J Young Pharm, 2019;11(1):51-55
A multifaceted peer reviewed journal in the field of Pharmacy
www.jyoungpharm.org | www.phcog.net

Original Article

Anti-cancer Activity and Gene Expression Responses to Methanol Extract of *Gladiolus segetum* in THP-1 Human Monocytic Leukemia Cells

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ABSTRACT

Objective: To Evaluate the viability of cells against exposure to the Methanol Extract of *Gladiolus segetum* (MEGS) by various colorimetric detection tests (Alamar Blue, WST-1) and the trypan blue exclusion test in Human Leukemia Monocytes THP-1 and Using reverse transcription-polymerase chain reaction assays. **Methods:** Human THP-1 monocytic cell line was exposed to 25 to 800 µg/mL concentrations of MEGS for 24 h and cellular viability was estimated using Alamar Blue, WST-1 and trypan blue tests. Gene expressions were performed using reverse transcription-polymerase chain reaction assays and the measurement of Caspase-3 enzyme activity. **Results:** A decrease of viability was observed with a dose-dependent effect of MEGS on THP-1. In addition, a differential expression involved in the different genes tested. Moreover, it has been shown overexpression of casp3 for an exposure to 25 µg/ml and 100 µg/ml of MEGS. **Conclusion:** Our results show that the MEGS present a toxicity to THP-1 cells, especially with regard to apoptosis processes.

Key words: Gene expressions, Viability, *Gladiolus segetum,*THP-1, Toxicity.

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DOI: 10.5530/jyp.2019.11.11

INTRODUCTION

Cancer is one of the most life-threatening diseases, with more than 100 different types occurring due to some molecular changes within the cell. It is the third leading cause of death worldwide following cardiovascular and infectious diseases.[1] It is estimated that 12.5% of the population dies due to cancer (WHO, 2004). The disease is widely prevalent and in the West, almost a third of the population develops cancer at some point of time during their life. Although the mortality due to cancer is high, many advances have been made both in terms of treatment and understanding the biology of the disease at the molecular level.[2] Cancer may affect people of all ages, but risk tends to increase with age, due to the fact that DNA damage becomes more apparent in aging DNA. Statistics indicate that men are largely plagued by lung, colon, rectum and prostate cancer, whilst women increasingly suffer from breast, colon, rectal and stomach cancer.

Leukemia is a cancer of the blood or bone marrow. It is most likely to affect people over the age of 55 years, but it is also the most common cancer in those aged under 15 years. In a person with leukemia, the bone marrow produces abnormal white blood cells that are called leukemia cells and leukemic blast cells. The abnormal cells can't produce normal white blood cells. Leukemia cells divide to produce copies of themselves. The copies divide again and again, producing more and more leukemia cells. THP-1 cells are a line of human monocytes from patients with leukemia. These tumor cells multiplying very rapidly, their use is widespread when it comes to performing in vitro cell tests, requiring a human cell model. They express the HLA A2, A9, B5, DRw1 and DRw2 antigens, as well as the complement C3 receptor.[3]

Despite many therapeutic advances in the understanding of the processes in carcinogenesis, overall mortality statistics are unlikely to change until, it is believed, there is a reorientation of the concepts for the use of natural products as new chemopreventive agents.[4] The use of medicinal plants has been increasing steadily with notable use in the pharmaceutical, cosmetic and food industries.[5] From a long period of time medicinal plants or their secondary metabolites have been directly or indirectly playing an important role in the human society to combat diseases.[6] Even though there are number of synthetic antitumor agents available, efforts are still on to search for effective naturally occurring anticarcinogens that would prevent, slow or reverse cancer development. Moreover, secondary metabolites are recognized by their numerous biological activities which include antibacterial, anticancer, antifungal, analgesic, anti-inflammatory, diuretic, gastrointestinal and antioxidant activities.[7-8]

Gladiolus segetum (Iridaceae)[9-10] is used in Algeria as a traditional medicine. The aerial parts of *Gladiolus segetum* have been used for the treatment of various diseases of digestive system, infertility, ulcer, tumor and other inflammatory affections in the Mediterranean region. Previous investigations of *Gladiolus segetum* have led to the isolation of saponins[11] and anthraquinones.[12] For this study, we have evaluated cell viability using both the Alamar Blue and WST-1 tests which may give different results and cell growth with trypan blue. The early expression (at 4 h) of seven relevant genes involved in the pathways leading to toxicity was assessed: Inflammation (TNF-α), oxidative stress (NCF1, OPA1, SDHA) and apoptotic balance (BCL2, PDCD4, CASP8). Furthermore, the measurement of THP-1 Caspase 3 Protein Activity Exposed to methanol extract of *Gladiolus segetum* for estimating anticancer properties.

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MATERIALS AND METHODS

Collection of Plant Material

Gladiolus segetum was collected in May 2015 from Batna, Algeria and identified by Professor Bachir Oudjehih (Department of Agronomic, University Batna1). The voucher specimen number is 183DAUB2004 was deposited in the herbarium of the above-mentioned department.

Preparation of extract

The aerial parts of *Gladiolus segetum* were shade dried and powdered; 600 g of powder was macerated sequentially using hexane, chloroform, ethyl acetate and methanol (Solvents of increasing polarity).

Cell Culture

Human THP-1 monocytic cell line was obtained from American Type Culture Collection (ATCC, TIB-202, Manassas, VA, USA). Cells were grown at 37°C under 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% of heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin. They were split every 3 days.

Evaluation of cell viability

Cells were 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 25, 50, 100, 200, 400 and 800µg/mL of MEGS. 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. Fluorescence and Absorbance were measured at 570/585 nm and 415nm, respectively. Numbers of dead and alive cells were enumerated 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 the following genes by human cells was measured: tumor necrosis factor-alpha (TNF-α), B-cell CLL/lymphoma 2 (BCL2), caspase 8 (CASP8), succinate dehydrogenase complex flavoprotein subunit A (SDHA), Neutrophil Cytosolic Factor 1 (NCF1), Programmed Cell Death 4(PDCD4) and optic atrophy 1 (OPA1). Total RNA was extracted from 1.5×106 THP-1 cells unexposed or exposed for 4 h to 25 and 100 µg/mL of methanol extract of *Gladiolus segetum* 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 actin, beta (ACTB) housekeeping genes, used as references for THP-1.Fold changes (FC) of gene expression were calculated by 2–ΔΔCt method.[13]

Caspase-3 enzyme activity assay

Caspase-3 activity of MEGS was determined using an kit EnzChek® Caspase-3 Assay 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 30 min. The caspase-3 activity of MEGS was then measured in a microplate reader at 342/441 nm.

Statistical analysis

Cell viability data are presented as means ± standard error of the mean (SE). Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's test, using the GraphPad Prism. 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. Values were considered statistically significant at $p < 0.05$.

RESULTS

Cell Viability

MEGS exposure for 24h reveals a dose-dependent effect, when the methanolic extract concentration of *Gladiolus segetum* reaches 800 µg/ml, the percentage of cell viability of THP-1 drops considerably with Alamar Blue test goes from 150% to less than 10% and from 250% to less than 70% with WST-1 test (Figure 1, 2). MEGS appear to exhibit cytotoxicity opposite THP-1 cells and the mitochondrial activity is increased from a concentration of 400 µg/ml. cells produce less formazan, which translates to a decrease in viability. In parallel, a significant decrease in the

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

Functional class	Gene	Sequence
	ACTB (internal control)	F: 5'-TTGGCAATGAGCGGTTCC-3' R: 5'-GTACTTGCGCTCAGGAGGAG-3'
Inflammation	TNF-α	F: 5'-TAGCCCATGTTGTAGCAAACC-3' R: 5'-GATGGCAGAGAGGAGGTTGA-3'
Oxidative stress	SDHA	F:5'-GCCAGGACCTAGAGTTTGTTC-3' R:5'-GCCTTGACTGTTAATGAGAATGC-3'
Oxidative stress	NCF1	F: 5'-AGAGTACCGCGACAGACATC-3' R: 5'-TAGTTGGGCTCAGGGTCTTC-3'
Oxidative stress	OPA1	F: 5'-CTTCCATGAGGGTCCATTTG-3' R:5'-CCG TTA GCC CTG AGA CCA TA-3'
Apoptosis	BCL2	F: 5'-GAGGATTGTGGCCTTCTTTG-3' R: 5'-GCATCCCAGCCTCCGTTAT-3'
Apoptosis	CASP8	F: 5'-GAAAGGGTGGAGCGGATTAT-3' R: 5'-GCTTCCTTTGCGGAATGTAG-3'
Apoptosis	PDCD4	F:5'AGACCAAATGAAAAGAGGTTATGAG-3' R: 5'-GCCCCTTGAAGGACAAAGAT-3'

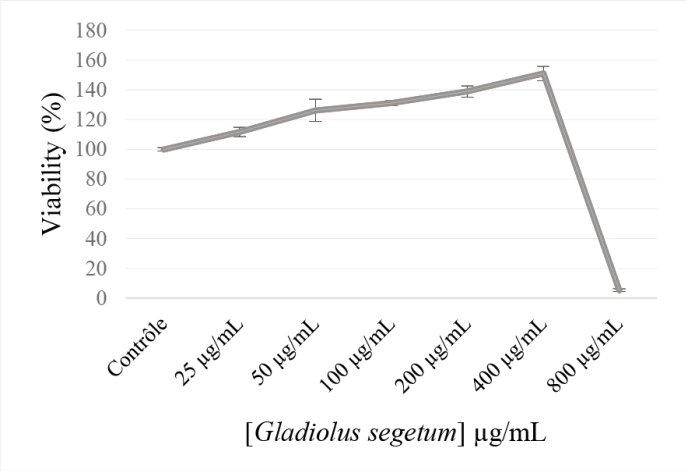

Figure 1: Influence of MEGS on cell viability assayed by the Alamar Blue assay. Cells were incubated with MEGS for 24h (n=12±mean SE).

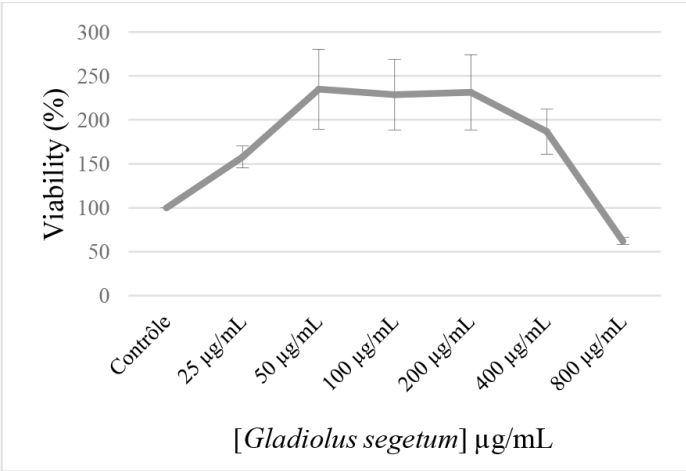

Figure 2: Influence of MEGS on cell viability assayed by the Wst-1 assay. Cells were incubated with MEGS for 24h (n=12±mean SE).

number of THP-1 human cells was observed with the trypan blue test, the percentage of cell viability collapses when the THP-1 cells are exposed to a concentration of 100 µg / ml in MEGS and goes from 100% to close to 0% which means that *Gladiolus segetum* extract may cause cell apoptosis (Figure 3).

Gene expression analysis

After 4 h, OPA1, NCF1, BCL2, PDCD4 and TNF-α genes were significantly up-regulated in human cells this for an exposure to 25 µg/ml of MEGS. On the contrary, pro-apoptotic CASP8 and oxidative stress SDHA genes were significantly down-regulated.

For an exposure to 100 µg/ml, OPA1, BCL2 and TNF-α were significantly up-regulated but less importantly, while expression of NCF1, CASP8, PDCD4 and SDHA was not significantly modified (Figure 4).

Caspase 3 Protein Activity

The activity of the protein caspase 3 is expressed at 140% for an exposure to 25 µg/ml and 120% for an exposure to 100 µg / ml. This overexpression tends to decrease slightly as concentrations increase (Figure 5).

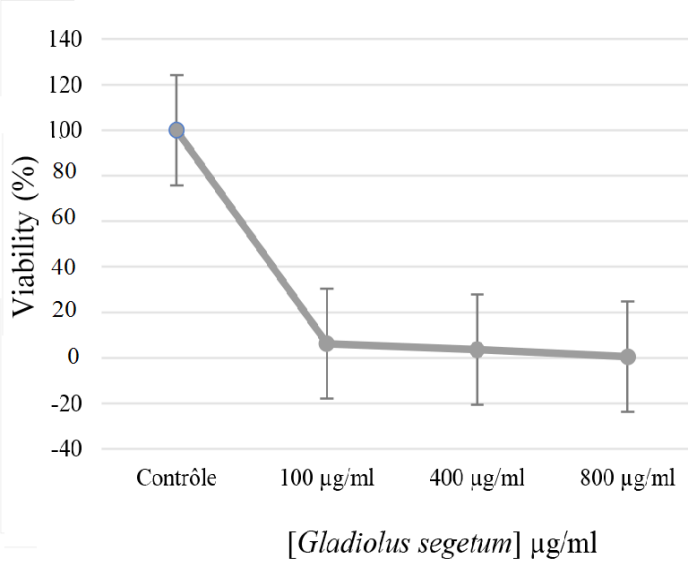

Figure 3: Influence of MEGS on cell viability assayed by the trypan blue assay. Cells were incubated with MEGS for 24h (n=12± mean SE).

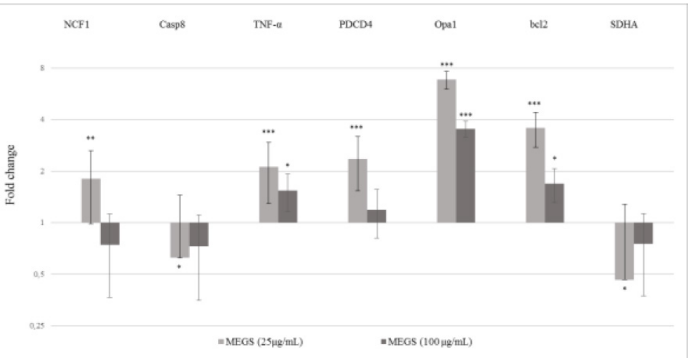

Figure 4: Gene expression changes in response to MEGS exposure of THP-1 human cells. Cells have been exposed to 25 and 100 µg/mL of MEGS for 4 h. Results are presented as fold change as compared to control±SE using ANOVA followed by Dunnett's test.

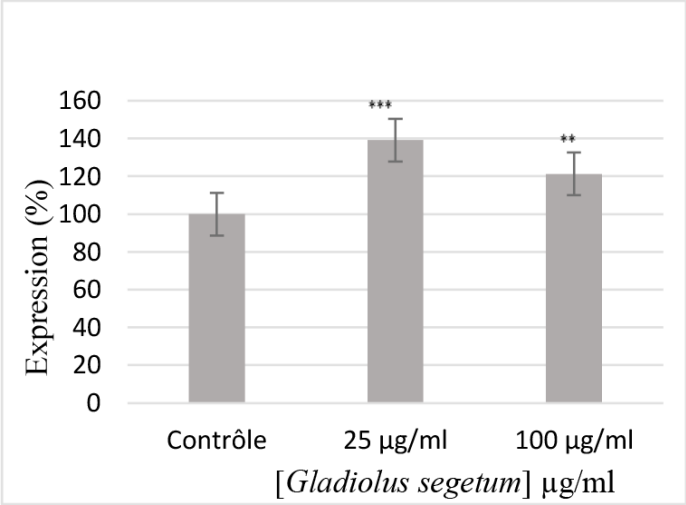

Figure 5: CASP 3 gene expression of THP-1 human cells exposed to MEGS. Cells have been exposed to 25 and 100 µg/mL of MEGS for 4 h. Values are represented as mean± SE.

DISCUSSION

There are several observations that overexpression of Bcl-2 can slow cell growth and promote cell death.[14-15] Overexpression of the OPA1 gene may be related to mitochondrial hyperactivity observed in cytotoxicity tests. Moreover, OPA1 overexpression did not protect against apoptosis, the toxicity may be related to an induced oxidative stress.[16] Interaction of Reactive Oxygen Species (ROS) with lipids, proteins, or DNA may explain cytotoxic effects of MEGS. Such an oxidative stress could activate specific signaling pathways, including MAP kinases and transcription factors sensitive to the redox potential as NFKB, leading to the synthesis of pro-inflammatory cytokines. This may explain the increase in the expression of TNF-α.[17]

Mitochondrial physiology is closely related to cellular apoptotic processes.[18] CASP3 is a well-known regulator of mitochondria-induced apoptosis, which cleaves many anti-apoptotic mitochondrial proteins.[19]

Caspase-3 is ubiquitously expressed in human tissue including liver and in human cell lines.[20-21] Overexpression and loss of expression of caspase-3 has been reported in a variety of human cell lines and malignancies.[22-24]

Herbal medicines have been appreciated and accepted all over the world and they have made an impact on both global health and international trade. Hence, medicinal plants continue to play an important role in the healthcare system of a majority of the world's population.[25] Moreover, there are number of synthetic antitumor agents available, efforts are still on to search for effective naturally occurring anticarcinogens that would prevent, slow or reverse cancer development. Plants have a special place in the treatment of cancer. It is estimated that plant derived compounds constitute more than 50% of anticancer agents. Extracts of plants were used for the treatment of various diseases and this forms the basis for all Indian systems of Medicine. Hence, secondary metabolites like polyphenols, terpenes and alkaloids have been reported to possess antimutagenic and anticancer properties in many studies.[26-27]

Gladiolus segetum contains a number of metabolites such as flavonoids, anthocyanins, saponins, anthraquinones, as well as ascorbic and fatty acids.[28-29] Many anthraquinones from plant sources are isolated from the Iridaceae family. The majority have been reported from the genus *Gladiolus*.[30-32]

The results of this experiment show that the treatment of THP-1 cells with the MEGS increased the caspase-3 activity. This suggests the involvement of caspase-3 in triggering apoptosis in MEGS-treated THP-1 cells. This plant is used in the context of traditional medicine to fight against dysentery syndromes, impotence and rheumatic pains.

CONCLUSION

The present study stating that the methanol extract of the aerial parts of this plant possess a potent toxic effect on THP-1 cells. Indeed, it causes genetic deregulation whose repercussions are observable at the cellular level. There is also an alteration of the mitochondrial functions; these being hyperstimulated, the consequences on the cells can only be harmful. This study confirms the use of the plant of *Gladiolus segtum* in traditional medicine.

ACKNOWLEDGEMENT

The authors wish to express thanks to the General Directorate for Scientific Research and Technological Development (DGRSDT) of the Algerian Minister of Higher Education and Scientific Research for providing a research grant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

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; **TNFα:** Tumor Necrosis Factor-Alpha; **MEGS:** Methanol Extract of *Gladiolus segetum*.

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Article History: Submission Date : 05-07-2018; Revised Date : 27-08-2018; Acceptance Date : 03-10-2018.
Cite this article: Eddine MS, Benkiki N, Akram MM, Joubert O, Doumandji ZM, Safar R. Anti-cancer Activity and Gene Expression Responses to Methanol Extract of *Gladiolus segetum* in THP-1 Human Monocytic Leukemia Cells. J Young Pharm. 2019;11(1):51-5.