# EFFECTS OF MULBERRY HEARTWOOD INFUSION ON T-BUTOOH -INDUCED EXPRESSION OF PROINFLAMMATORY GENES IN MACROPHAGE CELL CULTURE

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## ABSTRACT

**Oxidative stress is a key factor in the induction of chronic inflammation by activation of a variety of transcription factors and proinflammatory cytokines, which in turn could mediate different chronic pathological conditions. In the present study *Morus nigra* heartwood ethanol infusion was applied to oxidatively stimulated macrophage cells with the aim to investigate the effect of the plant on the expression of genes involved in proinflammatory response. The infusion markedly reduced the mRNA levels of the nuclear factor kB (NF-kB), tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) in a concentration-dependent manner. These results are a new contribution to the knowledge about the therapeutic potential of the mulberry tree, especially about its poorly examined heartwood.**

**Keywords:** *Morus nigra, heartwood infusion, oxidative stress, macrophages, gene expression*

## INTRODUCTION

Oxidative stress and inflammation are closely related phenomena, contributing to pathological conditions, such as cardiovascular diseases, metabolic syndrome and diabetes (15). Reactive oxygen species (ROS) may have a major impact on redox-sensitive signaling pathways in different types of cells, including macrophages and adipocytes (1,13,20). The activation of redox-sensitive transcription factors, such as NF-κB is a prerequisite of the induction of proinflammatory gene expression, including tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) (4,11,24,39).

Understanding the mechanisms behind the beneficial effects of medicinal and edible plants is the scope of many scientific investigations. It is known that plant extracts and plant phenolic compounds are able to modulate the production and function of cytokines and thus could be an alternative for inflammatory diseases counteraction (5,10).

*Morus nigra* L. (mulberry tree) is an arboreal plant traditionally applied by the folk medicine because of the various healing effects of its fruits, leaves and root bark (6,17,33). Along with the knowledge about the medicinal properties of the plant, mulberry is known to also have other traditional application – its heartwood is used as a material for barrels man-

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ufactured for storage of alcoholic beverages and respectively for their aging. Although, the ethanol extracts of mulberry heartwood have specified phytochemical composition (12), to our knowledge their biological effects still remain unrevealed. In our previous study, we estimated that the ethanol infusion from mulberry heartwood inhibited the expression of NF-κB and TNF-α in a model of oxidative stress in preadipocyte cell culture and we speculated about the effects of mulberry on adipose tissue metabolism and related diseases (22).

Now, the aim of this study is to explore the effect of ethanol infusion from *Morus nigra* heartwood on the expression of proinflammatory cytokines in oxidatively challenged macrophages, types of cells that are directly involved in low-grade inflammation of the fat tissue and associated metabolic diseases.

## MATERIALS AND METHODS

### *Infusion Preparation*

Ethanol infusion from *Morus nigra* heartwood was prepared following the traditional recipe for coloring high alcoholic beverages: 2g dried material from heartwood were placed in 1L 40% ethanol for 40 days. Heartwood samples were subjected to fumigation following the popular technology for aging of beverages: the wooden chips were boiled for 10 minutes and then saturated with cold water for 24 hours. Finally, the material was dried for 15 minutes at 150-190°C.

### *Cell Culture*

Mouse J774A.1 macrophages were obtained from American Type Culture Collection (ATCC). The cells were cultured in 75 $cm^2$ flasks at 37°C in a humidified chamber containing 5% $CO_2$. Nutrient medium comprised of phenol red-containing Dulbecco's modified Eagle's medium (DMEM, Lonza) with 4,5g/L glucose, L-glutamine and supplemented with fetal bovine serum (FBS) to a final concentration of 10% and penicillin/streptomycin mixture to a final concentration of 100U/ml each. Cells were grown in cell culture flasks up to 80% confluence.

### *Experimental Procedure*

The macrophages were collected and seeded in 6 well flasks at density $2x10^5$ cells/well for different treatments (Table 1). The *Morus nigra* infusion (M) or 40% EtOOH (Et) was dissolved as follows: 6.25µl, 12.5µl or 25µl to 2 ml in phenol red- free DMEM to a final content in the nutrient medium of 0.3%, 0.6% and 1.25%, respectively.

For an induction of oxidative stress, cells were treated for 24 hours with 100µM tertiary-butylhydroperoxide (t-ButOOH, tB) (32). At the end of the treatment the cells were collected for total RNA extraction.

*Table 1. Treatment groups of J774A.1 macrophages*

| Compound | Final content in the nutrient medium (%) | Groups | |
|---|---|---|---|
| | | without oxidant | with oxidant |
| 40% ethanol infusion from *M.nigra* (M) | 0.3 | M 0.3 | M 0.3+tB |
| | 0.6 | M 0.6 | M 0.6+tB |
| | 1.25 | M 1.25 | M 1.25+tB |
| 40% ethanol (Et) | 0.3 | Et 0.3 | Et 0.3+tB |
| | 0.6 | Et 0.6 | Et 0.6+tB |
| | 1.25 | Et 1.25 | Et 1.25+tB |

### *Quantitative Real-Time PCR Analysis*

Total RNA was extracted from J774A.1 macrophages using TRI Reagent according to the manufacturer's protocol (Ambion). In order to remove the contamination of genomic DNA, DNA-ase I treatment was performed using the recommended protocol of the manufacturer (Sigma). Complementary DNA was synthesized using Revertaid™ First Strand Synthesis Kit with oligo $(dT)_{18}$ primers and RevertAid™ reverse transcriptase (Thermo Scientific). The synthesis reaction was performed on GeneAmp PCR System 9700 (Applied Biosystems). Two-step real-time PCR analysis was performed (ABI PRISM 7500,

Applied Biosystems) to estimate gene expression level in cultured cells. Maxima SYBR Green qPCR Kit (Thermo Scientific) was used for sample analysis. The cDNA was amplified using forward and reverse primers of target genes (Table 2) commercially synthesized (Invitrogen Alpha DNA, Canada). Beta-actin was used as endogenous control. All samples were analyzed in triplicates. Gene expression levels were calculated by a $2^{-\Delta\Delta Ct}$ method (18) and expressed as relative units (RU) mRNA compared to the untreated controls where the level of gene expression of interest was considered to be equal to 1.

As demonstrated in Figures 1, 2 and 3, the addition of the oxidant to the nutrition medium (group tB) resulted in a significant increase in mRNA levels of the three tested genes, as compared with the controls (***p<0.001 vs. group C). Furthermore, a cumulative effect of ethanol and the oxidant was observed in stimulation of the expression of all three genes for the groups Et1.25+tB.

### *Expression of NF-kB*

Real-Time qPCR analyses demonstrated that none of the applied concentrations of the mulberry infusion changed the NF-kB expression (Fig. 1).

*Table 2. Sequences of primers used for RT-PCR analysis*

| Genes | Nucleotide sequence |
|---|---|
| β-Actin | F: 5'-ACG GCC AGG TCA TCA CTA TTG-3'<br>R: 5'-CAA GAA GGA AGG CTG GAA AAG- 3' |
| NF-kB | F: 5'-ATGGCAGACGATGATCCCTAC- 3'<br>R: 5'- TGTTGACAGTGGTATTTCTGGTG- 3' |
| TNFα | F: 5'- CCCTCACACTCAGATCATCTTCT -3'<br>R: 5'- GCTACGACGTGGGCTACAG -3' |
| IL-1β | F: 5'- TTCAGGCAGGCAGTATCACTC- -3'<br>R: 5'- CCACGGGAAAGACACAGGTAG -3' |

### *Statistical Analysis*

Data are presented as mean ± standard error of mean (*SEM*). Differences between the means of the groups were analyzed by Student's *t*-test or one-way ANOVA with Dunnett's multiple comparison test (GraphPad Prism 5.0). Values of P<0.05 were considered to be statistically significant.

## RESULTS

### *Effects of the Oxidant on Gene Expression*

The toxicity of t-ButOOH includes lipid peroxidation, depletion of cellular storage of glutathione and DNA damage (16,19,27). Its intracellular metabolism may cause mitochondrial oxidative stress (38).

In the present study t-ButOOH was applied in macrophage cell culture with the purpose of provoking oxidative stress. Three concentrations of mulberry ethanol infusion were applied to explore the effect of the preparation on genes related to the inflammatory response. The same concentrations of ethanol or ethanol along with the oxidant were applied in order to distinguish the effects of the plant-derived active compounds from the effects of ethanol solely.

Ethanol applied alone stimulated the expression of the gene in a concentration-dependent manner (**p<0.01). Pretreatment with the infusion (groups M0.6+tB and M1.25+tB) decreased significantly ox-

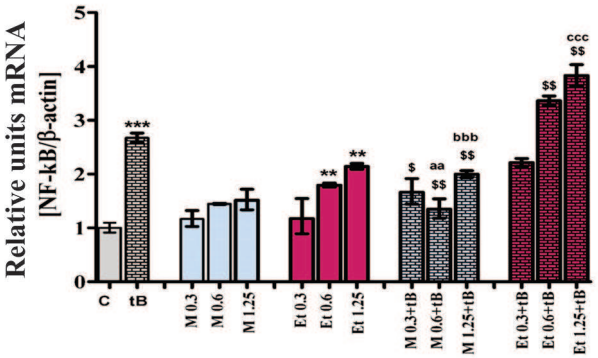


*Figure 1. Expression of NF-kB in J774A.1 macrophages, treated with different concentrations of M. nigra heartwood ethanol infusion in normal medium or under t-ButOOH-induced oxidative stress. Data are presented as mean±standard eror of mean (SEM); ***p<0.001, **p<0.01 vs. group C; $^{$$}$p<0.01 vs.groups tB; $^{aa}$p<0.01 vs.group E t0.6+tB; $^{bbb}$p<0.001 vs.group Et 1.25+tB*

idant-induced gene expression of NF-kB ($^{\$\$}$p<0.01). Furthermore, this inhibitory effect was confirmed in comparison with the cells treated with the same concentration of ethanol with the oxidant (Et0.6+tB and Et1.25+tB). Moreover, the ethanol and the oxidant applied together had a cumulative stimulatory effect on the mRNA levels of the gene.

### *Expression of TNFα*

Figure 2 summarizes the effects of the mulberry heartwood ethanol infusion on the TNF-α gene expression. The three applied concentrations of the infusion did not change the mRNA levels of the cytokine. Ethanol applied alone in concentrations 0.6 and 1.25 significantly stimulated the expression of the gene (**p<0.01). All of the applied concentrations of the infusion significantly inhibited the expression of TNF-α as compared with the tB treated cells ($^{\$\$}$p<0.01) upon induction of oxidative stress (groups M+tB). Furthermore, the highest concentration of the infusion, applied in the oxidatively stimulated cells significantly inhibited the expression of the cytokine as compared to the ethanol+oxidant treated cells ($^{cc}$p<0.01 vs. Et1.25+tB).

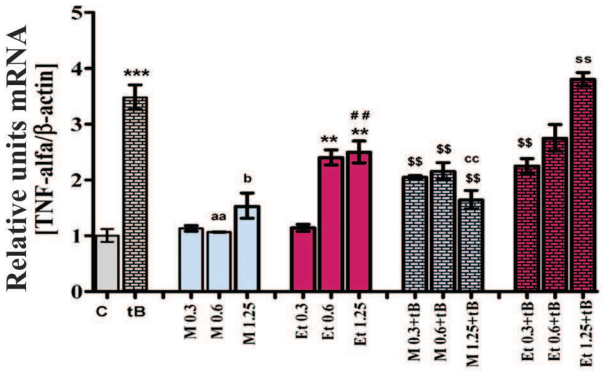


*Figure 2. Expression of TNFα in J774A.1 macrophages, treated with different concentrations of M. nigra heartwood ethanol infusion in normal medium or under t-ButOOH induced oxidative stress. Data are presented as mean±standard eror of mean (SEM); ***p<0.001 and **p<0.01 vs. group C; $^{\$\$}$p<0.01 vs. groups tB; $^{aa}$p<0.01 vs. group Et 0.6; $^{b}$p<0.05 vs. group Et 1.25; $^{cc}$p<0.01 vs. group Et 1.25+tB $^{ss}$p<0.01 vs. group Et 0.3+tB; $^{\#\#}$p<0.01 vs. group Et 0.3*

### *Expression of IL-1β*

The two higher concentrations of the mulberry infusion significantly inhibited the expression of IL-1β, compared with the controls (**p<0.01 for M0.6 and *p<0.05 for M1.25 versus group C). On the other hand, the ethanol applied alone in a concentration of 1.25 (group Et 1.25) significantly stimulated the cytokine expression (*p<0.05). Pretreatment with the two higher concentrations of the mulberry infusion (groups M 0.6+tB and M 1.25+tB) decreased significantly t-ButOOH - induced gene expression of IL-1β ($^{\$}$p<0.05 and $^{\$\$\$}$p<0.001 versus group tB). Data are presented in Figure 3.

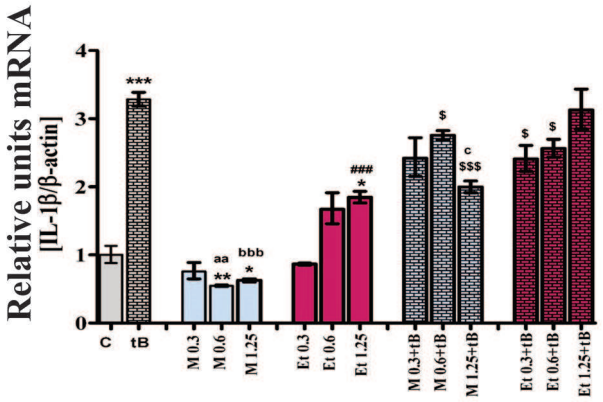


*Figure 3. Expression of IL-1β in J774A.1 macrophages, treated with different concentrations of M. nigra heartwood ethanol infusion in normal medium or under t-ButOOH-induced oxidative stress. Data are presented as mean±standard eror of mean (SEM); ***p<0.001, **p<0.01 and *p<0.05 vs. group C; $^{\$\$\$}$p<0.001 and $^{\$}$p<0.05 vs. groups tB; $^{aa}$p<0.01 vs. group Et 0.6; $^{bbb}$p<0.001 vs. group Et 1.25; $^{c}$p<0.05 vs. group Et 1.25+tB; $^{\#\#\#}$p<0.001 vs. group Et 0.3*

## DISCUSSION

The aim of the study was to explore the potential of mulberry heartwood ethanol infusion to affect the expression of three genes involved in the inflammatory response in a model of oxidative stress. Scientific data point that ROS could play a crucial role as intermediates in the development of chronic inflammation (21). Several studies reported that the impairment of redox balance in the cells could be related to induced expression of proinflammatory factors (13,26). The heterodimeric protein NF-kB is a redox-sensitive transcriptional factor involved in immune and inflammatory response, cell proliferation and apoptosis (7). Its activity could be affected by ROS in different ways (21). The target genes acti-

vated by NF-kB encode the synthesis of various cytokines, including TNFα, cytokines and their receptors as well as cell adhesion molecules (9). As a crucial factor involved in inflammatory responses, the activity of NF-kB is associated with both aging process and age-related diseases, for example, cancer, obesity, diabetes and atherosclerosis (2).

The results obtained in this study showed that mulberry infusion applied under conditions of oxidative stress (groups M+tB), markedly inhibited the expression of the three genes. Moreover, a pronounced concentration dependency was estimated in this effect. In a recent study, similar effect of the infusion on the expression of NF-kB and TNFα was observed in an oxidatively stimulated preadipocyte cell culture (22).

The described effects could be attributed to the active compounds extracted in the solution from the plant. Previously, we estimated that the mulberry heartwood infusion possessed high antioxidant activity *in vitro*, correlating with high total polyphenol content (23). Many authors reported similar effects of the polyphenols or polyphenol-rich extracts on the proinflammatory cytokines (including TNFα) in various experimental models of oxidative stress and inflammation (28,37). As potent antioxidants, polyphenols can affect the function of nuclear factor-erythroid 2-related factor 2 (NRF2) and AMPK signaling pathways. Polyphenols can also directly repress NF-kB signaling (29).

TNFα, a proinflammatory cytokine involved in the pathogenesis of many chronic diseases, could provide the link between inflammation and the development of insulin resistance (3). The mechanisms which connect the increased production of ROS, inflammation and obesity involve increased production of proinflammatory cytokines from macrophages such as TNFα and IL-1β (30).

IL-1β is produced by macrophages and it is responsible for the acute phase of inflammation (14). Several studies reported that polyphenols and polyphenol-rich extracts had the potential to inhibit both, the IL-1β-stimulated expression of NF-KB and directly the expression of pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6 in cell cultures (8,34). Proinflammatory cytokines produced by macrophages are implicated in the pathogenesis of metabolic disorders such as metabolic syndrome and insulin resistance (35). The infiltration of TNFα, IL-1β and IL-6 could play a role of a paracrine stimulus, activating NF-kB signaling pathway and thus increasing the systemic levels of cytokines in adipocytes observed in overweight subjects (25,31). On the other hand, naturally derived inhibitors of the NF-kB are considered to be a promising approach for the prophylaxis and management of pathological conditions that are due to prolonged activation of this signal pathway (36).

## CONCLUSION

The results reported in this study are another small step in clarifying the biological activities and therapeutic potential of *Morus nigra* heartwood infusion. Its antioxidant and anti-inflammatory action observed in models of induced oxidative stress indicate that this preparation from the plant could be a potential source of active ingredients applicable for prevention and treatment of various chronic diseases.

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