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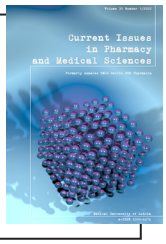

The effect of ethyl acetate extract from *Atractylis flava* Desf. on the gene expression of pro-inflammatory cytokines and oxidative stress markers in NR8383 alveolar rat macrophage cells

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ABSTRACT

Atractylis flava Desf. (*AF*) is common plant that is widely used for its anti- inflammatory and antioxidant properties. The purpose of this study was, therefore, to evaluate the cytotoxic effect and the molecular basis of antioxidant and anti-inflammatory effects of the ethyl acetate extract (*AFEAE*) obtained from the whole plant *A. flava. This was accomplished through* the use of NR8383 alveolar rat macrophage cells. Cultures of alveolar rat macrophage cells were treated with *AFEAE* (25–800 µg/mL), and cell viability was determined via WST-1 and LDH tests. In turn, the gene expression levels of nuclear factor κB (NF-κB), tumor necrosis factor-alpha (TNFα), interleukin 1 beta (IL1-β), interleukin 6 (IL-6), mitochondrial dynamin like GTPase (OPA1), Succinate dehydrogenase complex subunit A (SDHA) and neutrophil cytosolic factor 1 (NCF1) were assessed by applying RT-qPCR. The results show that ethyl acetate extracts of *A. flava* have non-cytotoxic effects, and the gene expression analysis demonstrates that AFEAF extracts generate significant downregulation of NF-κB, TNFα, IL-1 β, IL-6, NCF1, OPA1 and SDHA, compared to untreated cells. This study reveals that *Atractylis flava* ethyl acetate extract administration may be considered as a potential therapeutic strategy for inflammatory related diseases.

INTRODUCTION

Atractylis flava Desf. (AF), is one of the traditional medicinal plants that are extensively distributed in the Mediterranean zone. AF has been used for treating many circulatory disorders, inflammation, ulcers, intestinal parasites and snakebite poisoning [1]. Its therapeutic capacity is reported to be due to its high phytochemical content, among others, phenolic compounds and hydroxylated phenolic substances called "flavonoids". These are strong anti-inflammatory and antioxidant molecules that mitigate/prevent oxidative cell damage [2]. Besides the aforementioned, several studies have demonstrated the presence of triterpenes, steroids, saponins, and flavonoids, and, according to some authors, narcissin, tiliroside, ladaneine, and vicenin3 [3,4]. Additionally to its uses in folk medicine, it may be considered as a good candidate for the elaboration of new drugs [5]. Indeed, several studies have been performed showing its anti-inflammatory (by modulating pro-inflammatory cytokines expression), anti-oxidant/oxidative stress, antidiabetic, antipyretic and anticancer effect on a monocytic leukemia cell line model (THP-1 cells). In all of these studies, the beneficial effects of AF were concluded as the outcome of its therapeutic capacity due to the abundant active polyphenolic constituents.

In this study, the effect of ethyl acetate (*AFEAE*) extract of *A. flava* on cell viability, gene expression levels of inflammatory NF-κB, pro-inflammatory cytokines TNF-α, IL1-β and IL6 and oxidative stress gene OPA1, NCF1 and SDHA was investigated on a murine macrophage cell model [1].

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MATERIALS AND METHODS

Collection of plant and extraction procedure

The collection of AF was done in Biskra District in southern Algeria in May 2015 (flowering season, at Ain Ben Naoui, Biskra, 34°48'51.15''N; 5°39'17.93''E). Taxonomic identification was achieved at Agronomic Institute, University of Banta, Algeria (by botanist Pr. Bachir Oudjehih, Voucher Number: 660/LCCE). Whole plant was ground after being dried naturally. The plant was powdered by using a laboratory mill.

Extraction techniques

To obtain the ethyl acetate (EtOAc) extract, a maceration technique was applied. The powdered whole plant was stirred in a methanol/water 80:20 (v/v) mix (in a magnetic shaker for 24 h at ambient temperature). The obtained hydroalcoholic extract was then dried by using a vacuum evaporator. Afterwards, the obtained extract was submitted to liquid–liquid fractioning using (petroleum ether, dichloromethane, ethyl acetate, and *n*-butanol). Ethyl acetate fraction was employed to investigate the molecular basis of anti-inflammatory and anti-oxidant effects.

Cell culture

The NR8383 alveolar rat macrophage cell line (ATCC® CRL2192™, USA) was grown in Dulbecco's Modified Eagle Medium, supplemented with 4 mM of l-glutamine (SIGMA-G7513), 15% of heat-inactivated foetal calf serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin (SIGMA-P0781) and 0.25 µg/mL of amphotericin B (SIGMA-A2942) and incubated in a humidified mixture of air (95%) and CO_2 (5%) at 37°C and split every 4 days [6].

Viability test

WST-1 assay

Metabolic activity was performed by applying WST-1 assay (Roche, 11644807001, USA), according to manufacturer's protocol. Cells (NR8383) were seeded in 96-well plates and exposed to different concentrations (25 to 800 µg/mL) of *AFEAE* and incubated for 24 hours, the cells were again incubated with 5 µL of WST-1 Cell Proliferation Reagent at 37°C, 5% CO_2 for h. The absorbance was measured at 480 nm using a microreader (BioRad-iMARK).

Lactate dehydrogenase (LDH) cytotoxicity assay

Lactate dehydrogenase leakage assay was performed using the LDH Kit (Roche Roche-4744934001, Germany). Briefly, NR8383 cells were seeded in 96 well-plates with 5×104 cells/mL/well and treated different concentrations (25 to 800 µg/mL) of *AFEAE*. LDH assay was conducted following the manufacturer's instruction. A lysis solution of triton (10%) served as positive control. LDH in the supernatant was quantified using a microreader (BioRad-iMARK) at 490 nm and 630 nm. Medium and lysates were used as negative and positive control respectively [7].

Gene expression analysis by real-time PCR (qRT-PCR)

The expression of the following genes by NR8383 cells was assessed: nuclear factor kappa-light-chain-enhancer, the gene expression of nuclear factor κB (NF-κB), tumor necrosis factor-alpha (TNFα), interleukin 1 beta (IL1-β), interleukin 6 (IL-6) and mitochondrial dynamin-like GTPase (OPA1), succinate dehydrogenase complex subunit A (SDHA) and neutrophil cytosolic factor (NCF1).

In undertaking this task, total RNA was extracted from 1.5×106 NR8383 cells unexposed or exposed for 24 h to 600 µg/mL of *AFEAE* by TRIzol® Reagent (Invitrogen, La Jolla, CA). The degradation and purity of isolated RNA were determined by measuring optical densities using BioSpec-nano (Shimadzu Corporation, Kyoto, Japan) and capillary electrophoresis using RNA 6000 Nano® kit and the Bioanalyzer™ 2100. Moreover, iScript™ complementary Synthesis Kit (Bio-Rad, France) was employed for reverse transcription. In addition, cDNA synthesis was performed with 100 ng total RNA. Gene expressions were explored by RT-qPCR via iQ™ SYBR Green® Supermix in a Stratagene Mx3000p system (Agilent Technologies). Herein, 4 µL of each cDNA sample was amplified in a PCR reaction (final volume of 20 µL) containing 10 µL of PCR reagent and 300 nM of each gene specific primers (Table 1).

For all the samples, the following conditions were used: an inimical heat-denaturing step at 95°C for 5 min followed by 40 cycles of 95°C for 15 s, annealing at 60°C for 40 s, elongation and signal acquisition at 72°C for 40 s. To approve the amplification of specific transcripts, melting curve profiles were produced at the end of each reaction, and if two or more peaks were present, the results were excluded. Water was used as negative controls for each PCR run. For each gene, amplifications were performed from three prepared samples. Gene expression levels were normalized by comparison to ribosomal protein (RPL13) housekeeping genes. Fold change (FC) of gene expression was calculated by applying the $2^{-\Delta\Delta Ct}$ method [8].

Table 1. Primers used for gene expression in NR8383 rat cells

Functional class	Gene	Sequence
Internal control	*RPL13*	F: 5'CCCTCCACCCTATGACAAGA-3' R: 5'-GGTACTTCCACCCGACCTC-3'
Oxidative stress	*NCF1*	F: 5'-CTTGTAATTCCCGCATTGCT-3' R: 5'-GCCTCGTATGTCTTTGATGC-3
	OPA1	F: 5'-TCCTGTGCATTCAAGATGGA-3' R: 5'-GAGCTTTCATTGGGAAGAGC-3'
	SDHA	F: 5'-GAGGATTGTGGCCTTCTTTG-3' R: 5'-GCATCCCAGCCTCCGTTAT-3'
Inflammation	*NF-κB*	F: 5'-TTCGGAACTGGGCAAATGTT-3' R: 5'-ACACGTAGCGGAATCGAAAT-3'
	TNFα	F: 5'-TAGCCCATGTTGTAGCAAACC-3' R: 5'-GATGGCAGAGAGGAGGTTGA-3
	IL1-β	F: 5'-AGCAGCTTTCGACAGTGAGG-3' R: 5'-AAAGAAGGTGCTTGGGTCCT-3'
	IL6	F: 5'-TAGTCCTTCCTACCCCAACTTCC-3' R: 5'-GGTTTGCCGAGTAGACCTCA-3'

STATISTICAL ANALYSIS

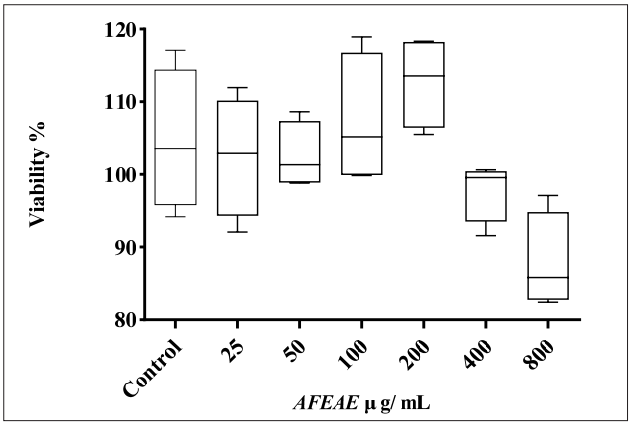
All data are presented as the means ± standard error (SEM) using GraphPad Prism version 8. (N – which represent the number of biological replicates as four; n – which represent the number of technical replicates as 6). Regarding

qRT-PCR (N=4; n=6), fold change was calculated by the ratio exposed/ unexposed cells and results were expressed as means±SE. Statistical differences between control and exposed cells were determined by applying ANOVA test, followed by Dunnett's. Significance was considered when $p<0.05$.

RESULTS

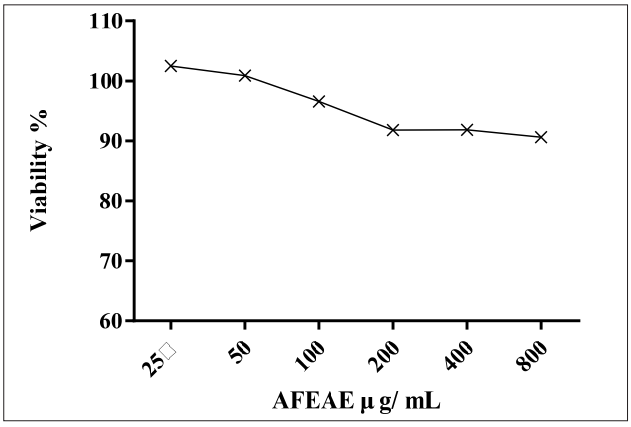
Cytotoxicity assays

The effects of the *AFEAE* on cell viability were determined using two independent approaches, i.e., the WST-1 assay and the LDH assay. The WST-1 assay showed that no cytotoxic effects were observed in any of the test samples, and more than 80% of cell viability was observed after 24h of exposure to 800 µg/mL of *AFEAE* (Figure 1). *AFEAE*, however, showed a tendency for a concentration-dependent decrease of formazan formation, indicating lower cytotoxicity, nonetheless, this did not reach statistical significance in the concentration range tested. No significantly enhanced LDH release was detected after 24 h following treatment with *AFEAE*, and 90% of cell viability was observed after 24h of exposure to 800 mg/mL (Figure 2). No morphological alterations were detected in cells after 24 h following treatment with 100-600 µg/mL *AFEAE* (Figure 3).



Data represents the means±SEM (N=4; n=6)

Figure 1. Cytotoxicity of *AFEAE* on NR8383 cells. The cytotoxicity was evaluated through WST-1 assay



Data represents the means±SEM (N=4; n=6)

Figure 2. Cytotoxicity of *AFEAE* on NR8383 cells. The cytoxicity was evaluated via LDH release measurement

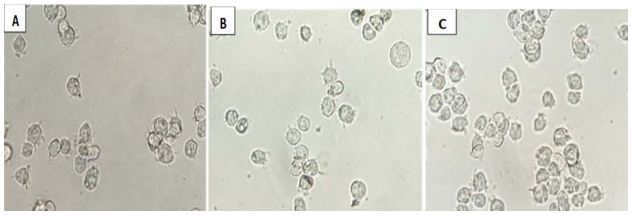


Figure 3. The effects of the *AFEAE* on the morphology of NR8383 cells. A: control, B–C: treated with 100-600 µg/mL *AFEAE*

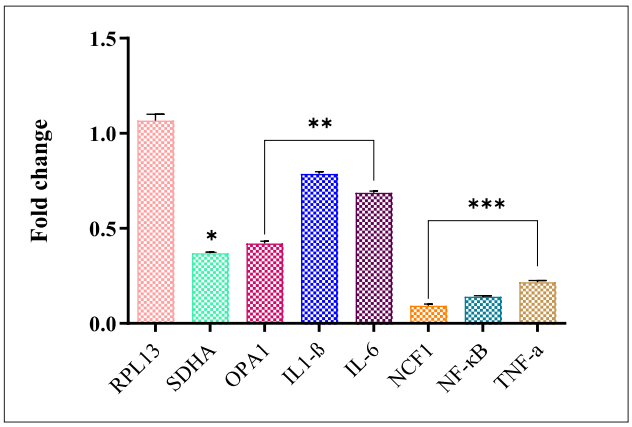
Gene expression analysis (real-time qPCR)

Results were represented as "Fold Change". Gene expression analyses via RT-qPCR revealed that AFEAE induced statistically significant downregulation of NF-κB ($p<0.001$), TNF-α ($p<0.05$), IL1-β ($p<0.05$), IL-6($p<0.05$), NCF1 ($p<0.001$), OPA1 ($p<0.05$), and SDHA ($p<0.0001$) gene expressions, compared to untreated control cells (Table 2). In Figure 4, the values are illustrated as percent of the control (RPL13).

Table 2. Gene expression changes in response to AFEAE exposure of NR8383 alveolar rat macrophage cell. Cells were exposed to 600 µg/mL of AFEAE for 24 h

Gene	Fold change	P value
NCF1	0.09±0.057	<0.001
OPA1	0.42±0.015	<0.05
SDHA	0.37±0.057	<0.01
NF-κB	0.14±0.057	<0.001
TNF-α	0.22±0.008	<0.001
IL1-β	0.79±0.012	<0.05
IL-6	0.68±0.008	<0.05

Results were presented as fold change as compared to control ± SEM (N=4; n=6) using ANOVA, followed by Dunnett's test



For quantitation of RT-qPCR results: * p<0.05, ** p<0.01, *** p<0.001

Figure 4. Gene expression levels of the selected genes as percent of the control

DISCUSSION

Although the pharmacological studies performed with *Atractylis flava* (AF) extracts showed this plant's beneficial effects, the exact mechanism of these results still remains unclear. Among the phytoconstituents found in our extract, vicenin-2, tiliroside and schaftoside were shown to have potent inhibitor effect upon pro-inflammatory cytokines

in different studies [9,10], while in clinical studies, quercetin, stigmasterol, chrysin, apigenin and oleolic acid were revealed to have antioxidant effects [11]. In demonstrating the suppressive effects of *AFEAE* as a whole on inflammatory *NF-κB*, pro-inflammatory cytokines *TNF-α*, *IL1-β* and *IL6* and oxidative stress gene *OPA1*, *NCF1* and *SDHA*, it may be more logical to discuss its anti-inflammatory and anti-oxidant properties.

In spite of the fact that previous publications have reported the pharmacological activities, as well as the health benefits of *A. flava* [1,11,12], no toxicological effect has currently been indicated. In the present investigation, alveolar rat macrophage cell NR8383 as an *in vitro* bio-indicator were exposed to the *AFEAE* to identify whether it has cytotoxic potential, and macrophage treatment with the extract was done at final concentrations of 25, 50, 100, 200, 400 and 800 µg/mL. The results of the LDH and WST-1 assays indicated that *AFEAE* was non-cytotoxic in NR8383 cells These results are corroborated and attested. The safety of *A. flava* has also been confirmed *in vivo* [13].

Macrophages are key cells in the process of inflammation. They are involved in the innate and adaptive immune response of which overactivation could lead to tissue destruction throughout an exacerbated inflammatory response. These cells initiate the transcription of several inflammatory genes, including many cytokines, via the NF-κB pathway. Therefore, inhibition of NF-κB, TNF-α, IL1-β, IL6, OPA1, NCF1 and SDHA could be considered one of the key anti-inflammatory and antioxidant mechanisms of *A. flava*.

In the process of inflammation, a variety of cells are incriminated. Among these are four crucial types of cells that mediate inflammation: macrophages, neutrophils, lymphocytes and mast cells. After these cells are attracted to the injury site, they release various inflammatory mediators, histamine, cytokines, NO, leukotrienes, prostaglandins and other substances [10]. Inflammatory cells also produce soluble mediators, chemokines and cytokines, which act by further inducting inflammatory cells to the site of inflammation and producing more reactive species (ROS). These pro-inflammatory mediators can activate signal transduction cascades, as well as induce changes in transcription factors, such as nuclear factor Kb (NF-κB) (Figur. 5), signal transducer of transcription 3, activator protein-1 (AP-1), hypoxia-inducible factor-1α (HIF-1α), NF-E2 related factor-2 (Nrf2) and nuclear factor of activated T cells, which mediate immediate cellular oxidative stress responses. Induction of cyclooxygenase-2 and inducible nitric oxide synthase (iNOS), aberrant expression of pro-inflammatory cytokines (tumor necrosis factor (TNF α), interleukin-1 (IL-1), IL-6) and chemokines (IL-8, CXC chemokine receptor 4 (CXCR4)), other than generating alterations in the expression of specific microRNAs, have also been reported to play a crucial role in oxidative stress-induced inflammation [14]. This sustained inflammatory/oxidative stress leads to many physiological and pathological processes, which can, over a long period lead, to carcinogenesis [15].

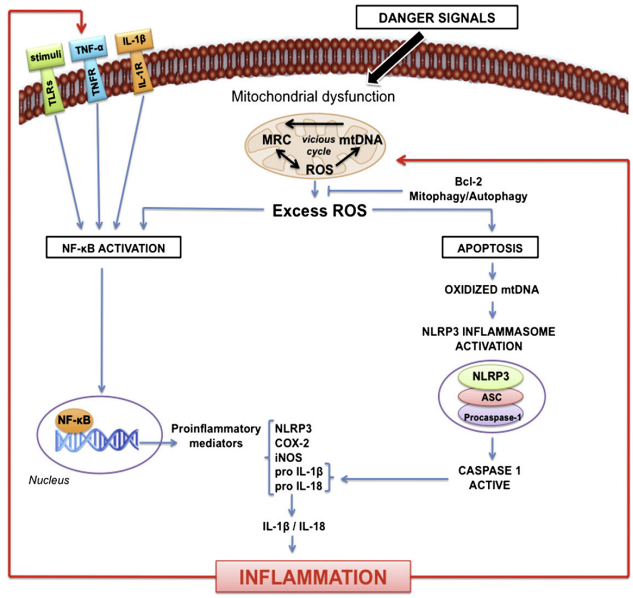


Figure 5. The interrelation between mitochondrial dysfunction and inflammatory response [16]

Altered mitochondrial function plays a main role in inflammatory mechanisms, as mitochondria have a crucial role in inflammatory signaling. Similarly, pro-inflammatory cytokines may also modify mitochondrial function. Both of these systems increase oxidative stress, advancing a complex inflammatory cycle. Furthermore, damage-associated molecular arrangement extrapolated among mitochondria could induce caspase-1 activation and inflammasome formation. Hence, strategies attempting at controlling oxidative stress may represent therapeutic and preventive effect in situations of inflammation [15].

Inflammation is another action that alters cell viability. Mitochondrial fusion and OPA1 protein are directly affected in this action via the OPA1–TNFα-NF-kB and stress genes regulatory pathway [17,18]. This pathway works via augmenting mitochondrial fusion and improving respiratory chain ability in response to cellular oxidative stress. TNFα is a pro-inflammatory cytokine produced by macrophages during acute inflammation. It is a signaling cytokine that is passed on to other cells. NF-κB is a protein complex that controls transcription of DNA and is affected in cellular responses to oxidative stress. Typically, inflammatory increases TNFα, which activates NF-kB, and eventually increases the production of OPA1 protein. The inflammatory response is usually regulated via complex multi-gene signaling editing the homeostatic balance of organelles in response to cellular oxidative stress [19,20].

Atractylis flava ethyl acetate extract, by causing the downregulation of the gene expressions of all factors studied in NR8383 cells, offers a wide array of opportunities for in vivo investigation to detect its efficacy on various inflammatory diseases. While there may be many mechanisms of action to be depicted on molecular basis, considering together the described anti-inflammatory and antioxidant effects of the demonstrated gene expression in alveolar rat macrophage cell NR8383, these preliminary results may partially explain the effectiveness of AF in the traditional medicine.

In light of these results, the cytotoxicity assays undertaken in this study demonstrated the safety of *Atractylis flava* ethyl acetate extract. This evidence has been confirmed *in vitro* in alveolar rat macrophage cells. In addition, *Atractylis flava* ethyl acetate extract may be beneficial for inflammatory diseases. Further *in vivo* studies are required to support these findings at the cellular level.

ETHICAL STATEMENT

ACKNOWLEDGEMENTS

Not applicable.

FUNDING

Not applicable.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable as rat cell lines were obtained from the ATCC®.

CONSENT FOR PUBLICATION

Not applicable as cell lines were obtained from the ATCC®.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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