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Efficacy of mullein extracts, *Verbascum thapsus* L. (Lamiales, Scrophulariaceae) in preventing and treating LPS induced inflammation in mouse granulosa cells

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ABSTRACT

***Verbascum thapsus* L., common mullein, has been used in traditional folk medicine as an antibiotic, antioxidant, and anti-inflammatory agent. To validate use as an anti-inflammatory, hot acidic aqueous extracts of *V. thapsus* L. leaves were introduced into control cultures of mouse granulosa cells; as well as cell cultures pre and post treated with lipopolysaccharide (LPS). Anti-inflammatory properties were demonstrated visually via immunocytochemistry (ICC) staining for transforming growth factor beta receptor II (TGFBRII). Cells subjected to mullein only, stained slightly darker than control cells and had few projections. LPS treated cells stained considerably darker and exhibited fewer projections. Those cells treated with mullein extracts prior to LPS administration were comparable to those that had received mullein after LPS administration. Those treated with LPS, pre and post treated with mullein were darker than the negative control but appreciably lighter than the LPS positive control. Quantification was achieved via IL-6 ELISA performed on the cell culture supernatant. IL-6 production following LPS was significantly decreased in both mullein pretreated (p=0.01) and post-treated cells (p=0.03) suggesting a role for mullein as an anti-inflammatory preventative and treatment.**

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INTRODUCTION

Interleukin 6 (IL-6) is a cytokine which acts as a pro-inflammatory mediator by triggering activation of the JAK/STAT signaling cascade in many cell types and plays a critical role in immune responses, inflammation, and hematopoiesis (Morris et al., 2021); and its production is associated with both pathogen-associated molecular patterns (PAMPs) (Kumar et al., 2011) and damage-associated molecular patterns (DAMPs) (Tanaka et al., 2014). Consequently, IL-6 production can be stimulated by bacterial lipopolysaccharide (LPS), which is found in gram-negative bacterial walls. In mice cardiomyocytes, LPS has been demonstrated to be a ligand for toll-like receptor 4 (TLR4) induced inflammation via IL-6 (Tarasiuk et al., 2020). A similar pathway has been demonstrated in bovine granulosa cells (Bromfield and Sheldon, 2011).

Verbascum thapsus L., common mullein, has been used in traditional folk medicine as an anti-inflammatory (Dalar et al., 2014). It contains three major chemical groups with known antioxidant properties: glycosides, flavonoids, and phenolics with glycosides as the predominant type (Mihailović et al., 2016) and of these, verbascoside are the most common (Hussain et al., 2009; Khuroo et al., 1988; Luca et al., 2019). Verbascoside has been linked to antitumor, antimicrobial, antioxidant, anti-inflammatory, and antinociceptive actions (Leitão et al., 2015).

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Figure 1. Mullein plants growing along a rural road in Ada, Ohio.

Nearly 60% of Americans have least one chronic condition, and worldwide, 3 of 5 people die due to chronic inflammatory diseases (Pahwa et al., 2022); thus, natural anti-inflammatory phytochemicals may be an important resource which should be explored. Hot acid extracts of mullein were used as both pre and post treatments to assess efficacy against LPS induced inflammation.

MATERIALS AND METHODS

Plants

Mullein samples were collected May through September 2016, from a stand growing wild along a back road in Ada, OH (Figure 1). The largest leaves in good condition were collected near the base of adult plants. Leaves were classified by reproductive status of the plant as preflower, in flower or post flowering. Leaves were dried on a flat screen and stored in the dark in plastic containers. A voucher specimen (VAM-01-006) was submitted to the Ohio Northern University Herbarium under the direction of Dr. Robert Verb.

Animals

7-9 week-old (littermates) C57BL/6 wild type female mice of reproductive age were purchased from Charles River Laboratory and maintained in the ONU animal facility. Mice were allowed to acclimate for 1 week prior to use. All mice were sacrificed within 9 weeks of arrival. Vaginal smears were collected and viewed microscopically to determine the stage of the estrous cycle. Mice were sacrificed only in the estrus stage of the estrus cycle. The estrus stage could be distinguished by vaginal cells appearing big, puffy, cornified, translucent, and anucleate (Byers et al., 2012). Cell culture preparation (animal sacrifice) and cell culture

experiments are described below.

Extraction protocol

3.0 g of each dried mullein sample was covered with 0.1 M potassium phosphate buffer pH 4 (Suomi et al., 2000) at 100°C for 1 h. Following vacuum filtration, the samples were evaporated at room temperature for one week and reconstituted with distilled water to 1 gram of plant material/ml of extract. Extracts were stored at 4°C. Although we did not assay our samples for verbascoside, this is a standard extraction to access the verbascoside, and it has been shown to be very stable in an acid buffered solution (Vertuani et al., 2011).

Cell culture preparation and treatment

To assess inflammation, granulosa cells were collected from C57BL/6 wild type mice in estrus (natural inflammation occurs within these cells during this phase). Estrus mice were anesthetized with 1-4% of Isoflurane (Allivet) through inhalation and then cervically dislocated (IACUC approved Protocol Number 22521). Mouse ovaries were grossly dissected and transferred to DMEM/F12 (Gibco) growth media. The surrounding fat was micro dissected, and the cleaned ovaries were transferred to EGTA (Sigma; 6.8 mM EGTA in M199/10 mM HEPES + 1x antibiotic) media at room temperature for 15 min followed by sucrose media (Sigma; 0.5 M Sucrose in 1.8 mM EGTA in M199/10 mM HEPES + 1x antibiotic) for 10 min. Follicles were punctured and shredded to collect granulosa cells. Cells were strained, counted, plated (300,000 cells/ml) onto coverslips in a 24 well plate and incubated for 24 h at 37°C.

Granulosa cell culture

Negative control wells (N=8) were left untreated and incubated for 24 h at 37°C. Positive control wells (N=8) were treated with 1 µl of LPS 1 µg/ml and incubated for 24 h at 37°C. To determine preventative anti-inflammatory action, for each seasonal extract, twelve wells were treated with 100 µl mullein extracts (100 ng/ml of plant material in DMEM). After 1 h, 4 of these wells were post treated with 1 µl of LPS 1 µg/ml (N=48). To determine efficacy of mullein extracts in treating existing inflammation, for each seasonal extract, 12 wells were initially treated with 1 µl of LPS 1 µg/ml and allowed to incubate for an hour. Four of these were post treated with 100 µl mullein extracts (100 ng/ml of plant material in DMEM) (N=48). The plates were incubated for 24 h at 37°C.

Supernatant was removed and stored at -80°C until the IL-6 ELISA (Millipore Sigma Aldrich) was performed. Wells were washed with sterile phosphate buffered saline (PBS), incubated for 20 min at room temperature in 300 µl of paraformaldehyde [PFA; (Electron Microscopy Sciences, EMS) prepared 4% in PBS], rinsed, and then stored in PBS, wrapped with paraffin wax, and placed at 4°C for ICC.

Immunocytochemistry (ICC) protocol

300 µl of diluted hydrogen peroxide [3% H_2O_2 in H_2O (Fisher Scientific)] was added to the wells for 10 min and washed twice with 300 µl of PBS. 250 µl of Avidin D (Vector Laboratories; Vectastain Kit) was added to the wells for 15 min and washed twice with 300 µl of PBS. 250 µl of Biotin (Vector Laboratories; Vectastain Kit) was added to the wells for 15 min and washed once with 300 µl of PBS. 250 µl of blocking buffer (Superblock; Pierce Chemicals: 37515) was added for 30 min. All incubations were at room temperature and after the last wash with PBS, 250 µl of primary antibody [TGFBR2, Goat IgG (R and D Systems)] was added to every well, except the negative control. The plate was then refrigerated at 4°C for 24 h.

The primary antibody was pipetted off and the wells received three, 3-min washes of PBS with triton [T-PBS; 1% solution of triton X100 in PBS (Sigma)]. The following substances were added in this order with the T-PBS wash in between each chemical: 250 µl of secondary antibody [Donkey Anti-Goat IgG (Abcam)] for 30 min, 250 µl of Avidin-Biotin Complex (ABC) reagent (Vector Laboratories; Vectastain Kit) for 30 min, DAB (3,3'-Diaminobenzidine; Vector Laboratories; Vectastain Kit) for 1 min. Well contents were pipetted off and cells were washed with distilled water, halting the color changing process. Coverslips containing the cell culture samples were dipped into hematoxylin four times, washed with distilled water, and then mounted onto slides.

Interleukin-6 (IL-6) ELISA

Cell culture supernatants were tested using the Mouse IL-6 ELISA Kit (Millipore Sigma Aldrich). Kit protocol was followed, samples were left undiluted. The colorimetric assay was read at an absorbance of 450 nm. Results of all duplicated samples were pooled.

Statistical analysis

Data were compared by one-way ANOVA with post hoc Student's t-tests. Significance was determined at $p<0.05$.

RESULTS AND DISCUSSION

Control cells (Figure 2A) maintained their normal fibroblast looking shape with projections and stained lighter than

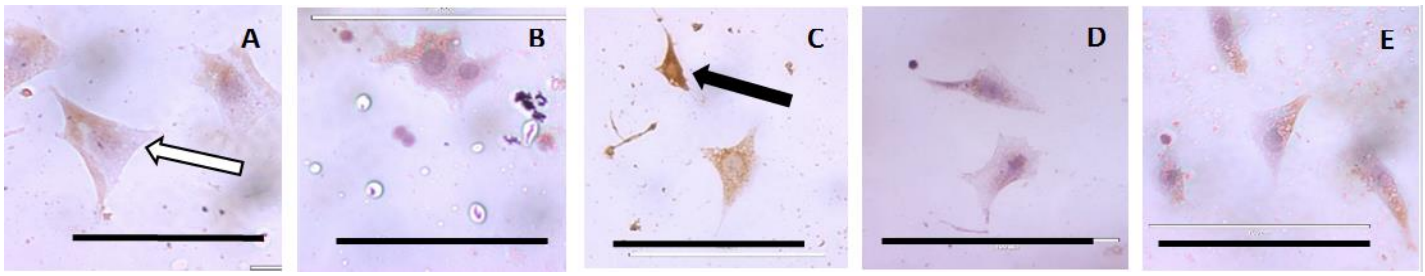

Figure 2. Immunocytochemistry of mouse granulosa cells stained for TGFBRII with DAB. Brown staining indicates presence of inflammation (TGFBRII). A. control- white arrow indicates clear cytoplasm; B. mullein; C. LPS positive control - black arrow indicates brown granular cytoplasm; D. Mullein treatment of LPS induced inflammation; E. Mullein prevention of LPS induced inflammation (Black bars indicate 1 mm).

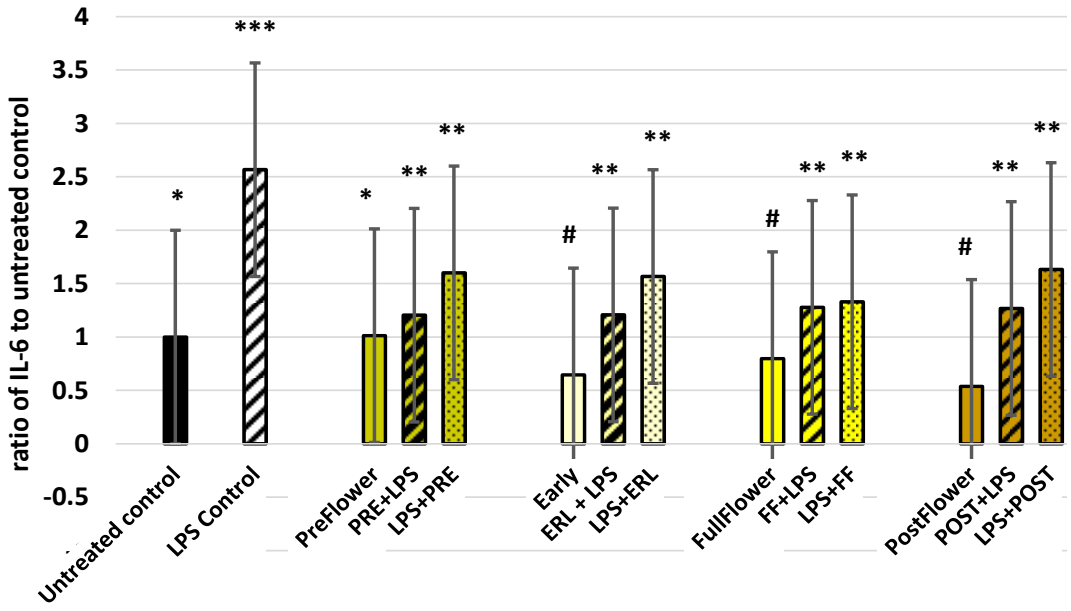

Figure 3. Seasonal variation in efficacy of mullein extracts in preventing and treating LPS induced inflammation. Solid bars received no LPS. Striped bars received mullein prior to LPS treatment, dotted bars received mullein after LPS treatment. Asterisks indicate significance see text for values.

LPS treated cells or cells treated with mullein alone. Mullein treated cells with no LPS demonstrated minimal rounding and retraction of extensions suggesting minimal stress (Figure 2B). Positive control cells subjected to LPS (Figure 2C) presented with dark spots within the cytoplasm and more appeared rounded which is associated with LPS-induced stress. LPS treated cells post-treated with mullein extracts (Figure 2D) also displayed minimal rounding and slight granule formation. Cells pretreated with mullein extract (Figure 2E) prior to LPS exposures were comparable to those that had been post-treated with mullein extracts.

IL-6 ELISA was performed to quantify interleukin secretion. IL-6 production was measured via optical density (OD) analysis at 450 nm and quantified using a standard curve. Since the assay was performed multiple times, experimental data were normalized to their individual controls and IL-6 quantification was expressed as an experimental to control ratio, where values greater than 1 indicate that there was more IL-6 present in the sample than in the control, and values less than 1, indicate less IL-6 present in the sample than in the control and therefore, less inflammation.

Mullein treated samples all had significantly lower IL-6 than the positive LPS control indicating a reduction in LPS induced inflammation (ANOVA $F_{56,12}$= 6.35; p=0.003). Both samples treated with mullein extracts prior to LPS (Figure 3) and those treated post LPS induced inflammation (dotted bars) exhibited significantly reduced inflammation as compared to LPS alone (t-test p=0.01, p=0.03 respectively), and did not differ significantly from each other (t-test=0.34). Cells treated with mullein

extracts from plants with any formed flower demonstrated significantly reduced inflammation compared to samples treated with extracts from preflowering plants (ANOVA $F_{47,12}$= 4,65, p<0.0001). However, they did not differ significantly from each other ($F_{22,2}$ = 1.276, p=0.298), potentially indicating that the active constituents are either concentrated in the flowers or that constituents aimed at protecting plants during their reproductive phase are responsible for the anti-inflammatory effects. Interestingly, those cells treated with mullein alone had reduced inflammatory markers compared to the untreated control (p<=0.003).

This study highlights two unique findings. First, we successfully introduce a new model system for investigating inflammatory responses. Mouse granulosa cells, which are key for the inflammatory process of ovulation, can be grown in culture, treated with experimental extracts, and monitored for IL-6 production. Subsequently, we also show that mullein extracts can both prevent and treat LPS induced inflammation. This is significant as previous studies have primarily focused on only the treatment of inflammation (Alipieva et al., 2014).

As no dose has been established for mullein, and specifically its verbascoside content, future studies will explore a dose response for this anti-inflammatory action. This will allow us to verify that mullein extracts/verbascoside can completely prevent LPS induced inflammation at higher doses. Others have shown that LPS induces granulosa cell inflammation producing IL-6 via TLR4 pathway in bovines (Bromfield and Sheldon, 2011) and verbascoside is known to suppress TLR4 (Lai et al., 2019). Verbascoside, which is soluble in the hot aqueous extraction protocol used herein, is the likely anti-inflammatory agent working via the TLR4 pathway. Therefore, in subsequent experiments, we will analyze verbascoside treatment and pretreatment effects on LPS induced inflammation in mouse granulosa cells.

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REFERENCES

Alipieva K., Korkina L., Orhan I. E. & Georgiev M. I. (2014). Verbascoside-A review its occurrence, (bio) synthesis and pharmacological significance. Biotechnol. Adv. 32(6): 1065-1076. doi: 10.1016/j.biotechadv.2014.07.001

Bromfield J. J. & Sheldon I. M. (2011). Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and perturbs oocyte meiotic progression *in vitro*. Endocrinology. 152(12): 5029-5040. doi: 10.1210/en.2011-1124.

Byers S. L., Wiles M. V., Dunn S. L. & Taft R. A. (2012). Mouse estrous cycle identification tool and images. PloS one. 7(4): 1-5. doi: 10.1371/journal.pone.0035538.

Dalar A., Guo Y. & Konczak I. (2014). Phenolic composition and potential anti-inflammatory properties of *Verbascum cheiranthifolium* var. cheiranthifolium leaf. J. Herb. Med. 4(4): 195-200. doi: 10.1016/j.hermed.2014.04.004.

Hussain H., Aziz S., Miana G. A., Ahmad V. U., Anwar S. & Ahmed I. (2009). Minor chemical constituents of *Verbascum thapsus*. Biochem. Syst. Ecol. 37(2): 124-126. doi: 10.1016/j.bse.2008.12.007.

Khuroo M. A., Qureshi M. A., Razan T. K. & Nichols P. (1988). Sterones, iridoids and a sesquiterpene from *Verbascum thapsus*. Phytochemistry. 27(11): 3541-3544. doi: 10.1016/0031-9422(88)80764-7

Kumar H., Kawai T. & Akira S. (2011). Pathogen recognition by the innate immune system. Int. Rev. Immunol. 30(1): 16-34. doi: 10.3109/08830185.2010.529976.

Lai X., Xiong Y., Zhou J., Yang F., Peng J., Chen L. & Zhong W. (2019). Verbascoside attenuates acute inflammatory injury in experimental cerebral hemorrhage by suppressing TLR4. Biochem. Biophy. Res. Comm. 519(4): 721-726. doi: 10.1016/j.bbrc.2019.09.057.

Leitão G. G., Pinto S. C., de Oliveira D. R., Timoteo P., Guimarães M. G., Cordova W. H. & Leitão S. G. (2015). Gradient x isocratic elution CCC on the isolation of verbascoside and other phenylethanoids: Influence of the complexity of the matrix. Planta Medica. 81(17): 1609-1613. doi: 10.1055/s-0035-1546116.

Luca S. V., Czerwińska M. E., Miron A., Aprotosoaie A. C., Marcourt L., Wolfender J. & Skalicka-Woźniak K. (2019). High-performance countercurrent chromatographic isolation of acylated iridoid diglycosides from *Verbascum ovalifolium* Donn ex Sims and evaluation of their inhibitory potential on IL-8 and TNF-α production. J. Pharmaceut. Biomed. Anal. 166: 295-303. doi: 10.1016/j.jpba.2019.01.031.

Mihailović V., Kreft S., Benkovic E. T., Ivanovic N. & Stankovic M. S. (2016). Chemical profile, antioxidant activity and stability in stimulated gastrointestinal tract model system of three *Verbascum* species. Indust. Crop. Product. 89: 141-151. doi: 10.1016/j.indcrop.2016.04.075.

Morris R., Hilton D. J., Jarratt A. & Babon J. J. (2021). Dissecting the molecular control of Interleukin 6 signaling using M1 cell line. Cytokine. 146. doi: 10.1016/j.cyto.2021.155624.

Pahwa R., Goyal A. & Jialal I. (2022). Chronic Inflammation [StatPearls]. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK493173/.

Suomi J., Sirén H., Hartonen K. & Riekkola M. L. (2000). Extraction of iridoid glycosides and their determination by micellar electrokinetic capillary chromatography. J. Chromatogr A. 868(1): 73-83 doi: 10.1016/s0021-9673(99)01170-x.

Tanaka T., Narazaki M. & Kishimoto T. (2014). IL-6 in inflammation, immunity, and disease. Cold Spring Harbor Perspectives in Biology. 6(10): a016295. doi: 10.1101/cshperspect.a016295

Tarasiuk E., Bonda T. A., Dziemidowicz M., Winnicka M. M., Bernaczyk P. & Kamiński K. A. (2020). The effect of interleukin 6 deficiency on myocardial signal transduction pathways activation induced by bacterial lipopolysaccharide in young and old mice. Adv. Med. Sci. 65(2):386-393. doi: 10.1016/j.advms.2020.06.006.

Tatli I., Akdemir Z., Bedir R. & Khan I. (2004). Saponin, iridoid, phenylethanoid and monoterpene glycosides from *Verbascum pterocalycinum* var. mutense. Turk. J. Chem. 28: 111-122.

Vertuani S., Beghelli E., Scalambra E., Malisardi G., Copetti S., Dal Toso R., Baldisserotto A. & Manfredini S. (2011). Activity and stability studies of verbascoside, a novel antioxidant, in dermo-cosmetic and pharmaceutical topical formulations. Molecules. 16: 7068-7080. doi: 10.3390/molecules16087068.