Anti-Inflammatory and Acetylcholinesterase Inhibition Activities of Globularia Alypum

Daycem. Khlifi[a,b,c], Rabiaa Manel. Sghaier[c1], Dhafer. Laouni[c], Al Akrem. Hayouni[d], Moktar. Hamdi[b], and Jalloul. Bouajila[a]

[a] Université de Toulouse, Laboratoire des Interactions Moléculaires et Réactivité Chimique et Photochimique UMR CNRS 5623, Université Paul-Sabatier, 118 route de Narbonne, F-31062 Toulouse, France.

[b] Laboratoire d'Ecologie et de Technologie Microbienne, Institut National des Sciences Appliquées et de la Technologie (INSAT), B.P. 676, 1080 Tunis, Tunisie

[c] Laboratoire de transmission, contrôle et immunologie des infections Institut Pasteur Tunis.

[d] Centre de Biotechnologie à l'Ecopark de Borj-Cédria, Laboratoire des Substances Bioactives, C BP-901 Hammam Lif. Tunisia

Email: {biodaicem, rabiaa_sghaier}@yahoo.fr, jalloul.bouajila@univ-tlse3.fr

***Abstract*—The Globularia alypum methanolic extract (GAME) was evaluated for the anticholinesterase, anti 5-lipoxygenase, NO production inhibitory activities and the transcriptional regulation pathway. Interestingly, GAME showed an important anti-inflammatory activity in a dose dependant manner, and inhibited nitric oxide (NO) production via transcriptional regulation of iNOS gene by (66%) at 150mg/L in IFN-γ/LPS stimulated RAW 264.7 macrophages. In addition, both Globularia alypum showed a 5-Lipoxygenase inhibitory activity with IC_{50} value of 79 ±0.8mg/L. Acetyl-cholinesterase inhibition was assessed by modification of the Ellman's method. Globularia alypum exhibited a strong activity against cholinesterase with IC_{50} value 9.33 ±0.47mg/L. The data suggest that Globularia alypum extract could be used as a natural inhibitor of oxidation and alzheimer disease, and since GAME induced a potent anti-inflammatory suggest its potent use for the treatment of inflammatory diseases.**

***Index Terms*—Globularia alypum, anti-inflammatory activity, anti- cholinesterase.**

I. Introduction

Many plants contain natural antioxidants that act in metabolic response to the endogenous production of free radicals and other oxidant species. In recent years, there has been growing interest in finding natural antioxidants, including volatile chemicals, in plants because they inhibit oxidative damage and may consequently prevent inflammatory conditions [1] ageing and neurodegenerative disease [2].

Free radicals with unpaired electrons are generated under oxidative and nitrative stress [3]. These are not only derived from disequilibrium cellular metabolism, but also from pathological status such as inflammation [4]. During inflammation, reactive oxygen (ROS) and nitrogen (RNS) species are produced by inflammatory cells and can oxidize biomolecules including lipids. At the same time, peroxynitrite can attract a hydrogen atom from a polyunsaturated fatty acid and results in the production of nitrated lipids [5]. Therefore, decreasing NO production under inflammatory conditions is an important step in decreasing the threats of oxidative and nitrative stress as well as the damage of inflammation. Nitric oxide (NO) is synthesized from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues [6]. However, NO synthesized by iNOS is induced by a variety of stimuli, such as oxidants, lipopolysaccharide (LPS), bacteria, viruses, and proinflammatory cytokines [7]. NO can be directly cytotoxic but can also interact with superoxide anions and result in the formation of peroxynitrite (ONOO-), which is the most reactive RNS. Excess production of ROS, NO, and RNS can damage DNA, lipids, proteins, and carbohydrates, leading to impaired cellular functions and enhanced inflammatory reactions. In addition, certain plants modulate the enzyme activities of arachidonic acid (AA) metabolizing enzymes such as phospholipase A2 (PLA2), cyclooxygenase (COX), and lipoxygenase (LOX) and the nitric oxide (NO) producing enzyme, nitric oxide synthase (NOS). An inhibition of these enzymes by plants reduces the production of AA, prostaglandins (PG), leukotrienes (LT), and NO, crucial mediators of inflammation. Thus, the inhibition of these enzymes exerted by plants is definitely one of the important cellular mechanisms of anti-inflammation. Furthermore, in recent years, many lines of evidence support the idea that certain natural compounds are the modulators of gene expression, especially the modulators of pro-inflammatory gene expression, thus leading to the attenuation of the inflammatory response [8].

Alzheimer's disease (AD), the most common cause of dementia in aged population, whose symptoms are cognitive decline and mental deterioration, is the result of massive and progressive loss of neurons from serval different region of the brain it is still controversial but some studies suggest that dietary supplement with antioxidants and free radical- scavengers (including

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vitamin E) may display some benefits in slowing the mild cognitive impairment of AD. Until now, the only treatment for this disease is based on the ‘‘cholinergic hypothesis’’ which means that the drugs approved for the Alzheimer therapy must act by counteracting the acetylcholine deficit, enhancing its level in the brain. Acetylcholine is involved in the signal transfer in the synapses and, after being delivered in the synapses, is usually hydrolyzed, giving choline and acetate in a reaction catalyzed by the enzyme acetylcholinesterase. The molecular basis of the drugs used up to now is their action as acetylcholinesterase inhibitors [9]. This enzyme is associated with the extra-cellular membrane surface and it plays an important role as a safeguard of the brain cells [10]. Recently it was shown that the senile plaques seem to induce inflammatory process in which radical oxygen species are liberated [11].

The present study was planned to evaluate the methanolic extract of *Globularia alypum* for antioxidant, anti inflammatory, and cholinesterase inhibitory activities taking into account the chemical composition of the extract.

The genus *Globularia* (Family: Globulariaceae) consists of plants which are herbs, chamaephytes or shrubs, common in the Mediterranean regions, Europe and North Africa (Tunisia, Morocco, Libya and Algeria). They are a rich source of phenolic compounds. G. alypum is commonly used in North African folk medicine. G. alypum, named locally as ‘zriga or Ain Larneb’ is a wild plant belonging to the Globulariaceae family. Skim et al [12] confirmed the beneficial effects of *G. alypum* infusion against hypoglycemic agents. The hydromethanolic extract of *G. alypum* is used as a source of potential antioxidants and may promote the reasonable usage of this plant in food technology and processing as well as for medical use [13].

Continuing our search for new candidate from Tunsian medicinal and aromatic plants, used as farctions or purified compounds, the present study was planned to evaluate the methanolic extract of Globularia alypum for its antioxidant, anti inflammatory, and cholinesterase inhibitory activities taking into account the chemical composition of the extract.

II. Materials and Methods

A. Plant Material

The leaves of *Globularia alypum* was collected in January 2009 from the centre area of Tunisia, precisely from the Sidi Bouzid region. Specimens was identified by Dr. Bousaid Mohamed at the Department of Botany, National Institute of Applied Sciences and Technology (INSAT, Tunis) and voucher specimens were deposited at the Herbarium of the Department of Botany in the cited institute.

B. Preparation of Extract

The leaves of *G. alypum* was dried in air shade at room temperature, and the dry plant was powdered. 50g of powders were extracted in a Soxhlet system with 500mL of methanol/Water (3/1) during 48h at 65℃. Extracts were concentrated by rotary evaporation under vacuum at 35℃.

C. Cell Culture

The murine monocytic macrophage cell line (RAW 264.7) was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultivated in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 1% gentamycine and 2mM L-glutamine as a complete growth medium. Cells were maintained in 75cm3 flasks with 10mL of medium and were incubated at 37℃ in an incubator with 5% CO_2 in humidified atmosphere. Every 3 days the cells were subcultured by splitting the culture with fresh medium at about 80% confluence and placed down at needed density for treatment. The viable cells were counted by the trypan blue exclusion assay.

D. Measurement of Anti-Inflammatory Activity by Nitrite Quantification

Exponentially growing cells were plated in 24-well microplates (BD Falcon) at a density of 3×10^5 cells/mL and were allowed to adhere overnight. Cells were stimulated by using 200U/ml of IFN-γ and 1μg/mL of LPS with or without the presence of increasing concentrations GAME. Wells with methanol were used as negative control. Cells were then incubated at 37℃, 5% CO_2 for 24h. The inhibition of NO production was previously tested by using the specific NOS inhibitor L-NG-monomethyl Arginine citrate (L-NMMA). After 24h, the culture supernatant was subjected to Griess assay for nitrite determination and the cells remaining in the well were tested for cell viability assay by using MTT test. NO determination using the Griess reaction [14] with minor modifications. Briefly, a 50μL of cell supernatants were incubated with an equal volume of Griess reagent (SIGMA) 40mg/ml at room temperature for 15min at obscurity. Absorbance at 540 nm was then measured using an automatic 96well Variokson Ascent plate reader (Thermo Electron) and the presence of nitrite was quantified by comparison with the $NaNO_2$ standard curve.

E. Total RNA Isolation and Quantitative Real-Time PCR

Cells were washed twice with cold phosphate buffered saline (PBS) and 1mL of Trizol reagent (Invitrogen, Carlsbad, CA) was added to each well of a 24-well plate to isolate total RNA following the manufacturer's protocol. Subsequently, the RNA samples were reverse transcribed by High-Capacity cDNA Archive kit (Applied Biosystem Prism). Real time PCR analysis was performed using the TaqMan probes procedure. Primers and probes for iNOS F:CAGCTGGGCTGTACAAACCTT and R:CATTGGAAGTGAAGCGTTTCG Probe: FAM-CATTGGAAGTGAAGCGTTTCG-TAMRA and Porphobilinogen deaminase (PBGD) F: CGGCCACAACCGCGGAAGAA and R: GTCTCCCGTGGTGGACATAGCAATGA and Probe: FAM-AGCTGGCTCTTACGGGTGCCCA-TAMRA were designed according to GenBank database using the

Primer Express 3.0 software provided by ABI. Expression of mRNA values was calculated using the threshold cycle (Ct) value, that is, the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. For each sample, ΔCt, sample was calculated by subtracting the Ct value of PBGD, a housekeeping gene, from that of each gene of interest to normalize the data. The expression levels relative to control were estimated by calculating ΔΔCt (ΔCt sample -ΔCt control) and subsequently using the 2−ΔΔCt method [15].

F. Anti-inflammatory Activity Using the 5-Lipoxygenase Assay

5-lipoxygenase activity of extract was determined using the method as published by Evans [16] and Baylac and Racine [17] with linoleic acid as the substrate for the 5 lipoxygenase enzyme (Cayman). In normal biological systems, 5-lipoxygenase enzyme catalyses the oxidation of unsaturated fatty acids containing 1–4 pentadiene structures with arachidonic acid as the biological substrate converting them into conjugated dienes which result in the continuous increase in absorbance at 234nm. 150 µL buffer solution sodium hydrogenophosphate (pH 7.4) was added to 60 µL linoleic acid, 20 µL of different concentrations of extracts (in Buffer solution) and 20 µL enzyme. A control mixture was prepared similar to the sample mixture: 170 µL buffer solution sodium hydrogenophosphate (pH 7.4) was added to 60 µL linoleic acid and 20 µL of different concentrations of extracts (in Buffer solution) in a microplate of 96 wells, and the final volume of each well was 250 µL. similar to the sample mixture but with the respective solvent instead of extract. Nordihydroguaiaretic acid (NDGA) was used as the positive control. Inhibition was calculated in the following way:

$$I\ (\%) = 100 - (A_{sample} / A_{contol}) \times 100$$

where A_{sample} is the absorbance of the extract containing reaction and $A_{control}$ the absorbance of the reaction control. Tests were carried out in triplicate. Extract concentration providing 50% inhibition (IC_{50}) was obtained plotting the inhibition percentage against extract solution concentrations.

G. Acetyl Cholinesterase Inhibition Assay

The acetyl cholinesterase enzyme activity was measured by spectrophotometric observation of the increase in a yellow colour product from thiocholine when it reacts with the dithiobisnitrobenzoate ion. The enzymatic activity was assessed by a modified colorimetric Ellman's method [18]. 50 µL of Tris–HCl buffer (pH 8), 25 µL of a buffer solution of extract with different concentrations and 25 µL of an enzyme solution containing 2.8U/ml. The reaction were then initiated via the addition of 125 µL of 3mM 5-5'-thiobis-2-nitrobenzoic acid (DTNB). After incubation 15 min at 25 ℃, 25 µL of a solution of 15Mm ATCI (synthetic substrate for AChE) was added in a microplate of 96 wells and the final volume of each well was 225 µL. Absorbance of the mixture was measured at 412nm after 10min. A control mixture was prepared, using 75 µL of a solution, similar to the sample mixture but with the respective solvent instead of extract. Galanthamin was used as a positive control. Each experiment was performed at least three times. Inhibition, in% was calculated in the following way:

$$I\ (\%) = 100 - (A_{sample} / A_{contol}) \times 100$$

where A_{sample} is the absorbance of the extract containing reaction and $A_{control}$ the absorbance of the reaction control. Tests were carried out in triplicate. Extract concentration providing 50% inhibition (IC_{50}) was obtained plotting the inhibition percentage against extract solution concentrations.

H. Statistical Analysis

All data were expressed as means ± standard deviations of triplicate measurements. The confidence limits were set at $P<0.05$. Correlations were carried out using the correlation and regression in the EXCEL program.

III. Results

A. Evaluation of the Anti-Inflammatory Activity of Extract

Phenolic compounds have been reported to be beneficial in the treatment of chronic inflammatory diseases associated with overproduction of nitric oxide (NO) [19]. In the inflammation process, NO is produced from L-arginine by the inducible NO synthase (iNOS). Peroxynitrite, formed by the rapid reaction between superoxide and NO, is a toxic substance that contributes to tissue injury in inflammatory diseases [20]. Inhibition of NO production results in anti-inflammatory activity and was studied *in vitro* by analysing of the effect of *Globularia alypum* extract on chemical mediators released from macrophages. Once activated by a pro-inflammatory stimulus, macrophages produce a large number of cytotoxic molecules. Treatment of RAW 264.7 macrophages with in IFN-γ/LPS for 24h induces NO production, as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in the media by a colorimetric procedure based on the Griess reaction. NO, a macrophage – derived mediator, is considered to play key role in inflammatory response, based on its occurrence at inflammatory and its ability to induce many of the hallmarks in the inflammatory reponse. Results presented in Fig. 1 showed that L-NAME (2mM) significantly inhibited NO release by 96%. However, GAME showed a good inhibition percentage of NO release in a dose dependant manner ranging from 150 (45%) to 600mg/L (66%). There was no report about anti-inflammatory activity of extract of Globularia alypum and as far as our litterature survey permits it seems that this is the first time that such resulat is being reported.

NO is an essential bio-regulatory molecule within the nervous, immune, and cardiovascular systems [21]. However, increased level of NO derived from iNOS can result in the formation of peroxynitrite after reaction with oxygen free radicals during inflammatory responses [22]. This study demonstrated that the *Globularia alypum* extract inhibited NO production and iNOS expression in a dose-dependent manner. These results suggest that the the *Globularia alypum* extract from leaves reduced NO and production via transcriptional regulation of iNOS gene.

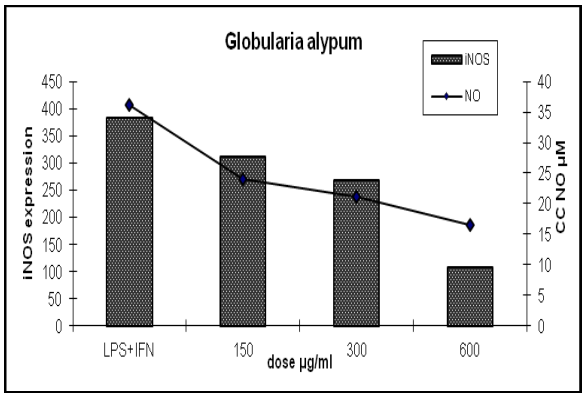

Figure 1. The effect of the treatment with *Globularia alypum* exctract of the murin macrophages Raw 264.7 into NO production and iNOS mRNA expression.

B. Evaluation of 5-Lipoxygenase Inhibitory Activity

Lipoxygenases are a family of enzymes which consists of 5-LOX, 12-LOX and 15-LOX. Leukotrienes, the fatty signals molecules are synthesized in the cell from arachidonic acid by catalyzation of 5-LOX. The over production of leukotrienes triggers contractions in the smooth muscles lining of the trachea to cause the inflammation in asthma and allergic diseases [23]. GAME showed 5-LOX inhibition activity with IC_{50} value 79mg/L. (Table I).

TABLE I. 5-LIPOXYGENASE AND ACETYLCHOLINESTERASE INHIBITORY AND ANTIOXIDANT ACTIVITY ACTIVITIES OF GLOBULARIA ALYPUM EXTRACT.

	IC_{50}(5-lipoxygenase)[a]	IC_{50}(acetyl cholinesterase)[a]
Globularia alypum	79 ±0.8	9.33 ±0.47
NDGA	2.23 ±0.06	
Galanthamine		1 ±1.1

[a]: mg/L . [b]R^{inh}. Standard deviations (SD) did not exceed 5%.

C. Evaluation of Acetyl Cholenesterase Inhibitory Activity

Acetyl cholinesterase plays an important role in the central nervous system. It is one of the fastest known enzymes and catalyzes the cleavage of acetylcholine in the synaptic cleft after depolarization. Inhibitors of AChE, such as galanthamine, are used frequently in the pharmacotherapy of AD. Since a large amount of evidence demonstrate that oxidative stress is intimately involved in age-related neurodegenerative diseases, there have been a great number of studies with have examined the positive benefits of antioxidants to reduce or to block neuronal death occurring in the pathophysiology of these disorders [24]. GAME exhibited an interesting activity against cholinesterase (Table I) with IC_{50} value 9,33mg/L. In the same order, Benamar and al [25] reported that the aqueous extracts from roots of *Globularia alypum* exhibit a moderate activity against acetyl cholinesterase with IC_{50} value 16,67mg/L (Table I).

IV. CONCLUSION

As an initial step to evaluate the beneficial health effects of *Globularia alypum*, we investigated the antioxidant, anti-cholinesterase and anti-inflammatory activities of *Globularia alypum* leaf extract. Next, we investigated the anti-inflammatory properties of the *Globularia alypum* by two methods: in LPS-stimulated RAW 274.7 cells and inhibition of 5-lipoxygenase. The *Globularia alypum* extract inhibited production of NO and iNOS in a dose-dependent manner and inhibited the 5-lipoxugenase activity. In addition, we investigated their anti-cholinesterase activity. The *Globularia alypum* extract inhibited the cholinesterase activity. These results indicate that the *Globularia alypum* extract exhibit anti-inflammatory properties via the inhibition of many pro-inflammatory mediators. Taken together, these results indicate that *Globularia alypum* leaf has potential for use as an antioxidant and anti-inflammatory and anti-cholinesterase agent.

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Daycem Khlifi PhD was born in November 22th, 1980 in Tunis (Tunisia). In fact, she got a Diploma of Medical Biotechnology from the Superior Institut of Biotechnology in Monastir (ISBM).
Since 2007, she started the postgraduate cycle and got the Master degree in humain genome at Pasteur Institute of Tunis. The topic of the work was "Study of microsatellite polymorphism of TLR2 gene immune response in tuberculosis in the Tunisian population".
The Ph-D in biology was performed in many Laboratories (University of Toulouse, Laboratory of Molecular Interactions and Chemical and Photochemical Reactivity, UMR 5623 CNRS, Pasteur Institute of Tunis, Laboratory of Transmission, Control and Immunobiology of Infections, LR11IPT02 (LTCII) and Laboratory of Microbial Ecology and Technology, National Institute of Applied Sciences and Technology (INSAT)), the topic was "Recovery of secondary metabolites of medicinal plants: phytochemical study and biological activities". Many papers were published mainly in: Food and Chemical Toxicology, Industrial Crops and Products, Molecules …

Rabiaa Manel Sghaier was born 1982 in Tunis. She received a Master degree in Biotechnology and Immunology Applied on Transmissible Diseases from the School of Pharmacy University of Monastir, Tunisia, in 2008. She is currently a PhD student at Institut Pasteur de Tunis.
She focuses on studying cellular immune responses during infectious diseases and cancer.

El Akrem Hayouni (Dr. Engineer), was born in November 26th, 1972 in Thala (Tunisia). His actually, a permanent Associate Professor at the Biotechnology Center at the Ecopark of Borj-Cedria (Tunisia). Dr. Hayouni worked on many topics such as "Purification and biochemical characterisation and industrial uses of lipases from various sources: microorganisms, hepatopancreas of primitive animals, birds and mammalians"; "DNA-based vaccination against rabies: therapeutic efficacy, biosecurity and large-scale production and purification of a high pharmacological quality plasmid" and his ongoing studies target the "Secondary metabolites from bioressources: bioprospection, phytochemical investigation and techno-functional valorisation in bio-industries". Many papers were published namely in Food Chemistry, International Journal of Food Microbiology, IOBC bulletins, Vaccine, Phytomedicine, Food Control, Industrial crops and products, Food Microbiology…As International experience Dr. Hayouni was invited as foreign visiting professor and as a plenaty speaker in Japan. He gave also lectures in many Tunisian institutions

Moktar HAMDI is a Professor in bioprocess engineering and industrial microbiology and he was Head of Department of Biological and Chemical Engineering (2002 – 2008) and doctoral school (2008 – 2012) at National Institute of Applied Sciences and Technology. He received a PhD degree in Microbiology from University of Aix Marseille (1991) and a DSc from University Paul SABATIER Toulouse (1997). He held postdoctoral experience in Biochemical and food engineering department at INSA Toulouse. He is a teacher of General Microbiology, Industrial and Food Microbiology, Environmental Bioprocess, and Bioreactor engineering and Bioseparations Engineering, .
He is head of The Laboratory of Microbial and Ecology and Technology (LETMi) which is constituted by three groups (50 persons). Hamdi's current research interests include (i) microbial ecology and microorganisms screening and application in foods fermentation and in wastewaters treatment, and (ii) scale down and scale up of food and microbial processes.
He has authored over 130 scientific publications, 9 patents and book chapters, and many conference contributions. He has an h-index of 23. He is Associate Editor of Natural and Environ. Science, and member of the Editorial Board of Journal chemical engineering, Bioprocess Engineering, African Journal of Biotechnology, Food Science Technology, Review of Scientific Env. Biotechnology, and Cell and plant Science.
He is consulting in Good Manufacturing Practices (GMP.HACCP) of Food manufactories and in Wastes Management and Treatment.
He was partner or director of many national and international projects in food and environmental processes.

Dhafer Laouini (Ph.D.) is a senior biologist at Pasteur Institute of Tunis. During the early 1990s, he served as Temporary Teaching staff at the Faculty of Sciences, Tunis. After completion of his Ph.D., he spent several years as a post-doctoral fellow at Paris Pasteur Institute, France where he worked on T cell repertoire and Signal transduction and at Harvard Medical School, Boston where he worked on Signal Transduction and Atopic Dermatitis Models. He served also as Temporary research and training Assistant at College de France, Paris from 1998 to 1999. He joined again the Institute Pasteur of Tunis in 2003 where he is presently investigating host-pathogen-vector relationships.

Jalloul Bouajila is a chemist analytician since 2002. It occupied of post-Doc during 3 years before becoming teaching researcher to the faculty of pharmacy of Toulouse in 2005. It directs the team "Molecules bioActives or Photo-activables for the Environment and Health". It directs several doctorates within the framework of industrial and academic collaborations in France and to foreign countries (Madagascar, Bénin, Tunisia, Egypt, Centre Africa…)