PROTECTION AGAINST ALZHEIMER'S DISEASE

USING OLIVE-POMACE OIL TO ATTENUATE

MICROGLIA ACTIVATION

FINAL ACTIVITY REPORT

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BACKGROUND

Microglia are resident macrophage cells and play a key role in most neurological diseases, including Alzheimer's disease. Microglial activity leads to the release of a large amount of pro-inflammatory mediators, mediated by changes in the brain's oxidative state. There is sufficient evidence to indicate that some of the fat-soluble components of olive-pomace oil could simultaneously modulate the oxidative stress and inflammatory response in microglia by being transported in the bloodstream as triglyceride-rich lipoproteins (TRL). The modulation of these phenomena could delay or slow down the development of neurodegenerative diseases like Alzheimer's.

Hypothesis

TRLs can activate microglia and lead to the release of pro-inflammatory substances, but their activation could be attenuated by adding the following bioactive components from olive-pomace oil: α-tocopherol, β-sitosterol, and oleanolic acid.

METHODS

The study involved two phase *in vitro* experimentation, modelling using type BV-2 microglia cells from mice.

In the first phase, the cells were pre-incubated for 1 hour with the bioactive components of olive-pomace oil, α-tocopherol, β-sitosterol, and oleanolic acid, in order to evaluate their potential protective effect against lipopolysaccharide activation (LPS, 100 ng/mL). The following test concentrations were used:

Oleanolic acid: 0.5 µM, 1 µM, 10 µM, 25 µM.

α-Tocopherol: 0.5 µM, 1 µM, 10 µM, 50 µM.

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β-Sitosterol: 0.5 µM, 1 µM, 10 µM, 25 µM.

The tested doses were chosen based on similar, previously published studies for those same compounds, although different cell types were used in those cases. The compounds were tested individually or in the form of a mix at different concentrations.

Concentration A: Oleanolic acid: 0.5 µM, α-Tocopherol: 0.5 µM and β-Sitosterol: 0.5 µM.
Concentration B: Oleanolic acid: 1 µM, α-Tocopherol: 1 µM and β-Sitosterol: 1 µM.
Concentration C: Oleanolic acid: 5 µM, α-Tocopherol: 10 µM and β-Sitosterol: 10 µM.
Concentration D: Oleanolic acid: 10 µM, α-Tocopherol: 50 µM and β-Sitosterol: 25 µM.

Additionally, free TRLs or TRLs containing α-tocopherol, β-sitosterol, and oleanolic acid were prepared at a 10 µM concentration, at which a greater protective effect was observed. The prepared TRLs were incubated with those same cells, which were added based on their triglyceride concentration (0.15 µmol per mL of culture medium).

The olive-pomace oil bioactive compounds were tested at different doses to see whether they caused any cell toxicity prior to testing for the capacity to attenuate glial activity. This involved using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method, which measures cell viability. The method was used on the three compounds four times.

The treatments were maintained for 24 hours for cells stimulated with both LPS and TRL, after which samples were taken of the cells and their culture mediums to analyse for the production of pro-inflammatory cytokines using immunological techniques (ELISA).

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RESULTS

1. Cell viability study

As can be seen in Figure 1 (Annex), **none of the tested vehicles (DMSO and ethanol) had a cytotoxic effect on the BV-2 cells**. This result derived in the decision to use DMSO as a vehicle. In any case, both vehicles slightly increased cell viability, to above 100%, although not to a significant amount. When it comes to oleanolic acid, the three lowest concentrations tested (0.5 µM, 1 µM, and 10 µM) offered a cell viability over 80%. However, **at the 25 µM concentration, the viability was around 50%**. For this reason, **this dose was eliminated from the test, with a 5 µM intermediate dose included**. α-tocopherol did not have a cytotoxic effect at any of the tested concentrations (Figure 2), with viability remaining above 80% in every case. All the same, a certain level of protective effect was encountered at the highest concentration (50 µM), indicating viability above 100%. Lastly, each β-sitosterol tested concentration resulted in 80% viability or higher (Figure 3). The lowest concentration (0.5 µM) produced a protective effect, although we don't consider it significant. No statistical analysis was undertaken during this phase as it was not necessary. On the other hand, the two highest concentrations (10 µM and 25 µM) caused 20% cell death, with viability remaining at 80%, the minimum acceptable level.

2. Protective effect after stimulation with LPS

2.1.Cytokine production

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Pre-treating BV-2 cells with oleanolic acid led to the production of the pro-inflammatory cytokines, interleukin 1β (IL-1β) (Figure 4), interleukin-6 (IL-6) (Figure 8), and tumor necrosis factor-α (TNF-α) derived from LPS stimulation (Figure 12). The response was dose-dependent for IL-1β and IL-6, **showing a greater effect at the maximum tested concentration (10 µM)**. In the case of TNF-α, all concentrations of oleanolic acid caused a very significant reduction in the production of this cytokine.

Adding α-tocopherol led to a decrease in IL-1β and IL-6 production, **which was also dose-dependent (Figures 5 and 9). In this case, significant differences were found at the 1 µM, 10 µM, and 50 µM concentrations**. However, α-tocopherol did not manage to reduce TNF-α release at any of the tested concentrations (Figure 13). In the case of β-sitosterol, it reduced IL-1β (Figure 6) and TNF-α (Figure 14) levels, but not IL-6, which increased as the dose increased in the culture medium (Figure 10), showing the opposite effect to what was expected.

When these compounds were analysed together, we observed a **significant reduction of IL-1β at the highest concentrations, and IL-6 and TNF-α at all concentrations**. In all cases, the mixes achieved at least a 50% reduction in cytokine production, which shows its additive capacity, although it is unlikely to have a synergic effect.

2.2. Reactive oxygen species production

The BV-2 cells were stimulated with LPS in the same way as in the previous experiment, but hydrogen peroxide was added in this case. ROS formation was subsequently determined using flow cytometry. The results are presented as a percentage of the positive control (LPS). Treatments with oleanolic acid (Figure 13) and α-tocopherol (Figure 14) gave rise to a **reduction in dose-dependent ROS formation,** which was statistically significant at the highest concentrations: 10 µM in the case of oleanolic acid and 50 µM in the case of α-tocopherol.

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On the other hand, **β-sitosterol again had an opposite effect**, given that the reductive effect on ROS release decreased as the dose in the culture medium increased (Figure 15). On using bioactive compounds as part of a mix, a **significant reduction in ROS was observed only at the highest concentration,** which did not surpass the combined reductions from the individual compounds.

2.3. Total glutathione

Adding LPS to the BV-2 microglia cells culture medium derived in a 100% reduction of the cellular content of total glutathione, a systemic peptide that provides antioxidant defence, compared to unstimulated cells (Figures 20-23). On the other hand, **treating cells with the three bioactive compounds under study managed to restore cellular glutathione levels**. The 10 µM concentration of oleanolic acid (Figure 20) and α-tocopherol (Figure 21) ended up being more effective. This same concentration and the next highest concentration (25 µM) had the greatest effect on cells treated with β-sitosterol (Figure 22). The mix of those three compounds also managed to restore cellular ROS levels, even **above levels in cells that were not activated with LPS** (Figure 23).

Given the results, the 10 µM dose was concluded as being most effective for the three bioactive compounds under study for studies with TRL.

3. Protective effect after stimulation with TRL

3.1.Cytokine production

Adding laboratory-prepared artificial TRLs to the microglia culture medium led to an **increase in the concentrations of cytokines IL-1β, IL-6, and TNF-α** (Figures 24-26). The increase was lower than previously observed after stimulation with LPS, but significant ($p<0.05$) compared to the negative control (saline solution) for the three cytokines, **showing for the first time that**

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TRLs can activate the mouse microglia BV-2 cell line, causing a pro-inflammatory effect. The tested compounds caused a reduction in IL-1β levels in the culture medium, but they weren't statistically significant, apart from with the mix of compounds (Figure 24). Adding α-tocopherol significantly reduced IL-6 concentration in the culture medium (Figure 25). In the latter case, no significant effect from the mix of compounds was found, probably because an opposite effect was observed with β-sitosterol again. In the same way, the reduction caused by oleanolic acid was not significant. On the other hand, this compound caused a very significant reduction in TNF-α release in the cells, resulting in a similarly very significant effect for the mix of compounds (Figure 27).

INTERPRETATION OF THE RESULTS

The cytotoxicity tests helped to understand which doses were suitable for treating BV-2 microglia cells with the bioactive compounds. Every tested dose was either non-toxic or very slightly toxic for the cells, except at the 25 µM oleanolic acid concentration, meaning that this dose needed to be replaced with a lower dose.

In the cell activation studies with LPS, it was observed that the three compounds, **α-tocopherol, β-sitosterol, and oleanolic acid, were capable of reducing IL-1β pro-inflammatory cytokine release** in the cells, and were dose-dependent, with the highest concentrations (above 10 µM) having a more significant effect. In the same way, treatment with **α-tocopherol and oleanolic acid had a reducing effect on IL-6 production**, also inflammatory in nature. On the other hand, β-sitosterol had a different effect. At lower concentrations, IL-6 release was observed to reduce, but this effect decreased as the treatment dose was increased. This effect could be due to a hormesis phenomenon, although more

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research is needed to confirm this. The effect of the compounds on TNF-α was very significant when the cells were treated with oleanolic acid, β-sitosterol, and the mix of compounds. **In short, the data shows that minor compounds of olive-pomace oil have a high capacity for attenuating inflammatory response in mouse microglia cells when activated with LPS.**

Stimulation with LPS led to an increase in ROS production. These reactive species were shown to be capable of acting as inflammatory process triggers, given that they activate the associated metabolic pathways. In our study, we confirmed that each bioactive compound from olive-pomace oil was capable of reducing ROS release, with the subsequent reduction in inflammatory cytokine production. This ROS reducing effect is probably due to the capacity of those bioactive compounds to restore antioxidant defence system levels. In fact, each tested compound managed to increase glutathione levels to some extent, which is one of the main neutralising peptides for toxic reactive species, such as ROS, and maintains cell redox balance.

It is important to point out that for the first time, **adding artificial TRLs was observed to increase the concentrations of IL-1β, IL-6, and TNF-α cytokines** in microglial cells, which would support our hypothesis that postprandial TRLs are involved in causing inflammation in the brain, mediated by microglia cells, which is noted with Alzheimer's disease. In this case, the effects of the bioactive compounds of olive-pomace oil were not as striking. The substances were released at reduced levels, but did not reach statistical significance in some cases. Even so, **α-tocopherol caused a very significant reduction in IL-6 release and oleanolic acid had the same effect on TNF-α. Adding the compounds together reduced the production of IL-1β and TNF-α.**

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CONCLUSIONS

Neuroinflammation and microglial activation are pathological markers for several neurodegenerative diseases, including Alzheimer's disease. ROS and pro-inflammatory cytokine release are induced by chronic microglial cell activation.

This study proposed two objectives. Firstly, to study whether TRLs, particles that transport fat-soluble type substances in the bloodstream during the postprandial period, had the capacity to activate microglia cells and induce ROS and cytokine release, giving rise to an inflammatory process. Secondly, to show that minor compounds with high biologic activity that may be present in olive-pomace oil, such as oleanolic acid, α-tocopherol, and β-sitosterol, can attenuate microglial activation, on reducing ROS production and cytokine release.

The results we obtained have confirmed the dual hypothesis. On the one hand, TRL particles activated BV-2 microglia cells up to 100% above basal conditions. On the other hand, when the TRLs contained the bioactive compounds from olive-pomace oil, particularly α-tocopherol and oleanolic acid, the activation attenuated, demonstrated by a reduction in cell cytokine production. This reduction was associated with lower ROS production and higher glutathione levels.

Therefore, our results suggest that the bioactive compounds from olive-pomace oil may have a protective effect against Alzheimer's disease by attenuating microglia activation. All the same, undertaking a clinical trial to determine whether the observed effects are replicable in humans is essential.

Seville, September 10th, 2018

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Signed Javier Sánchez Perona,

CSIC Chief Scientist

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ANNEX 1: FIGURES

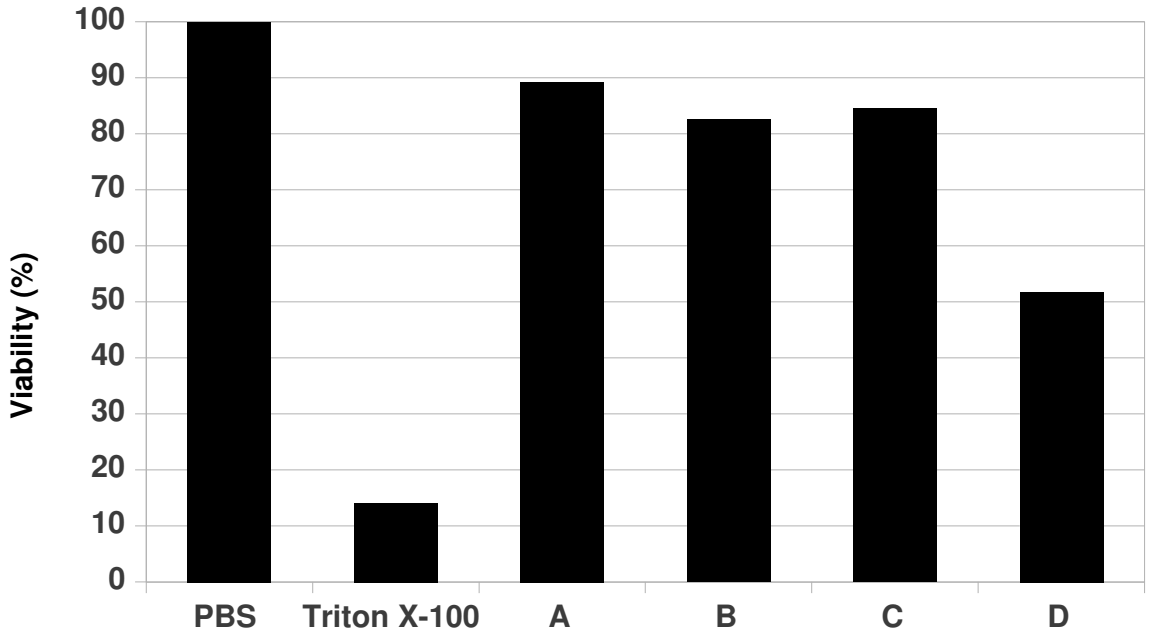

Figure 1. BV-2 cell viability after being treated with PBS (control), Triton X-100 (negative control), and different concentrations of oleanolic acid. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 25 µM.

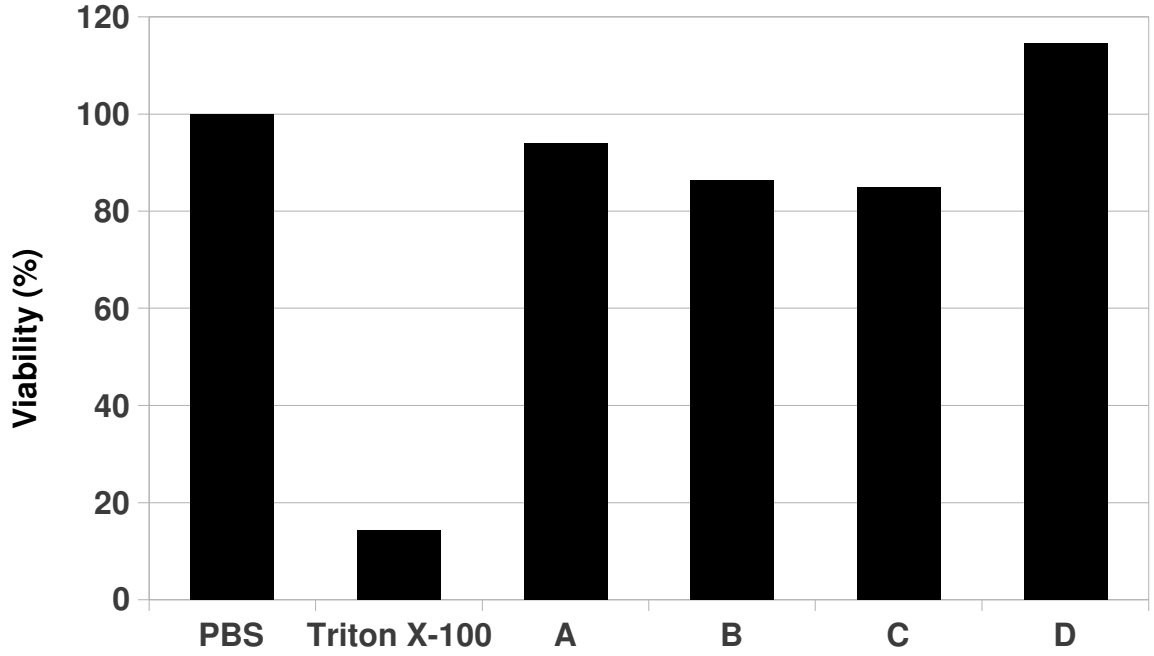

Figure 2. BV-2 cell viability after being treated with PBS (control), Triton X-100 (negative control), and different concentrations of α-tocopherol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 50 µM.

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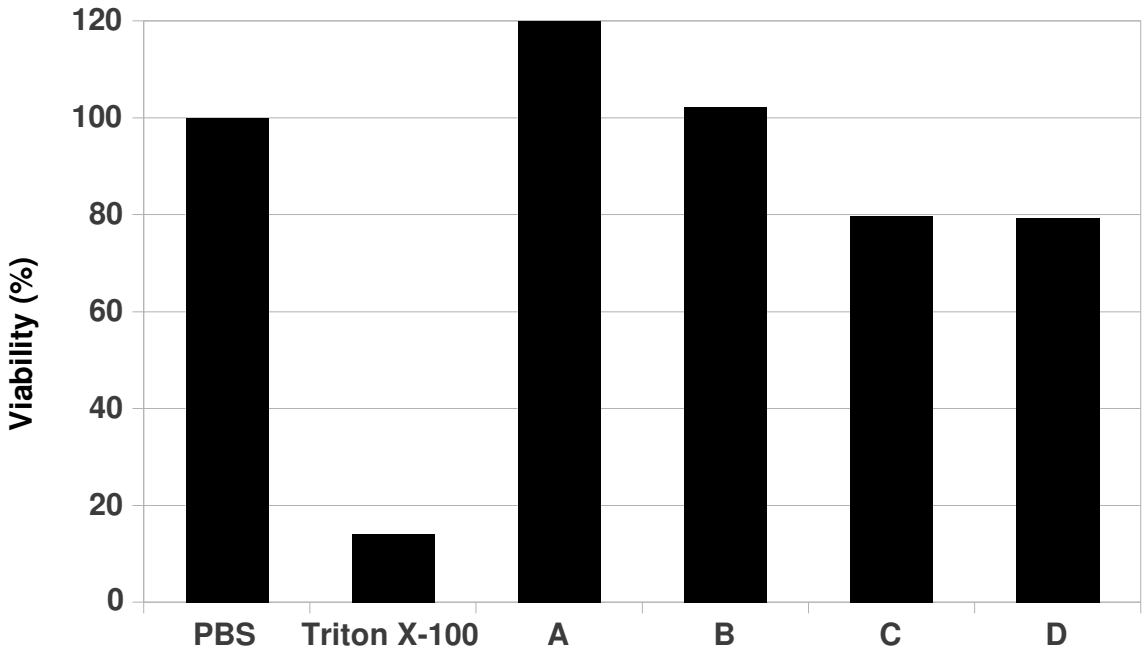

Figure 3. BV-2 cell viability after being treated with PBS (control), Triton X-100 (negative control), and different concentrations of β-sitosterol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 25 µM.

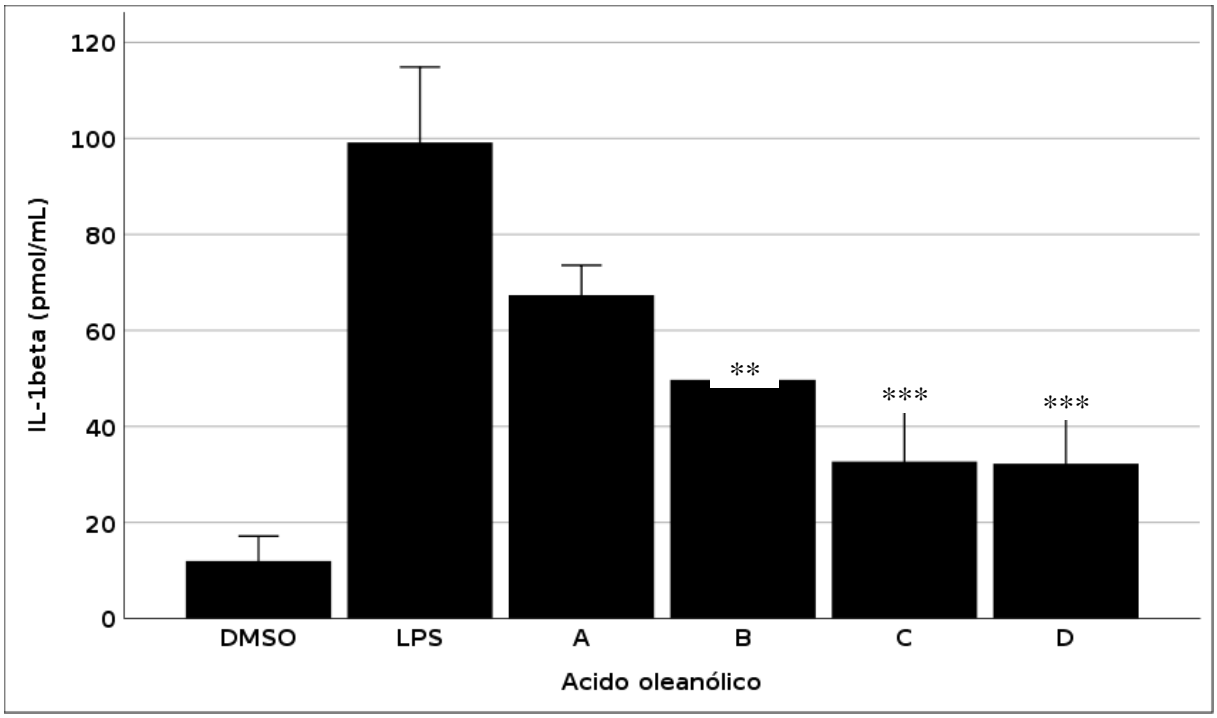

Figure 4. IL-1β concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of oleanolic acid. A: 0.5 µM, B: 1 µM, C: 5 µM, D: 10 µM. **: $p<0.01$, ***: $p<0.001$ vs. LPS.

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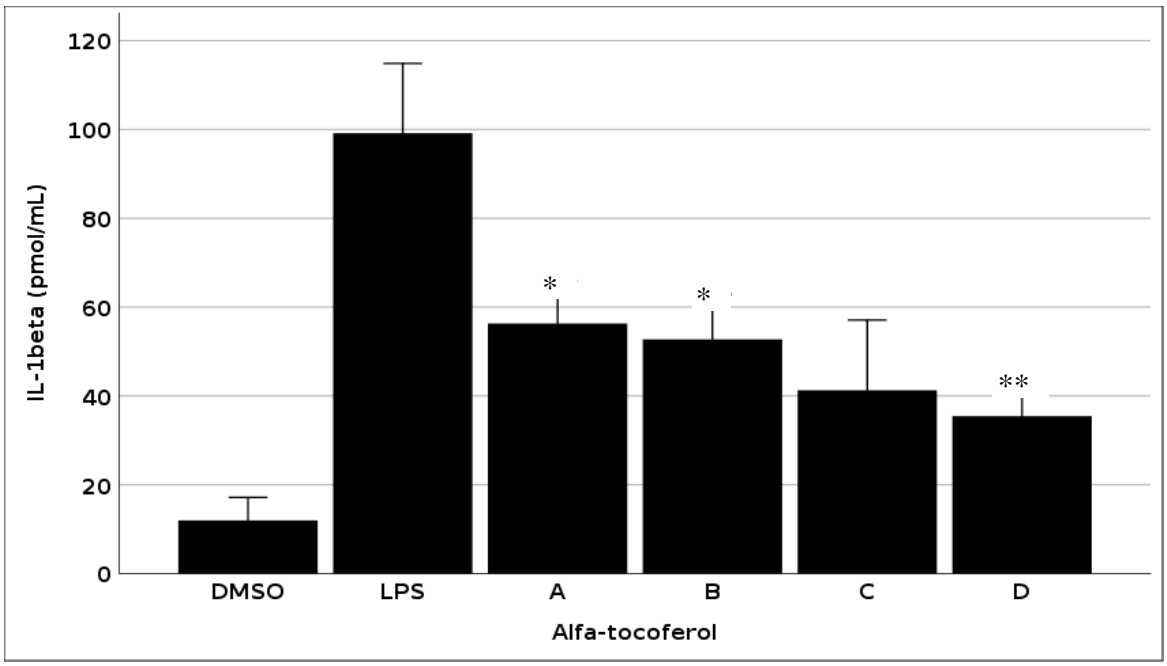

Figure 5. IL-1β concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of α-tocopherol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 50 µM. **: p<0.01, ***: p<0.001 vs. LPS.

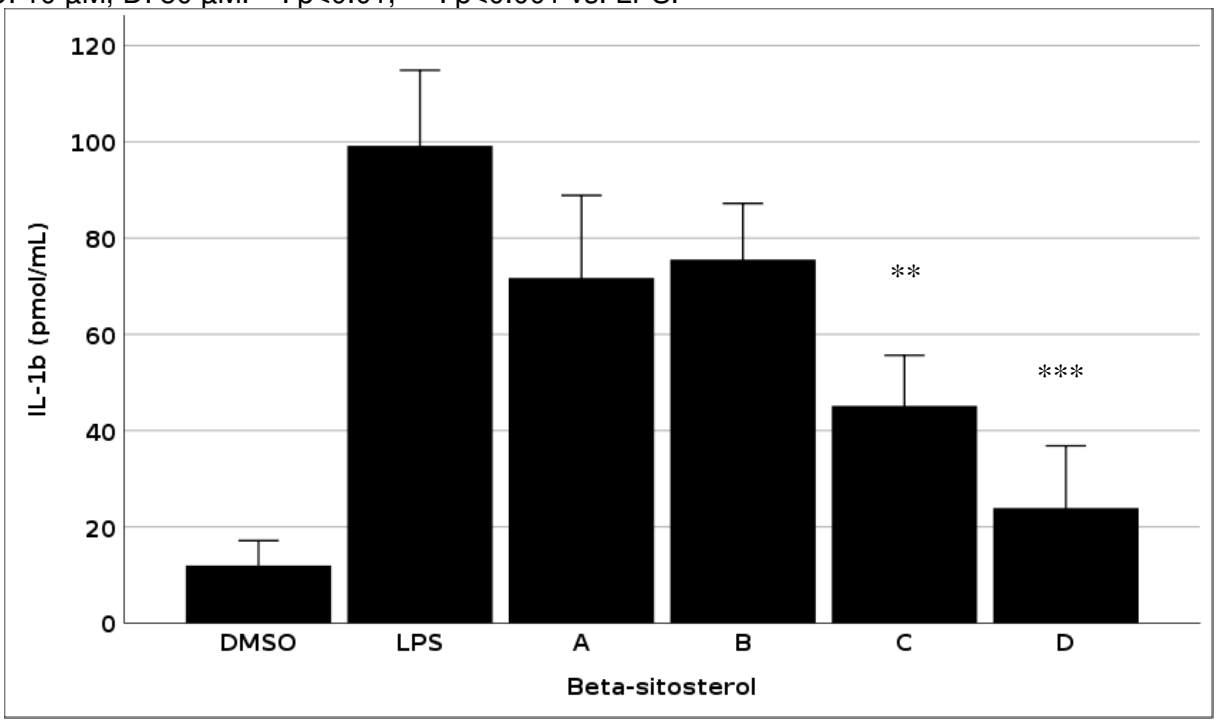

Figure 6. IL-1β concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of β-sitosterol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 25 µM. **: p<0.01; ***:p<0.001 vs. LPS.

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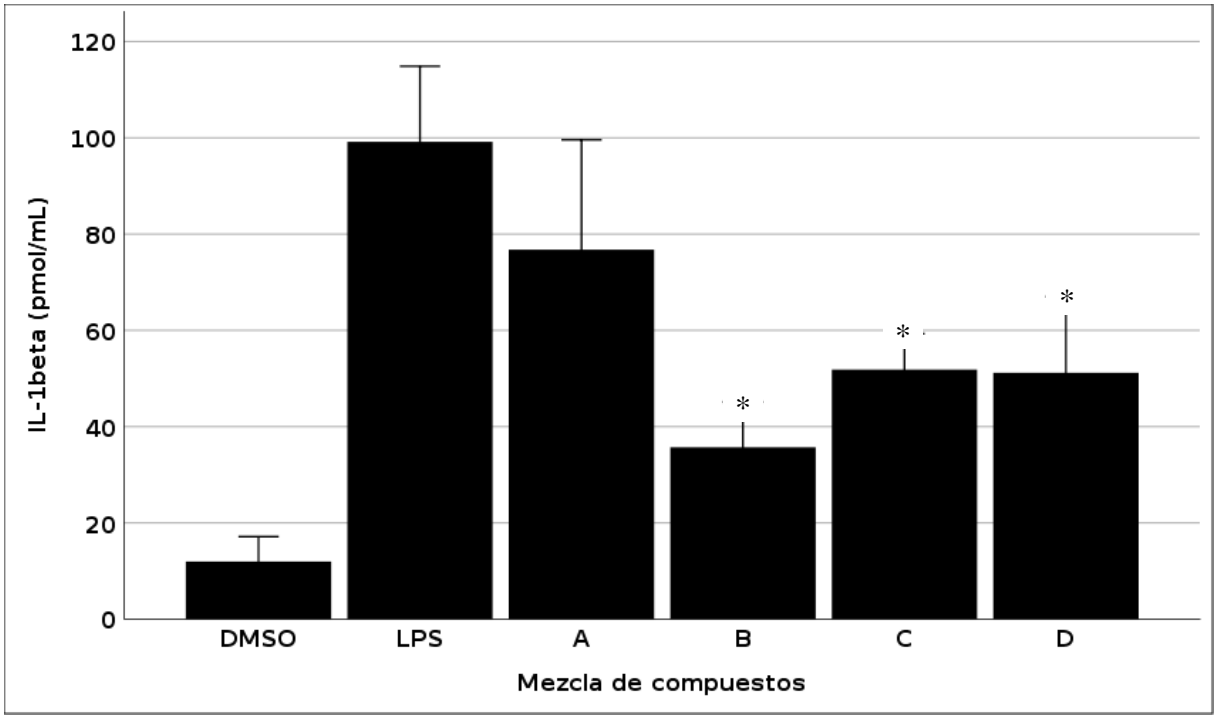

Figure 7. IL-1β concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations (see methods) of mixes of oleanolic acid, α-tocopherol, and β-sitosterol. **: p<0.01; ***:p<0.001 vs. LPS.

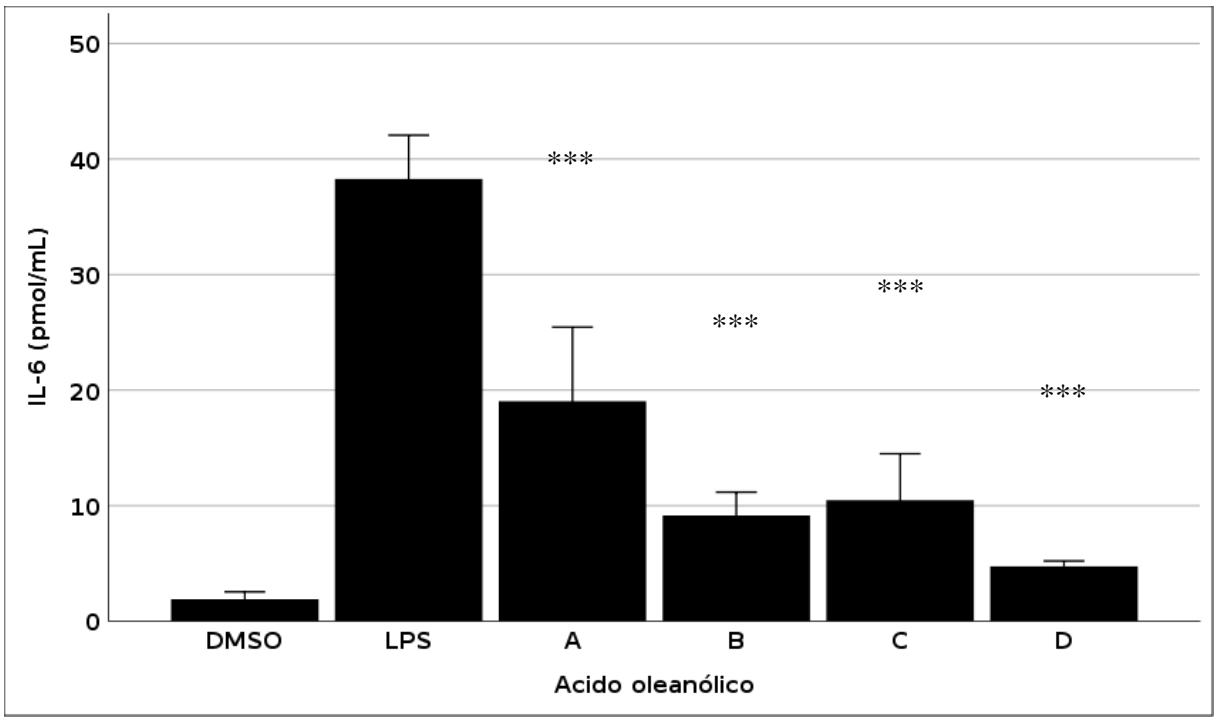

Figure 8. IL-6 concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of oleanolic acid. A: 0.5 µM, B: 1 µM, C: 5 µM, D: 10 µM. ***: p<0.001 vs. LPS.

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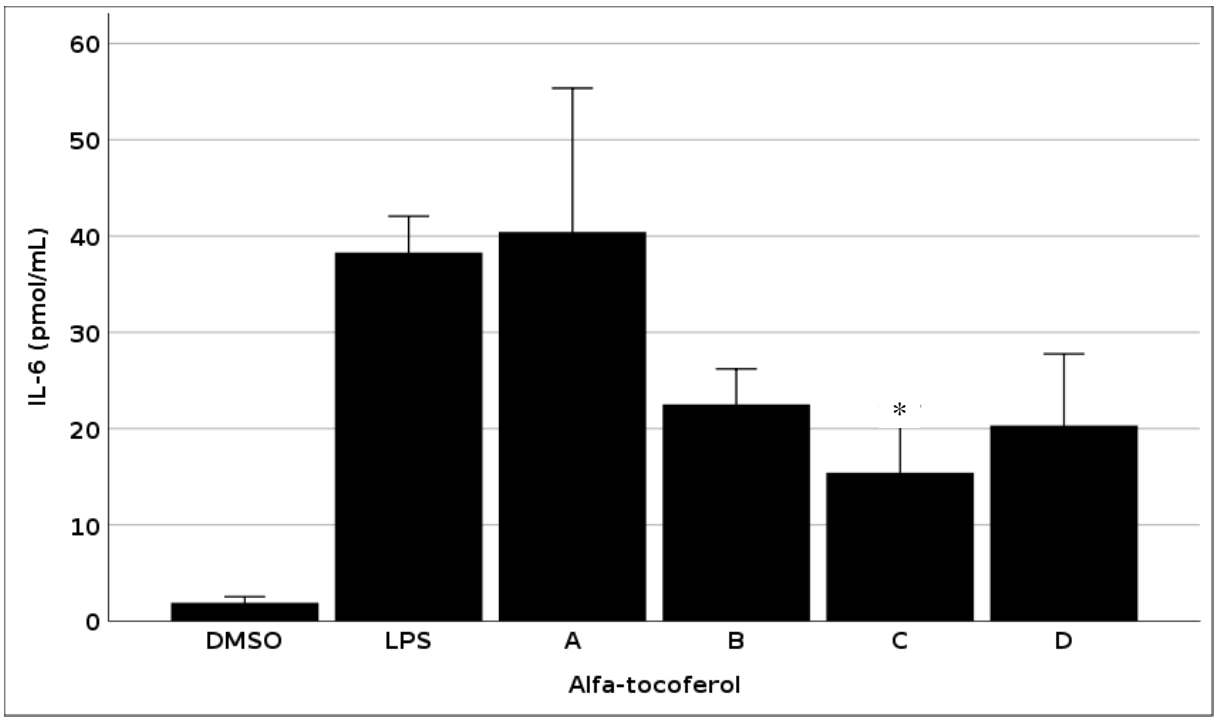

Figure 9. IL-6 concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of α-tocopherol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 50 µM. *: $p<0.05$ vs. LPS.

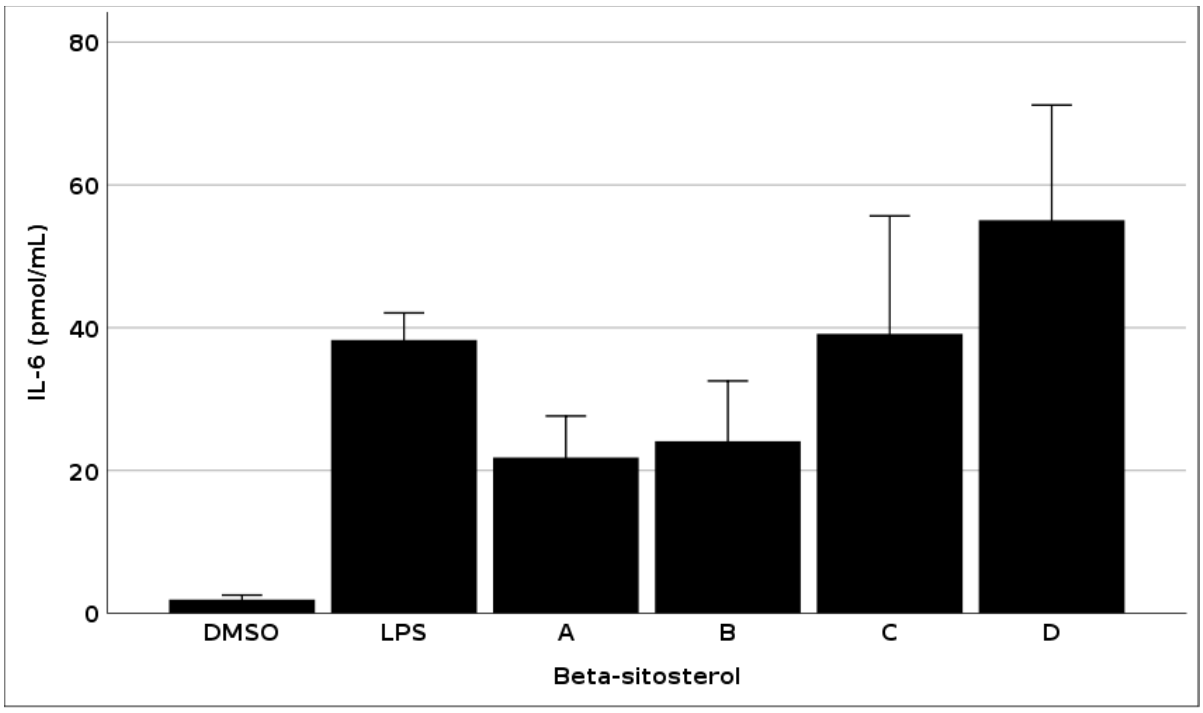

Figure 10. IL-6 concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of β-sitosterol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 25 µM.

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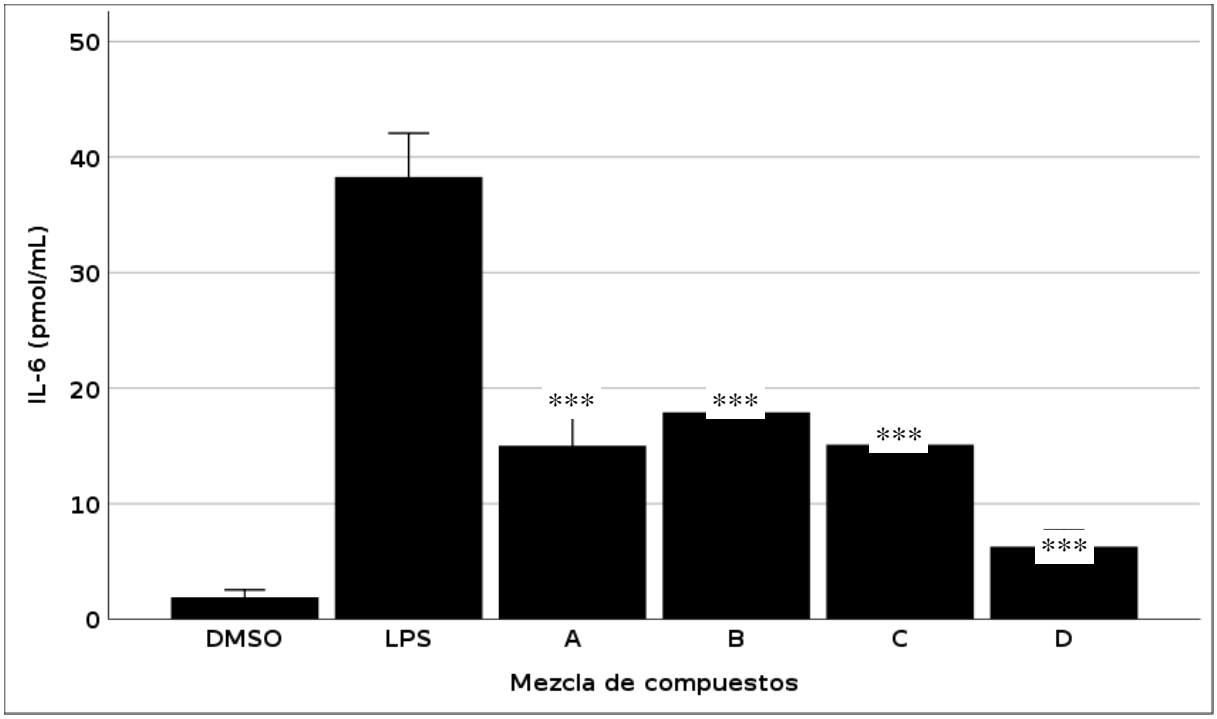

Figure 11. IL-6 concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations (see methods) of mixes of oleanolic acid, α-tocopherol, and β-sitosterol. ***:p<0.001 vs. LPS.

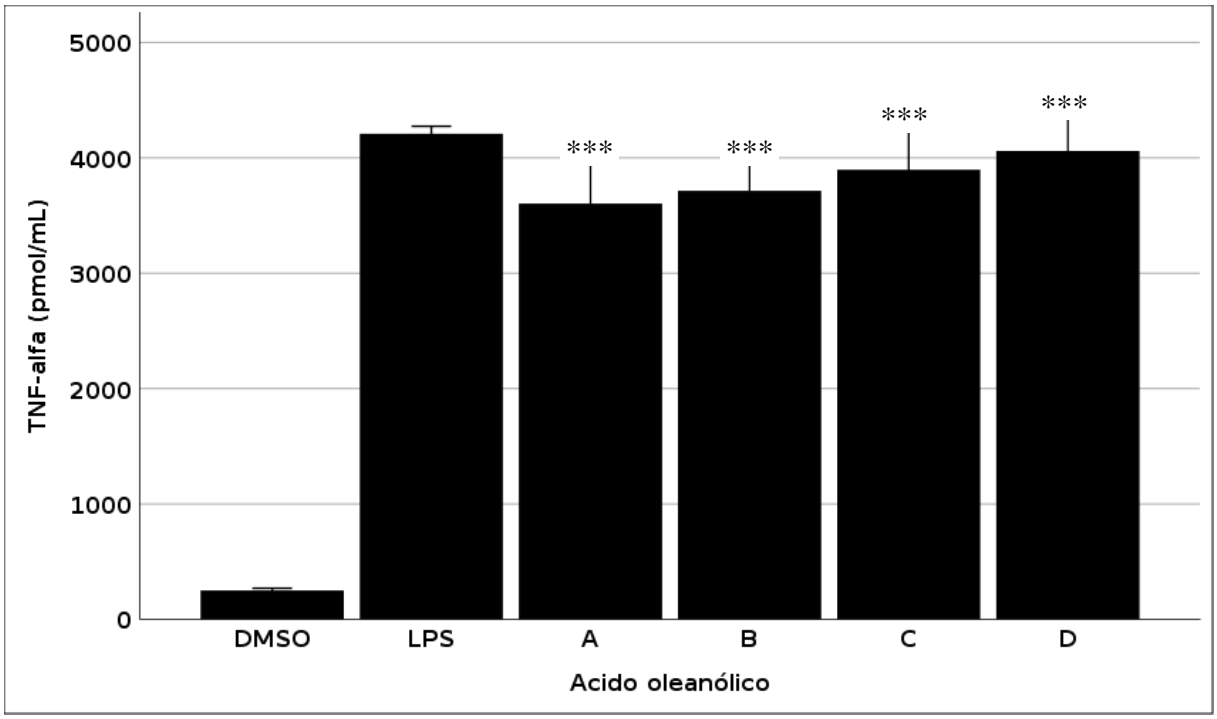

Figure 12. TNF-α concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of oleanolic acid. A: 0.5 µM, B: 1 µM, C: 5 µM, D: 10 µM. ***: p<0.001 vs. LPS.

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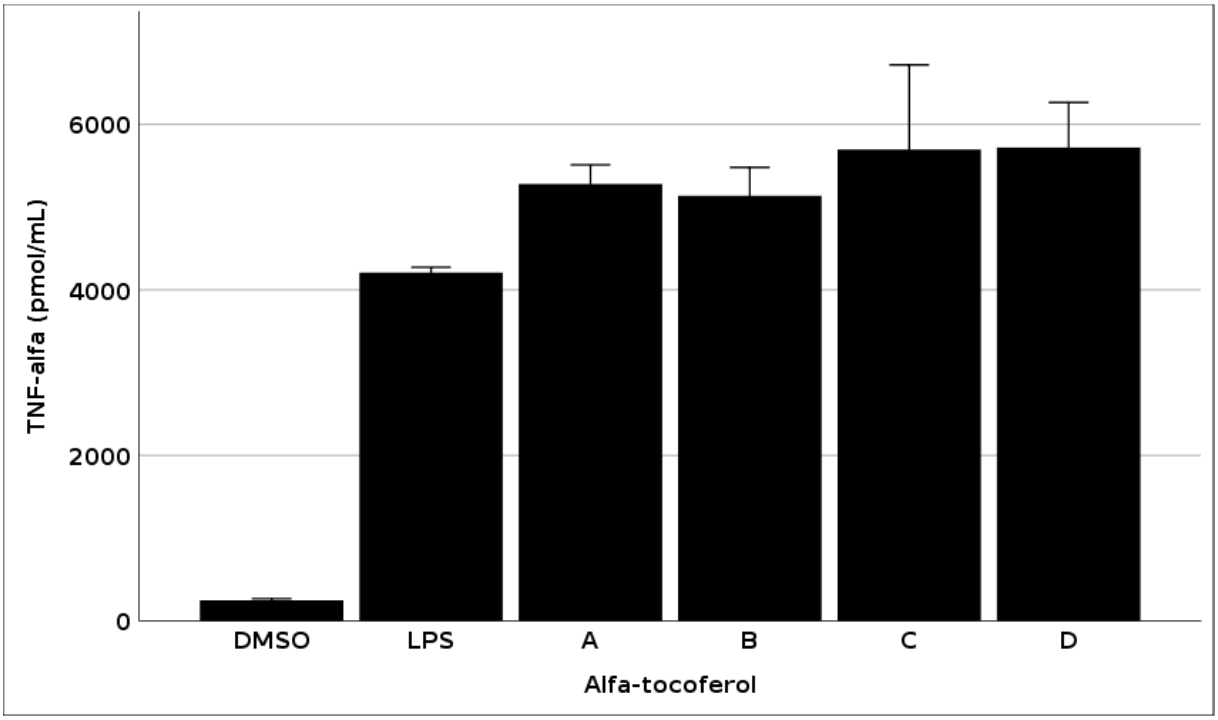

Figure 13. TNF-α concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of α-tocopherol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 50 µM.

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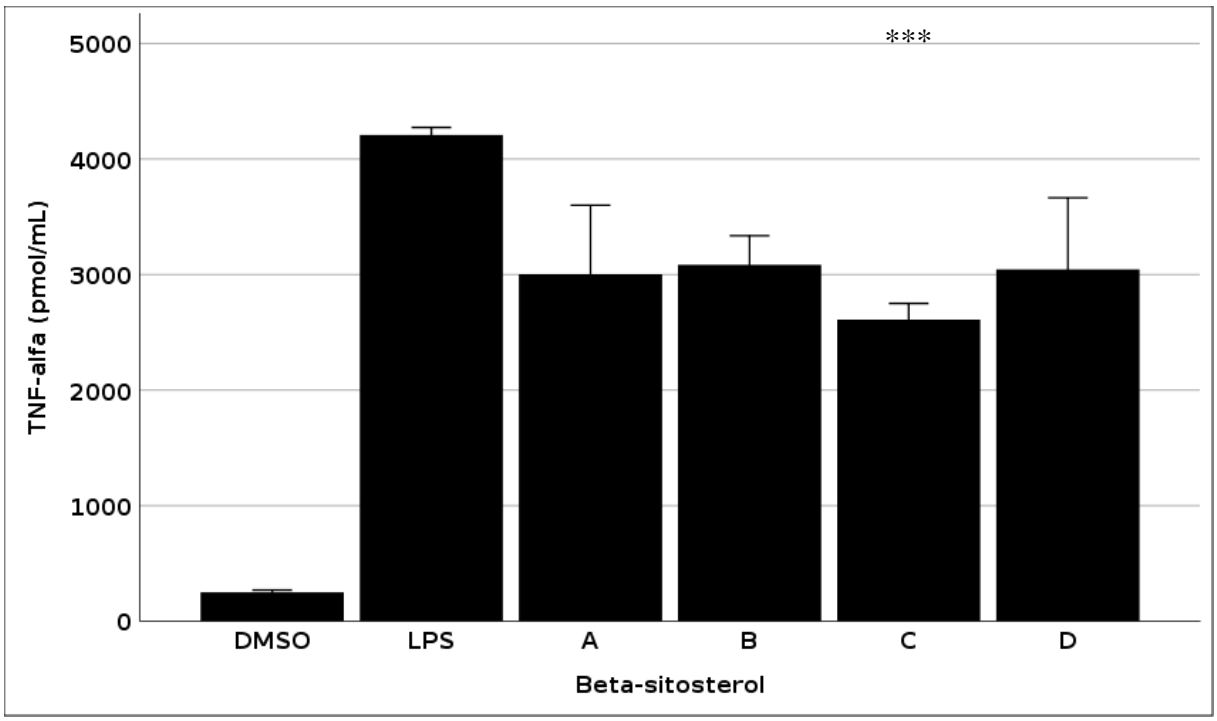

Figure 14. TNF-α concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of β-sitosterol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 25 µM. ***: p<0.001 vs. LPS.

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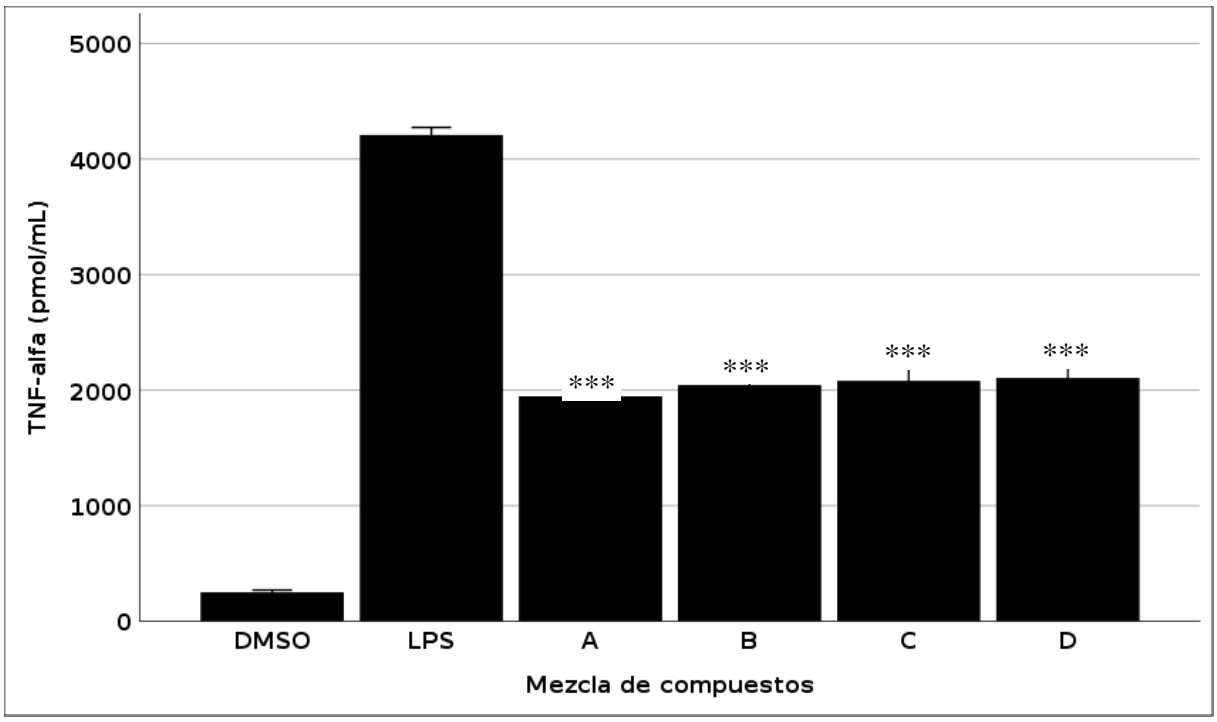

Figure 15. TNF-α concentration in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations (see methods) of mixes of oleanolic acid, α-tocopherol, and β-sitosterol. ***:p<0.001 vs. LPS.

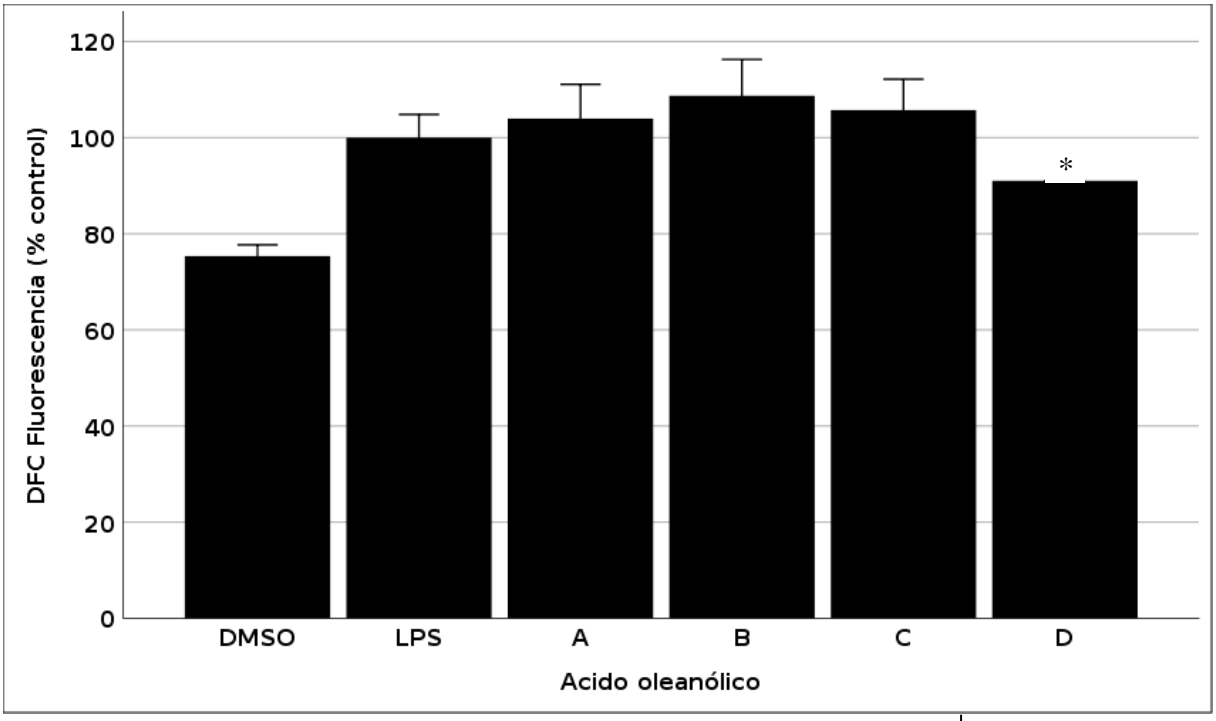

Figure 16. Reactive oxygen species (ROS) production in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of oleanolic acid. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 25 µM. Data expressed as a percentage of fluorescence emitted compared to the positive control. *: p<0.05 vs. LPS.

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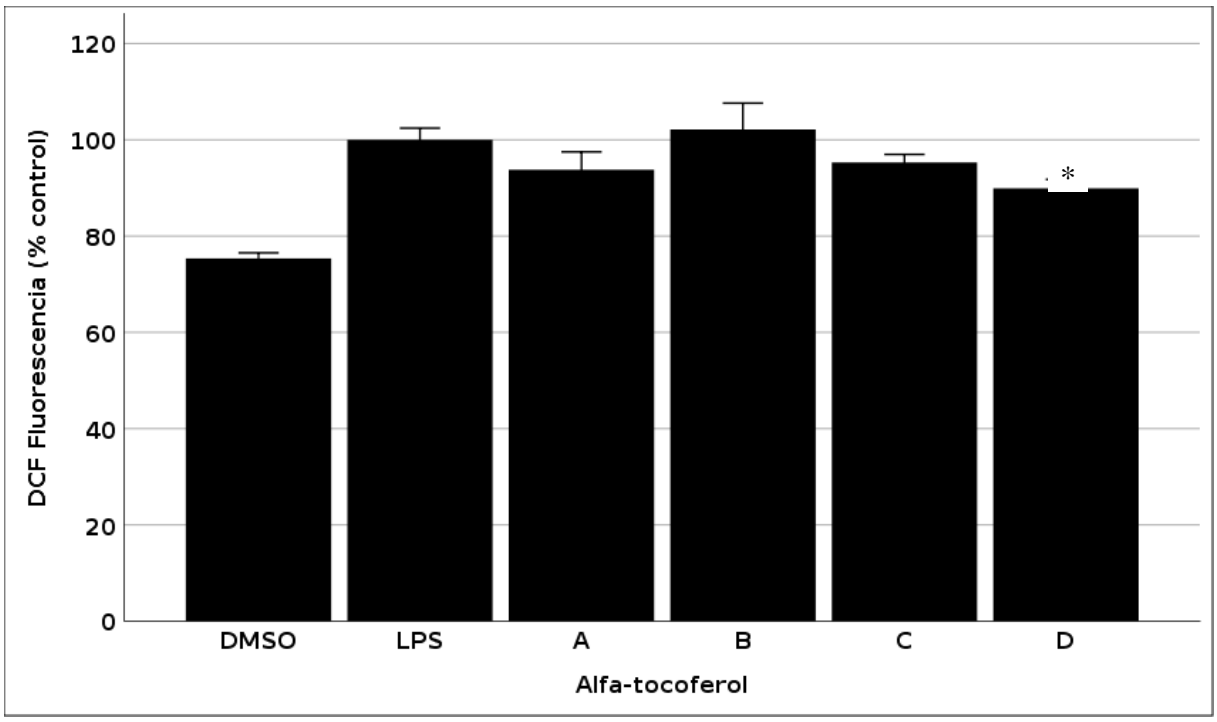

Figure 17. Reactive oxygen species (ROS) production in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of α-tocopherol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 25 µM. Data expressed as a percentage of fluorescence emitted compared to the positive control. *: p<0.05 vs. LPS.

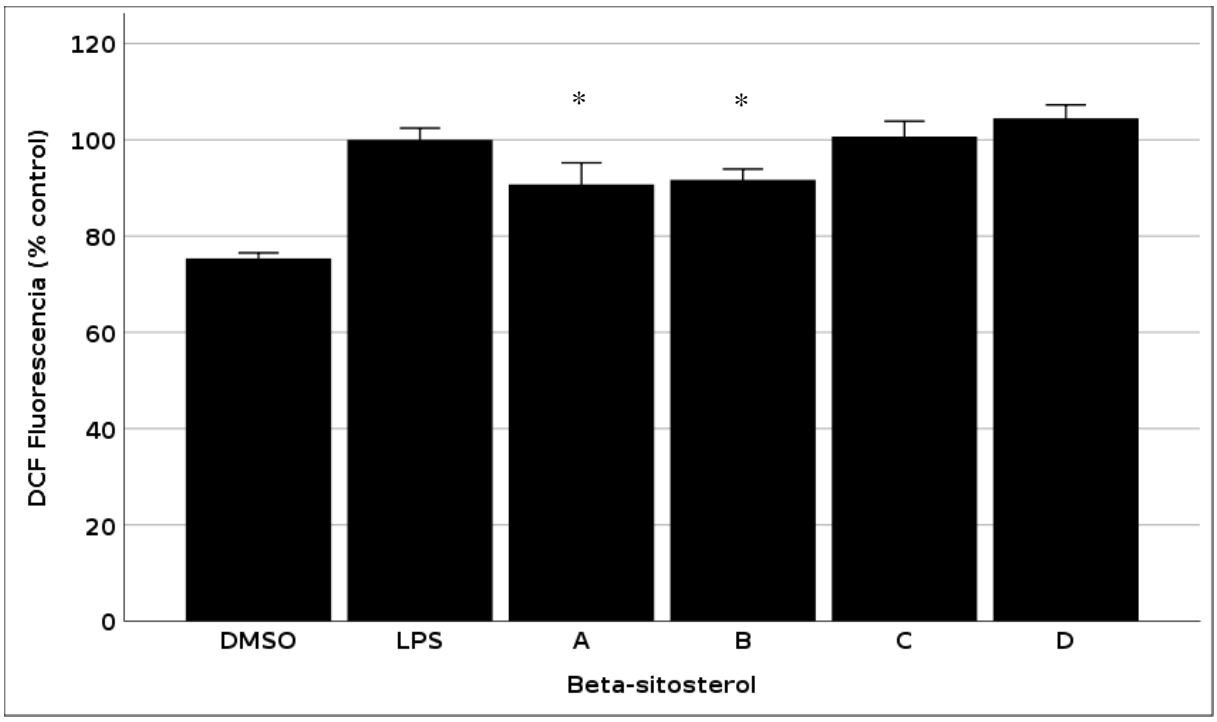

Figure 18. Reactive oxygen species (ROS) production in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of β-sitosterol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 25 µM. Data expressed as a percentage of fluorescence emitted compared to the positive control. *: p<0.05 vs. LPS.

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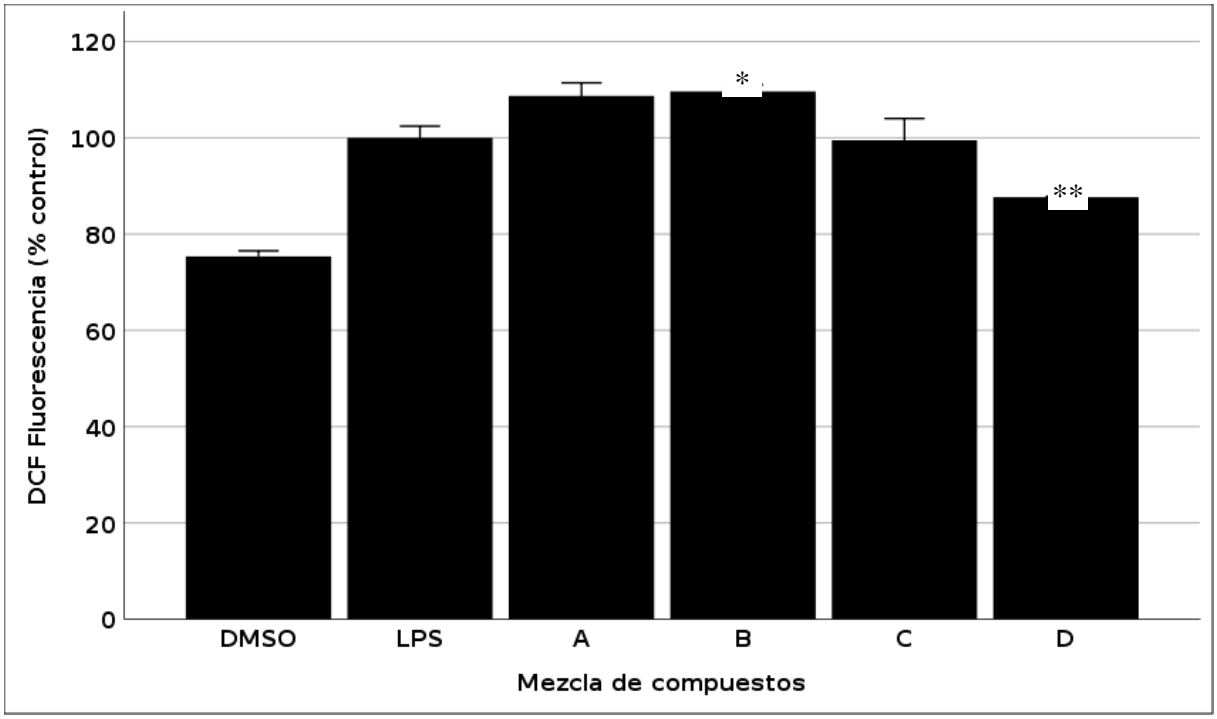

Figure 19. Reactive oxygen species (ROS) production in a BV-2 cell culture medium after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations (see methods) of mixes of oleanolic acid, α-tocopherol, and β-sitosterol. *:p<0.05; **:p<0.01 vs. LPS.

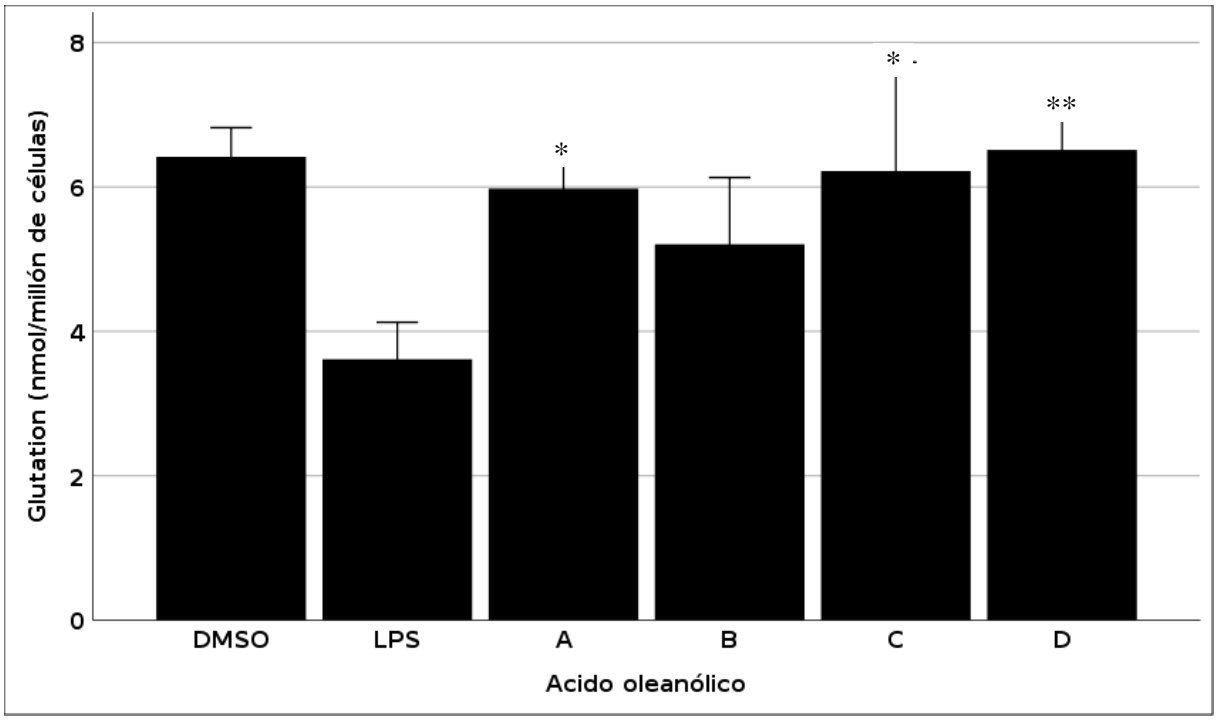

Figure 20. Total glutathione concentration in BV-2 cells after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of oleanolic acid. A: 0.5 µM, B: 1 µM, C: 5 µM, D: 10 µM. *: p<0.05, *:p<0.01 vs. LPS.

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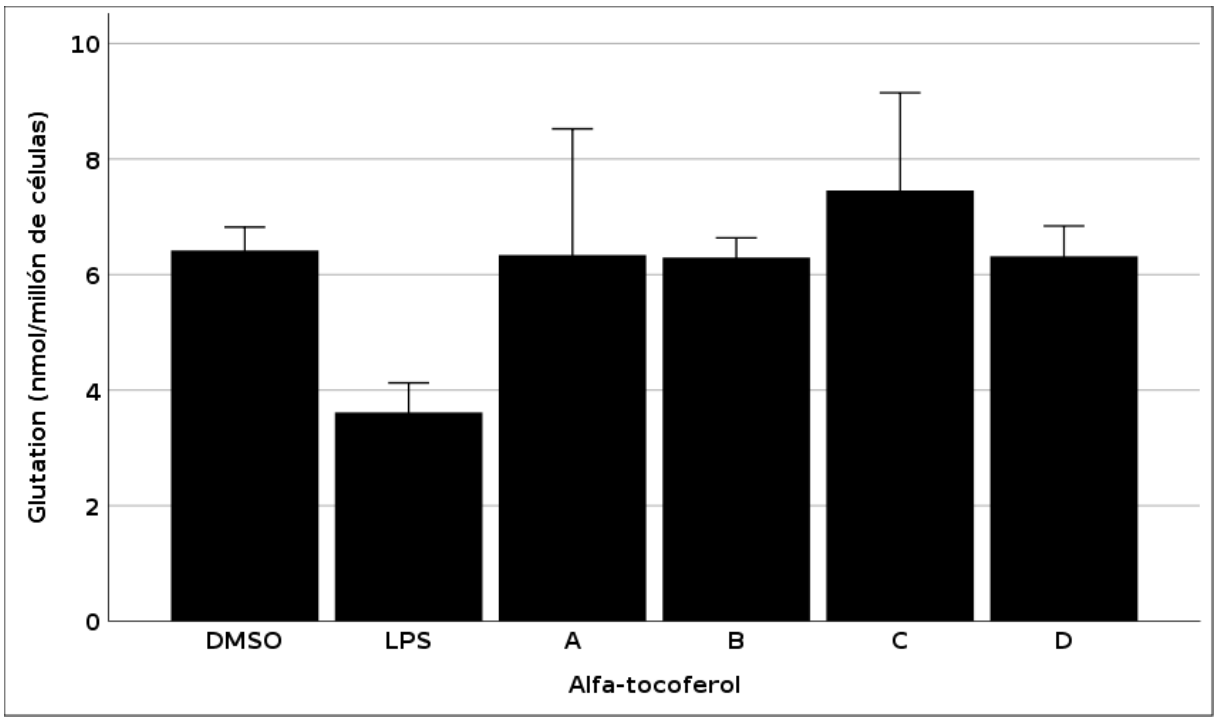

Figure 21. Total glutathione concentration in BV-2 cells after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of α-tocopherol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 50 µM. *: p<0.05 vs. LPS.

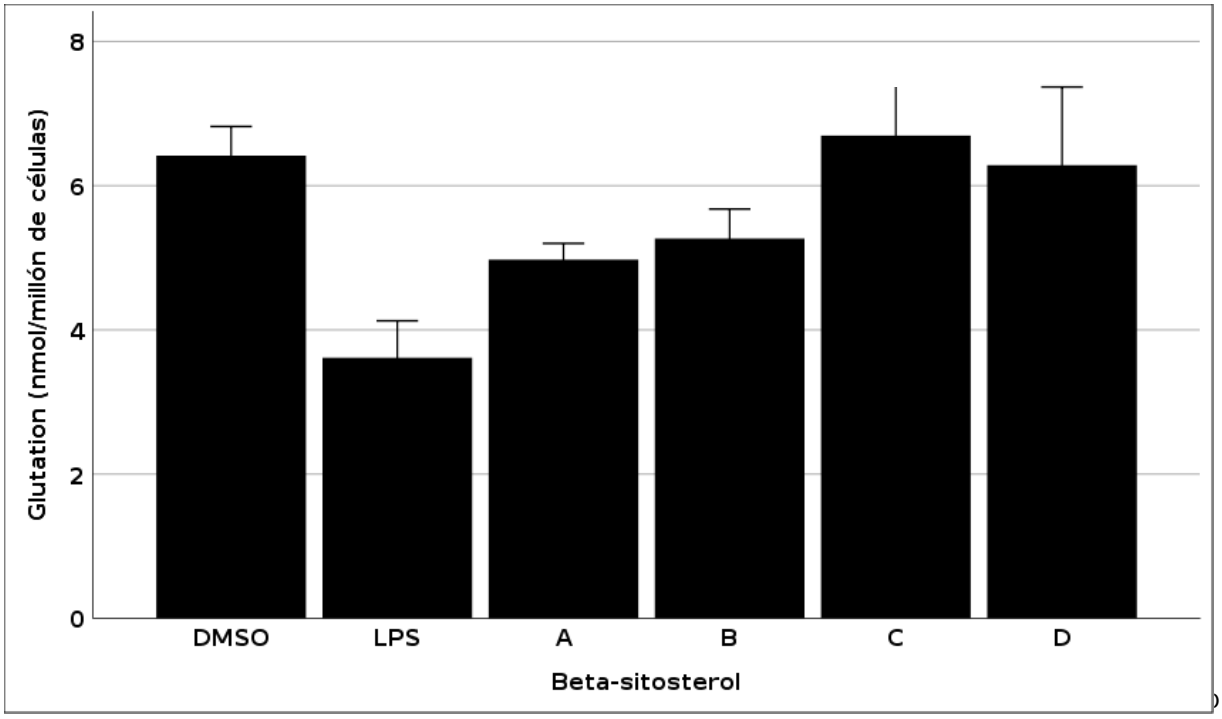

Figure 22. Total glutathione concentration in BV-2 cells after treatment with DMSO (negative control), LPS (positive control), and LPS plus different concentrations of β-sitosterol. A: 0.5 µM, B: 1 µM, C: 10 µM, D: 25 µM. **: p<0.01 vs. LPS.

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ERROR: syntaxerror
OFFENDING COMMAND: %ztokenexec_continue

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