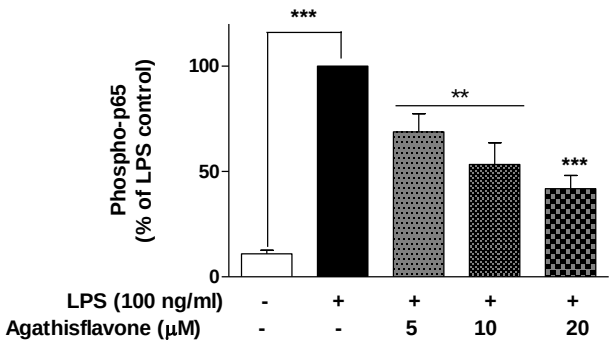


Figure 7

C


***
100
**
50
***
0
Phospho-p65
(% of LPS control)
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20

D

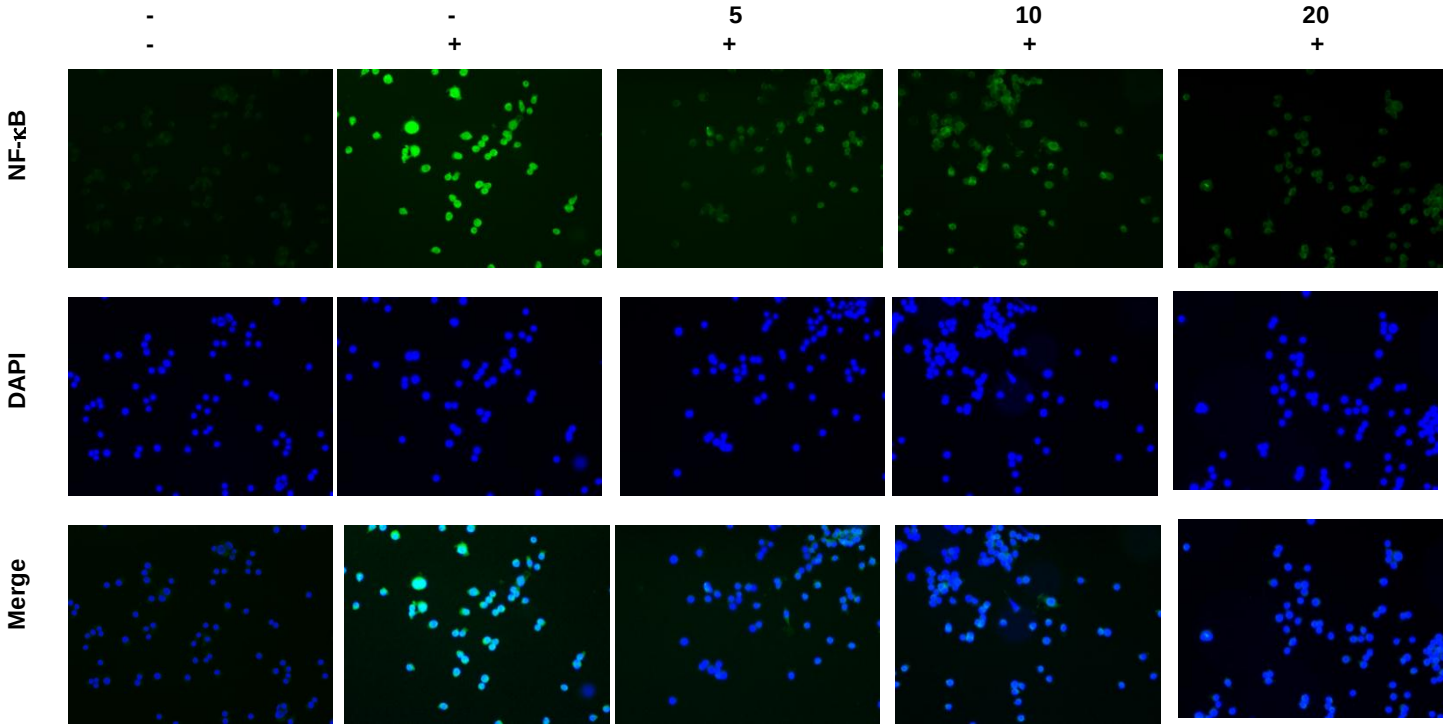


Figure 7

E

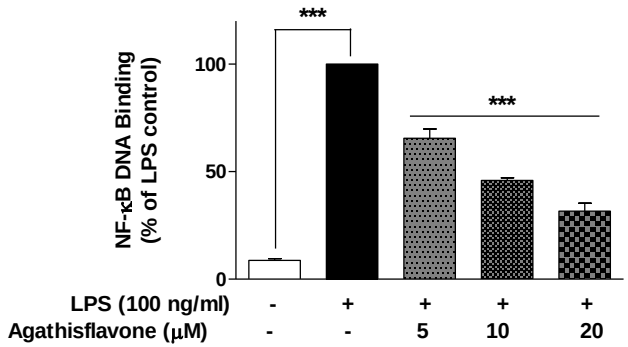

***
***
NF-κB DNA Binding
(% of LPS control)
100
50
0
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20

Figure 8

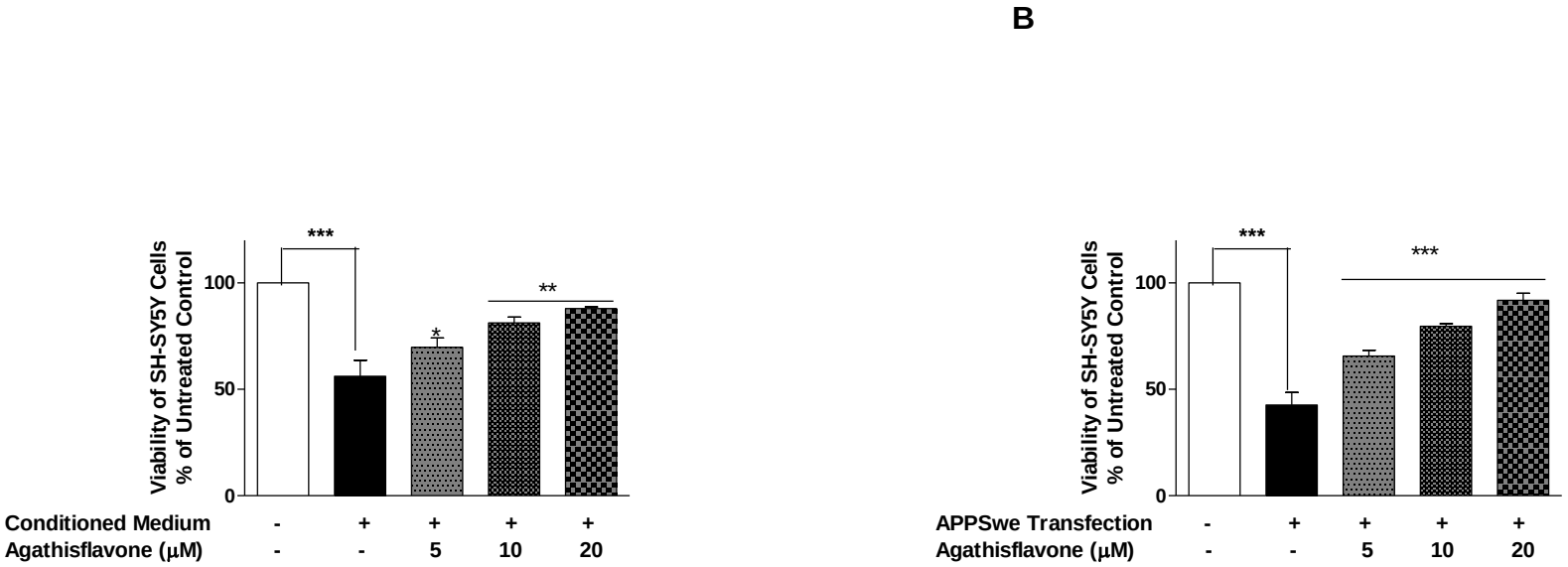
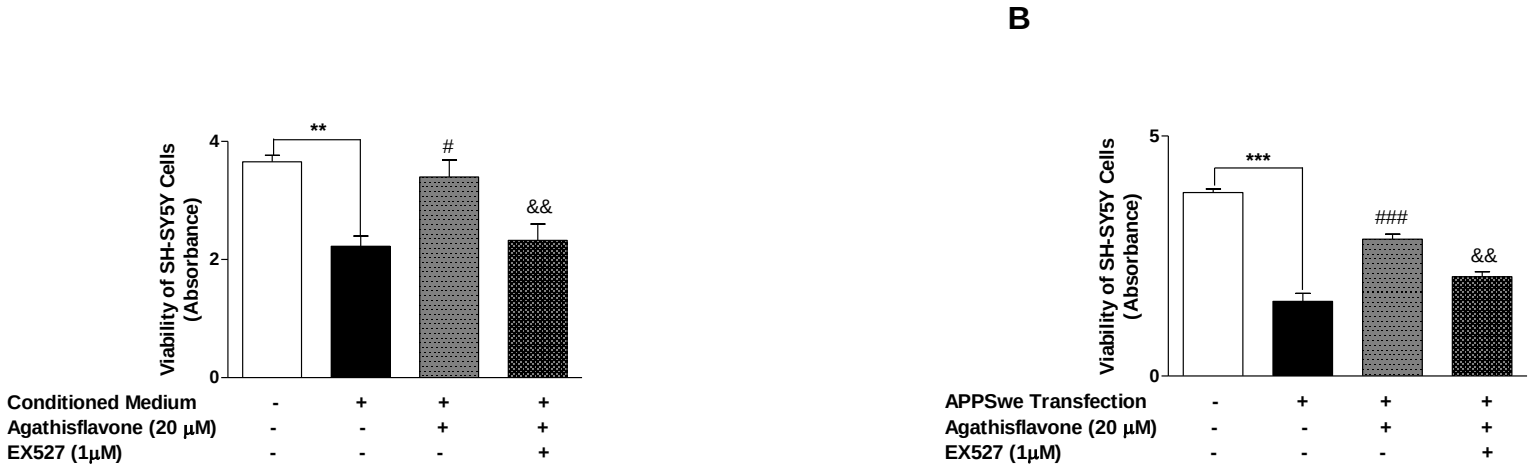

A
Viability of SH-SY5Y Cells
% of Untreated Control
100
50
0
***
*
**
Conditioned Medium - + + + +
Agathisflavone (μM) - - 5 10 20
B
Viability of SH-SY5Y Cells
% of Untreated Control
100
50
0
***
***
APPSwe Transfection - + + + +
Agathisflavone (μM) - - 5 10 20

Figure 9


A
Concentration of SIRT1 (μg/ml)
100
50
0
**
*
LPS (100 ng/ml) - + + + +
Agathisflavone (μM) - - 5 10 20
B
Concentration of SIRT1 (μg/ml)
100
50
0
**
*
**
APPSwe Transfection - + + + +
Agathisflavone (μM) - - 5 10 20

Figure 10


A
Viability of SH-SY5Y Cells (Absorbance)
4
2
0
**
#
&&
Conditioned Medium - + + +
Agathisflavone (20 μM) - - + +
EX527 (1μM) - - - +
B
Viability of SH-SY5Y Cells (Absorbance)
5
0
***
###
&&
APPSwe Transfection - + + +
Agathisflavone (20 μM) - - + +
EX527 (1μM) - - - +