

Gene Expression Levels of Selected Factors in Monocytic Leukemia Cell Line THP-1 Upon Treatment with *n*-butanol Extract of *Atractylis flava* Desf against Cancer

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ABSTRACT

Objective: The aim of the present study was to screen the anticancer activity of *Atractylis flava* Desf in butanolic extract (AFBE). In addition, we attempted to investigate the gene expression levels of seven relevant genes involved in the pathways leading to toxicity: oxidative stress (NCF1, OPA1, SDHA), inflammation (TNF α), apoptotic balance (PDCD4, BCL2, CASP8) and the measurement of caspase-3 activity on the acute monocytic leukemia cell line THP-1. **Material and Methods:** The cell viability was assessed using Trypan blue exclusion, alamarBlue® and WST-1 assays. The gene expression levels were tested by RT-qPCR. Activity of caspase-3 activity was performed using the EnzChek® Caspase-3 Assay kit to confirm the induction of apoptosis on tumor cells. **Results:** We observed significant growth inhibitory activity of the AFBE extract on the acute monocytic leukemia cell line THP-1. Moreover, the gene expression analysis showed that the plant extract caused statistically significant downregulation of selected genes compared to the untreated cells. Caspase-3 activity in the treated cells was

significantly elevated. **Conclusion:** The presents study states that the butanolic extract of *Atractylis flava* Desf showed a significant cytotoxic effect against THP-1 cells. Our results suggest that the AFBE may be beneficial for the treatment and prevention of cancer.

Key words: *Atractylis flava* Desf, Gene expression, RT-qPCR, Apoptosis, THP-1.

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INTRODUCTION

Cancer is a large group of diseases, all of which have one thing in common i.e. cells growing out of control or fundamentally a disease of tissue growth regulation failure. In order for a normal cell to transform into a cancer cell, the genes which regulate cell growth and differentiation must be altered.¹ Anticancer drugs can destroy tumors and arrest cancer progress but cancer treatment may damage healthy cells and tissues.² Cancer may be successfully treated with chemotherapy. However, chemotherapeutic agents are highly toxic to a wide range of normal body cells and thus are associated with diverse side effects. In addition, multiple drug resistance is a major determinant of chemotherapy failure. Therefore, natural products including, traditional medicinal plants, have emerged as a tempting more tolerated alternative with minimum side effects.³

The species *Atractylis flava* Desf. belongs to the genus *Atractylis* L. of the family Asteraceae(Compositae) is a kind of endemic plant of North Africa.⁴⁻⁵ A small number of *Atractylis* species have been studied for their phytochemical composition. Therefore, the knowledge about phytochemical investigations of *Atractylis* plants is very scanty, amongst these plants; *A. flava*, *A. macrocephala* and *A. gummifera* from which flavonoids, polysaccharide, triterpenes and diterpenes were identified.^{6,7} Moreover, *Atractylis* species are used as general remedies in traditional medicine for treatment of various diseases such as inflammation, ulcer, tumor and circulatory disorders, hepatitis, when *Atractylis flava* Desf is particularly known in traditional North African medicine for its diuretic effects.⁸⁻¹⁰

The objective of this study was to examine the cytotoxic effects of *Atractylis flava* Desf. Our investigation was based on the changes in the gene expression levels of selected factors such as B-cell CLL/ lymphoma 2 (BCL2), caspase 8 (CASP8), neutrophil cytosolic factor 1 (NCF1), mitochondrial dynamin like GTPase (OPA1), programmed cell death 4 (PDCD4), Succinate dehydrogenase complex subunit A (SDHA), tumor necrosis factor-alpha (TNF α) and the measurement of caspase-3 activity on the acute monocytic leukemia cell line THP-1 treated with AFBE.

MATERIALS AND METHODS

Plant Material

The whole plant *Atractylis flava* Desf was collected from Biskra Algeria in May 2015. The plant material was identified by Prof. Bachir Oudjehih of the Agronomic Institute of Banta 1 University, Algeria. A voucher specimen number (660/LCCE) was deposited in the herbarium of the mentioned department.

Extraction

The collected whole plant *Atractylis flava* was air-dried and powdered. 500 g powder was macerated with MeOH-H₂O (80: 20). After filtration, the filtrate was concentrated under vacuum at room temperature the hydro alcoholic extract was submitted to liquid-liquid fractioning using solvents with increasing polarities (petroleum ether, dichloromethane, ethyl acetate and *n*-butanol).

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Cell culture

The human monocytic THP-1 (ATCC[®], TIB-202[™], Manassas, VA, USA) non-adherent monocyte cell line was grown in RPMI 1640 medium. Supplemented with 2mM of L-glutamine, 10 % of heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B were added. Cells were grown at 37°C under 5 % CO₂ atmosphere and split every 3 days.

Evaluation of cell viability

The THP-1 monocytic cell line was seeded in 96 well plates with 5×10³ cells per well. After overnight incubation, plates were centrifuged (300×g, 20 min) and the medium was removed by aspiration. Fresh medium was added and cells were incubated for 24 h with 12.5, 50, 100, 200, 400 and 800 µg/mL of *AFBE*. Six wells were used per culture condition and experiments were repeated thrice. After 24 h, the cell viability was checked using alamar Blue[®] and WST-1 assays, performed according to manufacturer's protocols. The fluorescence and absorbance were measured at 570/585 nm 585 and 415 nm, respectively. Numbers of dead and alive cells were estimated by microscopy (objective ×40) in Glasstic[®] Slides 10 (Kova International, Garden Grove, CA) using trypan blue.

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

Expression of B-cell CLL/ lymphoma 2 (BCL2), caspase 8 (CASP8), neutrophil cytosolic factor 1 (NCF1), mitochondrial dynamin like GTPase (OPA1), programmed cell death 4 (PDCD4), Succinate dehydrogenase complex subunit A (SDHA) and tumor necrosis factor-alpha (TNFα) genes by leukemic cells (THP-1) was assessed. Total RNA was extracted from 1.5× 10⁶ THP-1 cells unexposed or exposed for 4 h to 25 and 100 µg/mL of *AFBE* by TRIzol[®] Reagent (Invitrogen, La Jolla, CA). RNA purity and degradation were checked by spectrophotometry using BioSpecnano (Shimadzu Corporation, Kyoto, Japan) and capillary electrophoresis using RNA 6000 Nano[®] kit and the Bioanalyzer[™] 2100 (Agilent Technologies, Santa Clara, CA). The complementary DNA (cDNA) synthesis was performed with 100 ng of total RNA using the iScript[™] cDNA Synthesis Kit (Bio-Rad, Marnes la Coquette, France) following the manufacturer's protocol. Gene expressions were determined by qRT-PCR with the iQ[™] SYBR Green[®] Supermix (Bio-Rad) in a Stratagene Mx3000p system (Agilent Technologies). Briefly, 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 two primers (Table 1). For all the samples, the following conditions were used: an initial 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 and elongation and signal acquisition at 72°C for 40 s. To confirm the amplification of specific transcripts, melting curve profiles were produced at the end of each reaction and if two or more peaks were presents, the corresponding results were excluded. Water was used for negative controls for each PCR run. For each gene, amplifications were performed from three independently prepared samples. Gene expression levels were normalized by comparison to β-actin (ACTB) housekeeping gene, used as references for THP-1. Fold changes (FC) of gene expression were calculated by 2^{-ΔΔCt} method.¹¹

Caspase-3 activity determinations

Caspase-3 activity of *AFBE* in cell lysates was measured using the EnzChek[®] Caspase-3 Assay kit and processed for caspase-3 enzyme activity assay following the manufacturer's protocol. In brief, the cells were washed with PBS and lysed using cold cell lysis buffer. Next, the cell suspension was centrifuged and the supernatant was collected and incubated with 50 µL of 2X reaction buffer along with 5 µL of 1 mM caspase-3 substrate (DEVD-pNA, 50 µM final concentration) at 37°C for

Table 1: Primers used for gene expression analysis in THP-1 human cells.

Functional class	Gene	Sequence
Apoptosis	ACTB (internal control)	F:5'-TTGGCAATGAGCGGTTC-3' R:5'-GTACTTGCGCTCAGGAGGAG-3'
	PDCD4	F: 5'-AGACCAAATGAAAAGAGGTTATGAG-3' R: 5'-GCCCTTGAAGGACAAAGAT-3'
	BCL2	F: 5'-GAGGATTGTGGCCTTCTTTG-3' R: 5'-GCATCCCAGCCTCCGTTAT-3'
Oxidative stress	CASP8	F: 5'-GAAAGGGTGGAGCGGATTAT-3' R: 5'-GCTTCCTTTCGGAATGTAG-3'
	NCF1	F:5AGAGTACCGCGACAGACATC-3' R: 5'-TAGTTGGGCTCAGGGTCTTC-3'
	OPA1	F: 5'-CTTCCATGAGGGTCCATTTG-3' R: 5'-CCGTTAGCCCTGAGACCATA-3'
Inflammation	SDHA	F: 5'-GCCAGGACCTAGAGTTGTTC-3' R: 5'-GCCTTGACTGTTAATGAGAATGC-3'
	TNFα	F: 5'-TAGCCCATGTTGTAGCAAACC-3' R: 5'-GATGGCAGAGAGGAGGTTGA-3'

30 min. The caspase-3 activity of *AFBE* was then measured in a microplate reader at 342/441 nm.

Statistical analysis

Data are presented as the means ± standard error (SE) of three independent experiments using GraphPad Prism version 5. The data were compared by one-way ANOVA, followed by Tukey's test in Cell viability assays. Regarding qRT-PCR, FC were calculated by the ratio exposed/unexposed cells and results were expressed as means± SE. Statistical differences between control and exposed cells were determined by ANOVA followed by Dunnett's test. The level of significance was considered when $p < 0.05$.

RESULTS

Effect of the *AFBE* on THP-1 Cells viability

After exposure to 25, 50, 100, 200,400 and 800 µg/mL of *AFBE* for 24 h, a significant decrease of 2 to 4% of cell viability compared to the control was observed by WST-1 test (Figure 1). The trypan blue test (Figure 2) showed a dose-dependent decrease of 60% of cell viability after incubation of cells with 100, 400 and 800 µg/mL of *AFBE*. In contrast, under the same conditions, a dose-dependent increase in mitochondrial activity of THP-1 human cells up to 250 % at 800 µg/ml was observed with Alamar blue test (Figure 3).

Gene expression analysis

We have evaluated the effect of *AFBE* (25 and 100 µg/ml) on the expression of PDCD4, BCL2, CASP8, NCF1, OPA1, SDHA and TNFα. Based on our RT-qPCR data, *AFBE* significantly decreases the gene expression levels of selected factors after 4 h of treatment (Figure 4).

Caspase-3 activity determinations

The measure of caspase 3 activity in the cells is a direct mean for the determination of caspase dependent apoptosis. Our results showed a significant increase in the caspase 3 activity ($P < 0.05$ for 25 µg/mL) (Figure 5).

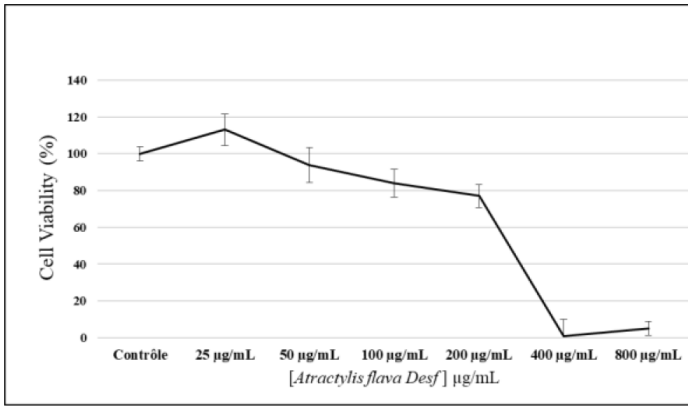


Figure 1: Cytotoxic effect AFBE on cell viability assayed by the WST-1 assay. Cells were incubated with AFBE for 24h (n=12± SEM).

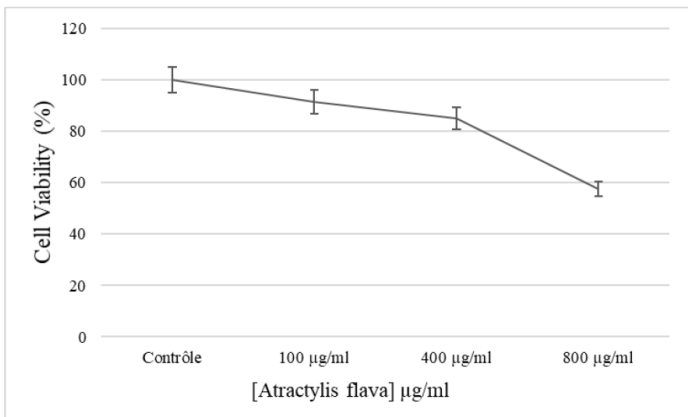


Figure 2: Cytotoxic effect AFBE on cell viability assayed by the Trypan blue assay. Cells were incubated with AFBE for 24h (n=12± SEM).

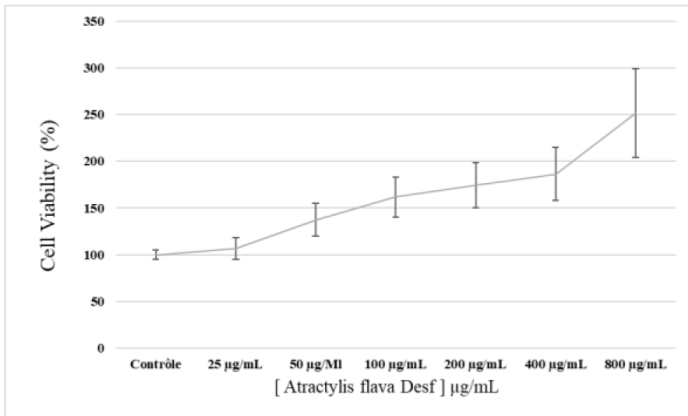


Figure 3: Cytotoxic effect AFBE on cell viability assayed by the Alamar blue assay. Cells were incubated with AFBE for 24h (n=12±SEM).

DISCUSSION

The study of mitochondrial activity of THP-1 treated with AFBE revealed a fall of THP-1 at 200 µg/ml. In addition, the down-regulation of the OPA1 and SDHA genes is said to be the cause, since these observations reflect morphological alterations in mitochondria and deregulation of the respiratory chain. Among the genes responsible of mitochondria

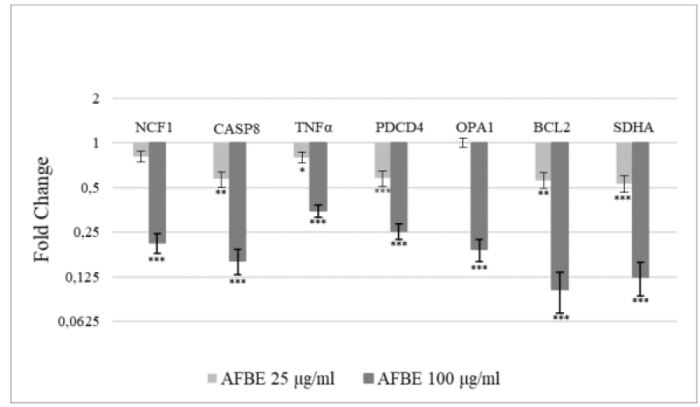


Figure 4: Gene expression changes in response to AFBE exposure of THP-1 human cells. Cells have been exposed to 25 and 100 µg/mL of AFBE for 4 h. Results were presented as fold change as compared to control ± SEM (N=4) using ANOVA followed by Dunnett's test. *, $p < 0.05$ **, $p < 0.01$ and ***, $p < 0.001$ indicating a statistical difference versus control.

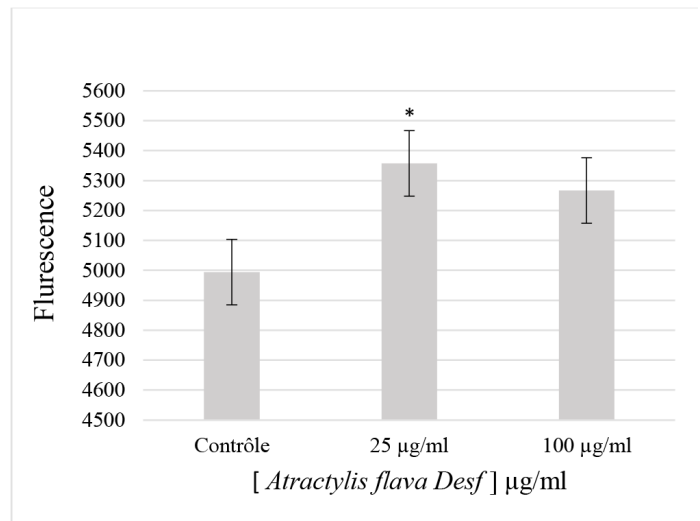


Figure 5: Detection of caspase-3 activity in macrophage THP-1 cell line treated with extract. Cells were treated with 25 and 100 µg/ml of plant extract for 4 h. Results are expressed as mean ± standard deviation using ANOVA followed by Dunnett's test. *, $p < 0.05$ indicating a statistical difference versus control.

functions, OPA1 gene plays a major role in the maintenance of the mitochondrial network morphology and dynamics and in regulating cell death signalling pathways.¹² A down regulation of OPA1 has been reported to promote mitochondrial network aggregation and to modify the structure of the mitochondrial inner membrane.¹³ In fact, SDH is an important ROS generation site.¹⁴ After succinate to fumarate oxidation, which occurs in the SDHA subunit, electrons are transported via the Fe/S sites in the SDHB subunit to the coenzyme Q-binding sites formed by the SDHB/C/D proteins, leading to the reduction of ubiquinone and electron flow to the respiratory complex III.¹⁵ Any interference in this chain of redox reactions results in leakage of electrons and superoxide formation following interaction with molecular oxygen. Indeed, several molecules that target the ubiquinone binding site of SDH eliciting superoxide generation cause apoptosis of cancer cells and are currently under study as potential chemotherapeutics.¹⁶ Conversely, upstream impairment of succinate oxidation by SDHA subunit can abrogate any elec-

tron flow to further SDH components, thus inhibiting ROS generation. Further, the TNF α gene, coding for a pro-inflammatory cytokine was down-regulated, this could indicate a reduction in inflammatory processes. Caspase-3 activation is a crucial component in the apoptotic signaling cascade. To understand how AFBE can be toxic to human monocytic THP-1, we examined the caspase-3 activity in the AFBE-treated macrophage cells using a caspase-3-specific substrate, DEVD-pNA, which is cleaved to produce a fluorescent product. That caspase-3 activity in the cells treated with AFBE was significantly elevated, which was consistent with the observed effect of this extract on cell viability. This clearly shows that the cytotoxic activity of *Atractylis flava* Desf can be attributed to apoptosis induction through caspase-3 activation. Induction of apoptosis in tumour cells is considered a valuable means of treating cancer.¹⁷ A wide variety of natural substances have been known to induce apoptosis in various tumor cells. It is therefore considered important to filter the apoptotic inducers of plants, either as raw extracts or as isolated components of them.¹⁸

CONCLUSION

In conclusion, the findings of the present study demonstrate, for the first time, to the best of our knowledge, that AFBE, causing the down regulation of the gene expressions of all seven factors studied and significantly decreased cell viability and proliferation in the cell lines tested. Indeed, we observe a degradation of mitochondrial activity. This is none other than the consequence of conformational alterations of the mitochondria accompanied by a dysfunction of the Krebs cycle. In addition, AFBE exhibits a down regulation of TNF α this effect may be beneficial for inflammatory diseases. This pathway should be further explored by studying interleukins and interferons.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AFBE: *Atractylis flava* butanolic extract; **BCL2:** B-cell CLL/ lymphoma 2 **CASP8:** Caspase 8; **NCF1:** Neutrophil Cytosolic Factor 1; **OPA1:** Mitochondrial dynamin like GTPase; **PDCD4:** Programmed cell death 4;

SDHA: Succinate dehydrogenase complex subunit A; **TNF α :** Tumor Necrosis Factor-alpha.

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