Quercetin combined with Concanavalin A as a safe vaccine's adjuvant that stimulates PBMCs proliferation, alleviates lipid peroxidation and boosts *i*NOS activity in the elderly.

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Authors' contributions

Dr. Samia Bouamama designed the study, contributed in running the laboratory work, analysis of the data and drafted the paper. Amina Bouamama contributed in critical reading and English language editing of the manuscript. All the authors have read the final manuscript and approved the submission.

Abstract

Background: Aging is associated with immunity decline and low vaccinal efficacy**.** The purpose of our research was to evaluate the possible *in vitro* stimulating effects of quercetin combined with concanavalin a on PBMCs immune cells.

Methods: PBMCs from healthy aged and young individuals were obtained by the gradient density centrifugation. PBMCs were cultured at a 96-well cell culture microplate in RPMI-1640 medium supplemented with quercetin in the presence of concanavalin a and incubated in a 5% CO2 humidified incubator at 37°C for 48 h. Cell proliferation was assessed with MTT colorimetric assay. Intracellular levels of MDA, carbonyl proteins, glutathione were assessed by spectrophotometric assays. IL-2 release was determined by ELISA method. *i*NOS activity was studied by measuring nitrite products levels using Griess reagent.

Results: As compared to their respective young controls; aged PBMCs showed a low proliferation potency and low IL-2 and NO release, while intracellular Malondialdehydes (MDA), carbonyl proteins were higher in these cells. Importantly, PBMCs from aged subjects treated by quercetin and con A display significantly a high proliferative response similar to young cells, a restored *i*NOS activity and a reduced cellular oxidative damages with mitigated MDA formation but with high CP levels compared to untreated PBMCS cells (data not shown).

Conclusion: According to our results, it can be concluded that quercetin combined with Con a lectin may be a safe promising vaccinal adjuvant and it has significant effects in reducing the complications of aging in immune cells , as well as mitigating the oxidative stress in PBMCs cells.

Key words: Aging, vaccinal adjuvant, PBMCs, Quercetin, Concanavalin a.

1-Introduction

Aging is a complex and polyetiological process, accompanied with gradual and spontaneous biochemical and physiological changes including increased susceptibility to diseases, adverse environmental conditions and loss of mobility and agility [1]. Over time, the major functions of the body such as the circulatory system and digestive system, the endocrine system and the immune system, become less performing. Similarly, the regression of cognitive and memory functions is one of the markers of aging [2]. As age advanced, a progressive attenuation of the quantitative and functional characteristics of various types of immune cells occurs, which manifests in the form of a tendency to inflammation, an increased susceptibility to infectious diseases as well as diminished ability to maintain tolerance to self antigens and a decrease in the response to immunization [3,4]. Previous studies have revealed a potent link between immune function and age reported that PBMCs cell proliferation was reduced in older individuals, compared with younger individuals. Subsequently [5] . Compared to other age groups, older adults are more prone to more frequent and prolonged infectious diseases. Hence, according to the US Center for Disease Control and Prevention (CDC), at the end of December 2020, older adults Americans accounted for more than 92.45% of COVID-19 deaths in the country [4]. Despite the important successes achieved with current vaccines, most available vaccines still fail to elicit long-lasting immunity in older adults [6]. Strategies for increasing the immunogenicity of existing vaccines proposed in recent years are accepted and rational choice of adjuvants in engineered vaccines may provide effective protection for older adults [4] . To date only two adjuvants (MF59 and AS01B) are currently licensed for persons older than 65 years [6] . Plant derived-adjuvants are relatively non-toxic and do not cause significant side effects, which are a major concern associated with synthetic compounds and second, they have proven to potentiate the immune response, making attractive the use of these compounds for the development of vaccines [7]. Research evidences increasingly reinforce the notion that natural lectins and

dietary polyphénols exert profound effects on immunity in particular Con a and quercetin; thus they would be candidates to be adjuvants [8]. Con a , a lectin from jack bean (*Canavalia ensiformis*) seeds showed immunomodulatory effects that are stimulated by their interaction with glycan's moieties present on the surface of immune cells ; as a result of this interaction, signal transduction mechanisms are triggered to produce cytokines. Many plant lectins induce Th1 immunity [7]. Quercetin, 3,3',4',5,7-pentahydroxyflavone, one of the most abundant vegetal flavonoid in the human diet, widely distributed as secondary metabolites in the plants . Significant food sources include apples, onions, berries, leafy green vegetables, hot peppers, red grapes, and black tea, Quercetin possesses antioxidant, free radical–scavenging, and anti-inflammatory properties that may influence immune system competence and resistance to pathogens [9]. Existing data report the in vitro senescence delaying activity in primary cells and rejuvenating effects of quercetin in senescent cells [10]. Previous studies demonstrating the results of immunization with plant phenolics report that Quercetin exhibits adjuvant activity by enhancing Th2 immune response in ovalbumin immunized mice [8].

To better understand the possible clinical uses and safety of quercetin and con a as a vaccinal adjuvants, we assessed in vitro quercetin + Con a treatment-induced changes on PBMCs cell proliferation, pro-/anti-oxidant state and IL-2 and nitric oxide release from both young and aged subjects .

2-Experiments

2.1-Reagents

Con a : Concanavalin A , MTT: (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), Histopaque 1077, DNPH (2,4-dinitrophenyl hydrazine), DTNB: (5,5-dithiobis-2-nitrobenzoic acid) , Trypan blue dye and all other chemicals were purchased from Sigma-Aldrich company (Sigma, St Louis, MO, USA). Incomplete Roswell Park Memorial Institute

1640 (RPMI 1640) medium was produced by Gibco Life Technologies Inc. (Paisley, U.K). it was aseptically supplemented with 25 mM HEPES buffer, 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), penicillin (100 UI/ml) and streptomycin (100 µg/ml).

Stock solution of quercetin (200 µg/ml) was prepared by dissolving quercetin (Sigma Aldrich, France) in DMSO (<1% in culture medium); the finale concentration of quercetin in the culture medium was 20 µg/ml (6,5 mol/L) . It was reported previously that DMSO concentration below than 1 % was neither cytotoxic nor genotoxic to mammalian cells.

2.2-Study population

PBMCs cells were isolated from peripheral blood of 16 healthy donors (10 elderly women and men and 06 young healthy subjects ; In brief, fasting venous blood were collected from healthy volunteers (young subjects aged between 20 to 33 years old) and the elderly (aged over than 65 years old) . All patients in this study are informed about the purpose of the work according to the statement of Helsinki and no investigation is conducted without prior consent signed by the participants. This is achieved by respecting the anonymity and confidentiality of information. The study was performed under the approval of the Ethics Committee of Tlemcen University Hospital.

2.3-PBMCs culture in vitro

Blood PBMCs from healthy donors were obtained by the gradient density centrifugation method as described by [11], in brief 4 ml of whole EDTA blood was layered into 3 ml of Histopaque 1077 solution, centrifuged at 4000 rpm for 40 minutes. The peripheral blood lymphocytes at the interface of plasma and Histopaque were collected and washed twice with

122 balanced saline solution, the cell clot was maintained in RPMI complete medium. Cell
123 viability was evaluated by the Trypan blue exclusion method.
124 $4 .10^6$ of lymphocytes were aseptically seeded at a 96-well cell culture plate in RPMI-1640
125 medium in presence of 5 µg/ml the mitogen Con A and 20µg/ml of quercetin. Treatment
126 with solvent only was used as negative controls. The cell culture was incubated in a 5% CO2
127 humidified incubator at 37°C.

128 **2.4-PBMCs cellular proliferation assay**

129 Cell proliferation was analyzed with cell viability assays that measure the rate of cellular
130 metabolism with MTT substrate as described earlier [12]. Briefly, after 48 of incubation cell
131 viability was assessed with an additional 10 µl/well of MTT (5 mg/ml). The plates were read
132 on microplates reader at a 570 nm wavelength. PBMCs Proliferation is expressed by The
133 ratio expressed as a percentage of absorbance of treated cells to untreated cells that served as
134 control. Or

135 **Cell viability % = {OD treated cells / OD untreated cells controls × 100} .**

136 **2.5-Determination of intracellular redox biomarkers**

137 The lipid peroxidation in lymphocytes was assessed by measuring the concentration of MDA
138 in cell homogenate which is performed by the thiobarbituric acid reactive species assay
139 described by **Nourooz –Zadeh et al.** [13] . Carbonyl proteins (markers of protein oxidation) in
140 lymphocytes homogenate were assayed by the 2,4-dinitrophenyl hydrazine (DNPH)
141 reaction[14] .
142 Reduced glutathione GSH level in PBMCs was measured by using , 5,5-dithiobis-2-
143 nitrobenzoic acid (DTNB) as described earlier [15]
144
145

2.6- IL-2 cytokine Elisa –assay

Released Levels of IL-2 cytokine was determined in PBMCs free supernatants using an enzyme-linked immunosorbent assay (ELISA) kit (Abfrontier, Multiplex Human Cytokine ELISA Kit).

2.7- Nitric oxide release in cells supernatants (iNOS activity)

NO release by PBMCs was checked by the method described by **Guevara et al. (1998)** [16]**,** which is based on the dosage of nitrites. After centrifugation, the supernatant obtained is mixed with the Griess reagent (sulfanilamide and N-1naphtylethylenediamine dihydrochloride in orthophosphoric acid). Coloration Pink-violet appears whose intensity is proportional to the concentration of NO. Optical densities are measured by spectrophotometer at 540 nm. Concentrations of NO are plotted in the calibration curve established using NaNO2 sodium nitrite (0-100µM).

2.8- Statistics

All statistical analyses were performed by Minitab 16 statistical software and Microsoft Excels, 2007. Data are expressed as mean ± SD. Differences between groups were performed by one way ANOVA test followed by Turkey grouping method. All *in vitro* cultures were repeated at least three times. Statistical significance was set at $p < 0.05$.

3- Results

3.1- In vitro effect of quercetin on cell proliferation of PBMCs

PBMCs from aged subjects have a significant reduced proliferative potency compared to the controls, interestingly, quercetin + Con a treatments in vitro restored cellular proliferation in the elderly.

3.2- Intracellular oxidant/antioxidant biomarkers in PBMCs

MDA and CP levels, were significantly increased in the PBMCs of elderly subjects compared to the control. Quercetin treatment significantly caused a decline in the lipid peroxides in both young and aged PBMCs (**p = 0,012) , but with a concomitant increases in CP solely in aged PBMCs (**p = 0,0008) . (Fig 3)

PBMCs from aged subjects presented equivalent GSH cellular levels compared to the PBMCs of the young controls , whereas treatment of PBMCs with RJ significantly increased the intracellular GSH amounts solely in the older subjects (Fig 4).

3.3- IL-2 and *i*NOS activity in PBMCs

The cellular release of interleukin-2 (IL-2) was significantly declined in the elderly subjects compared to the young controls. Quercetin + Con a treatment downregulates IL-2 secretion in both young and aged subjects.

PBMCs in the aged subjects released a lower NO cellular content which means low *i*NOS activity, than the PBMCs in the control subjects. Treatment of cells with quercetin + Con a restored *i*NOS activity (Table 1).

4- Discussion

The human immune system has as primary physiological role the elimination of potentially harmful compounds and the potential wide array of threatening organisms; it evolved to include many different cell types, many communicating molecules and multiple functional responses. It is evident that effective defense against pathogenic organisms requires a well functioning immune system. Consequently, individuals with weakened immune systems are at

195 increased risk of becoming infected and of infections being more serious [17]. In the public
196 health system, vaccination is one of the most effective measures to prevent the spread of
197 infections. The critical principle of vaccination is the stimulation of the body's immune
198 response and the formation of population immunity in the population to protect against
199 infectious or other diseases [4]. One of the most consequences of immunosenescence is an
200 impaired response to vaccines with advanced age [6]. The present study is concordant with
201 our previous published results, when stimulated by Con a alone, PBMCs proliferation is
202 significantly reduced in aged populations compared to younger controls. The mechanisms
203 underlying the Immunosuppression include a series of cellular and molecular events involving
204 the change of several biochemical pathways and different cell populations [3]. Interestingly,
205 Con a combined with quercetin enhanced significantly PBMCs proliferation in the elderly as
206 well as in young cells. Quercetin seems stimulate cellular proliferation by acting to the
207 estrogen receptor (ER) in PBMCs, accumulated evidence suggests that physiologically
208 relevant concentrations of quercetin can exert phytoestrogen-like activity [18]; it activate also
209 GC-A or other guanylate cyclase isoforms [19].

210 The theory of free radicals of aging proposes that several age-related changes in immune
211 cellular functions, which depend on the redox state of these cells, can be good markers of
212 health, biological age and longevity. At cellular scale, free radicals overproduction are
213 markedly associated with age related immunity decline; thus excessive amounts of ROS
214 cause damage, they can damage cellular components such as carbohydrates, polyunsaturated
215 fatty acids, DNA and proteins [20]. It is well established that the concentration of oxidized
216 biomolecules increases with age
217 including MDA, and CP [21]. Oxidized lipids occur when a hydrogen atom is removed from the
218 methyl group unsaturated fatty acid. MDA is a keto-aldehyde produced by the peroxidation of
219 unsaturated lipids. Excess MDA combines with free amino acids

by producing protein peroxidation. The results of our research are in accordance with **Dalle-Donne et al., (2006) [22];** we noticed in aged PBMCs cells, an increase of two pro-oxidant parameters: MDA and carbonyl proteins, however, cellular antioxidant potency GSH did not decrease compared with young PBMCs . Increased cellular oxidative stress plays an important role in immunosenescence [23]**.** When lipid peroxidation is increased, changes in the permeability of the cell membrane may occur.

In fact In our study, PBMCs of aged patients were exposed to an evident intracellular oxidative stress with a higher level of oxidant biomarkers. Several in vivo and in vitro studies suggest that dietary antioxidant molecules would also attenuate tissue damage caused by oxidative challenges [24]. In the current study, our findings showed that in the presence of quercetin , MDA decreased significantly in aged PBMCs ; Our results are in favor of previous reports suggesting, that the quercetin possess a powerful antioxidant effects. T lymphocyte, a subtypes of PBMCs are particularly important in the immune response against specific antigens, they fall into two classes main, T4 which carry the surface protein called CD4 and T8 which have CD8 at their surface T lymphocytes are the main cells that contribute to vaccination efficacy.
They secrete a panoply of cytokines; IL-2 an essential interleukin that mediate clonal expansion of T lymphocytes . Anterior studies have shown a decline in IL-2 release during aging [11, 25]. Quercetin down-regulates IL-2 release both in young and aged subjects.
NO a signaling molecule that has been implicated in antimicrobial cytotoxicity for a variety of microorganisms, including bacterial, parasitic, fungal, and viral pathogens and tumor cells [26] Literature background supports the use of NO as immunity boosting agent and hence, the nitric oxide releasing compounds could act as lucrative in this context [27]. Our results shew that quercetin combined with Con a boost NO release from PBMCs in the elderly as well as in young subjects.

5- Conclusion and study limits

According to our encouraging obtained results, quercetin combined with Con a may have significant immunostimulatory and it can reduce the complications of aging in immune cells, as well as mitigating the oxidant stress in PBMCs cells. Quercetin combined with con a seems to have application as potential vaccine adjuvant; however, it is not possible to conclude witch vaccines type is quercetin suitable for. So it is important to highlight as a limitation that as in any vaccination scheme, the effectiveness of an adjuvant molecule should be studied in specific antigen-adjuvant combinations, so future clinical trials are needed.

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Conflicts of Interest: As declared by authors, no conflict of interest

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References

[1] Mocchegiani E, Malavolta M. Role of Zinc and Selenium in Oxidative Stress and Immunosenescence: Implications for Healthy Aging and Longevity. *Handbook of Immunosenescence*. 2019;2539-2573. Published 2019 Apr 11. doi:10.1007/978-3-319-99375-1_66

[2] Ly A, Shevelev A, Andres C, Pan XY, Trojan J. Mécanismes et pathologies du vieillissement. *J Afr Cancer*. 2013; 5:103-113.

[3] Cannizzo ES, Clement CC, Sahu R, Follo C, Santambrogio L. Oxidative stress, inflamm-aging and immunosenescence. *J Proteomics*. 2011;74(11):2313-2323. doi:10.1016/j.jprot.2011.06.005

[4] Andryukov BG, Besednova NN. Older adults: panoramic view on the COVID-19 vaccination. *AIMS Public Health*. 2021;8(3):388-415. Published 2021 May 8. doi:10.3934/publichealth.2021030

[5] Li

[6] Pereira B, Xu XN, Akbar AN. Targeting Inflammation and Immunosenescence to Improve Vaccine Responses in the Elderly. *Front Immunol*. 2020;11:583019. Published 2020 Oct 14. doi:10.3389/fimmu.2020.583019

[7] Sander VA, Corigliano MG, Clemente M. Promising Plant-Derived Adjuvants in the Development of Coccidial Vaccines. *Front Vet Sci*. 2019;6:20. Published 2019 Feb 12. doi:10.3389/fvets.2019.00020

[8] Reyna-Margarita HR, Irais CM, Mario-Alberto RG, Agustina RM, Luis-Benjamín SG, David PE. Plant Phenolics and Lectins as Vaccine Adjuvants. *Curr Pharm Biotechnol*. 2019;20(15):1236-1243. doi:10.2174/1389201020666190716110705

[9]

[10] Malavolta M, Pierpaoli E, Giacconi R, et al. Pleiotropic Effects of Tocotrienols and Quercetin on Cellular Senescence: Introducing the Perspective of Senolytic Effects of Phytochemicals. *Curr Drug Targets*. 2016;17(4):447-459. doi:10.2174/1389450116666150907105104

[11] Bouamama S, Merzouk H, Latrech H, Charif N, Bouamama A. Royal jelly alleviates the detrimental effects of aging on immune functions by enhancing the in vitro cellular proliferation, cytokines, and nitric oxide release in aged human PBMCS. *J Food Biochem*. 2021;45(2):e13619. doi:10.1111/jfbc.13619

[12] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J.Immunol.Methods*. 1983; **65**: 55–63. doi:10.1016/0022-1759(83)90303-4

[13] Nourooz-Zadeh J, Tajaddini-Sarmadi J, Ling KL, Wolff SP. Low-density lipoprotein is the major carrier of lipid hydroperoxides in plasma. Relevance to determination of total plasma lipid hydroperoxide concentrations. *Biochem J*. 1996;313 (Pt 3)(Pt 3):781-786. doi:10.1042/bj3130781

[14] Levine RL, Garland D, Oliver CN, et al. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol*. 1990;186:464-478. doi:10.1016/0076-6879(90)86141-h

[15] Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959;82(1):70-77. doi:10.1016/0003-9861(59)90090-6

312 [16] Guevara I, Iwanejko J, Dembińska-Kieć A, et al. Determination of nitrite/nitrate in
313 human biological material by the simple Griess reaction. *Clin Chim Acta*. 1998;274(2):177-
314 188. doi:10.1016/s0009-8981(98)00060-6

315 [17] Miles EA, Childs CE, Calder PC. Long-Chain Polyunsaturated Fatty Acids (LCPUFAs)
316 and the Developing Immune System: A Narrative Review. *Nutrients*. 2021;13(1):247.
317 Published 2021 Jan 16. doi:10.3390/nu13010247

318 [18] Van der Woude H, Ter Veld MG, Jacobs N, van der Saag PT, Murk AJ, Rietjens IM.
319 The stimulation of cell proliferation by quercetin is mediated by the estrogen receptor. *Mol*
320 *Nutr Food Res*. 2005;49(8):763-771. doi:10.1002/mnfr.200500036

321 [19] Chen ZJ, Vetter M, Chang GD, Liu S, Chang CH. Quercetin, a phytoestrogen and dietary
322 flavonoid, activates different membrane-bound guanylate cyclase isoforms in LLC-PK1 and
323 PC12 cells. *J Pharm Pharmacol*. 2003;55(3):353-358. doi:10.1211/002235702685

324 [20] Höhn A, Grune T. Lipofuscin: formation, effects and role of macroautophagy. *Redox*
325 *Biol*. 2013;1(1):140-144. Published 2013 Jan 19. doi:10.1016/j.redox.2013.01.006

326 [21] Jacob KD, Noren Hooten N, Trzeciak AR, Evans MK. Markers of oxidant stress that are
327 clinically relevant in aging and age-related disease. *Mech Ageing Dev*. 2013;134(3-4):139-
328 157. doi:10.1016/j.mad.2013.02.008

329 [22] Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative
330 damage in human disease. *Clin Chem*. 2006;52(4):601-623.
331 doi:10.1373/clinchem.2005.061408

332 [23] **(Sandhu and Gurcharan, 2002).**

[24] Cao G, Booth SL, Sadowski JA, Prior RL. Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *Am J Clin Nutr*. 1998;68(5):1081-1087. doi:10.1093/ajcn/68.5.108

[25] **Meydani et al., 1990**

[26] **Kissin**

[27] Mir JM, Maurya RC. Nitric oxide boosters as defensive agents against COVID-19 infection: an opinion [published online ahead of print, 2020 Nov 30]. *J Biomol Struct Dyn*. 2020; 1-7. doi:10.1080/07391102.2020.1852969

Tables

Table 1 . IL-2 and NO releases from PBMCs

Groups	IL-2 (pg/ml)	NO (µM)
Young PBMCs + Con a	563,3 ± 6,12 [a]	14,38 ± 6,45 [a]
Aged PBMCs + Con a	526 ± 2,36 [b]	10,21 ± 7,53 [b]
Young PBMCs + Con a + Quercetin	464,68 ± 2,42 [c]	14,34 ± 6,50 [a]
Aged PBMCs + Con a + Quercetin	460,8 ± 9,83 [c]	14,32 ± 5,00 [a]
***P*- One-way ANOVA**	0,0006	0,013

Note: Each value represents the mean ± standard deviation. PBMCs: peripheral Blood Mononuclear Cells, Con A: concanavalin a lectin, IL-2: Interleukin-2, NO: Nitric oxide. Significant differences between groups are indicated by small letters (a,b,c,d) , with *P*- One-way ANOVA less than 0.05.

Figures

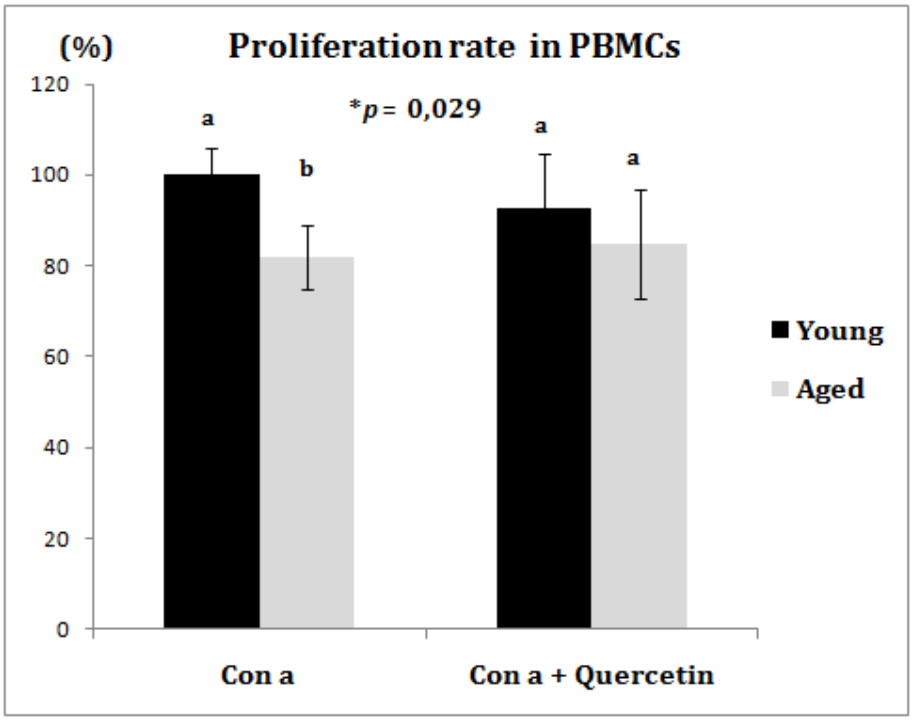

Fig 1: PBMCs cellular proliferation rate in response to quercetin and Con a treatments

Each value represents the mean ± standard deviation. PBMCs: peripheral Blood Mononuclear Cells, Con A: concanavalin a lectin. Significant differences between groups are indicated by small letters (a,b,c,d) , with *P*- One-way ANOVA less than 0.05.

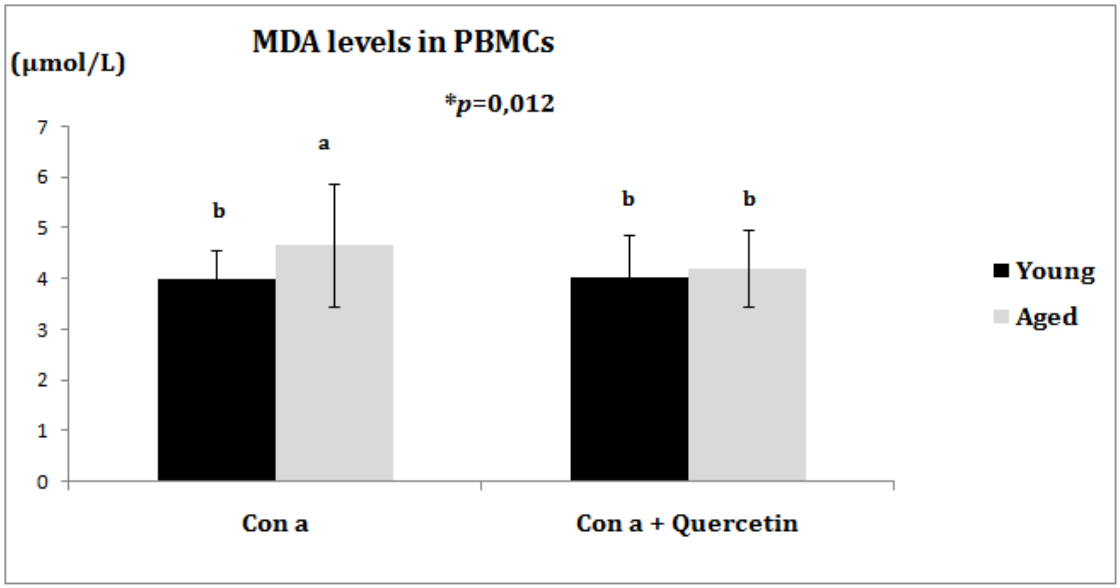

Fig 2: Intracellular Malondialdehydes levels in PBMCs

Each value represents the mean ± standard deviation. PBMCs: peripheral Blood Mononuclear Cells, Con A: concanavalin a lectin. MDA: Malondialdehydes. Significant differences between groups are indicated by small letters (a,b,c,d) , with *P*- One-way ANOVA less than 0.05.

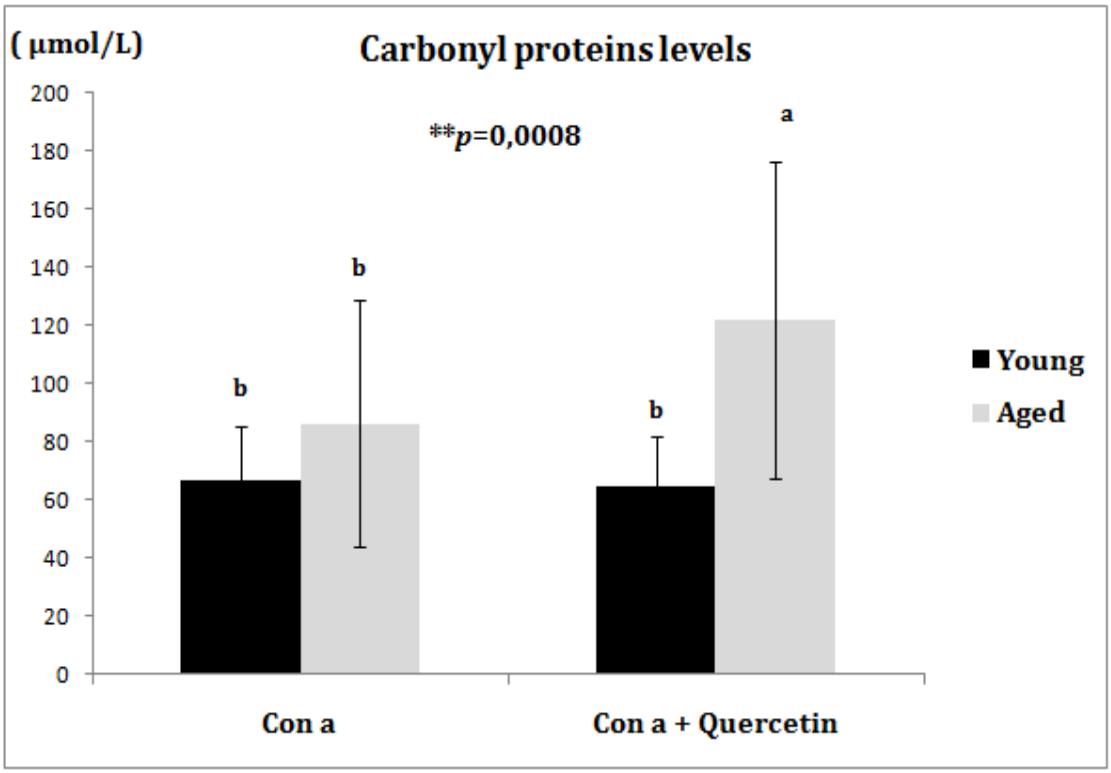

Fig 3: Intracellular carbonyls protein levels in PBMCs

Each value represents the mean ± standard deviation. PBMCs: peripheral Blood Mononuclear Cells, Con A: concanavalin a lectin. Significant differences between groups are indicated by small letters (a,b,c,d) , with *P*- One-way ANOVA less than 0.05.

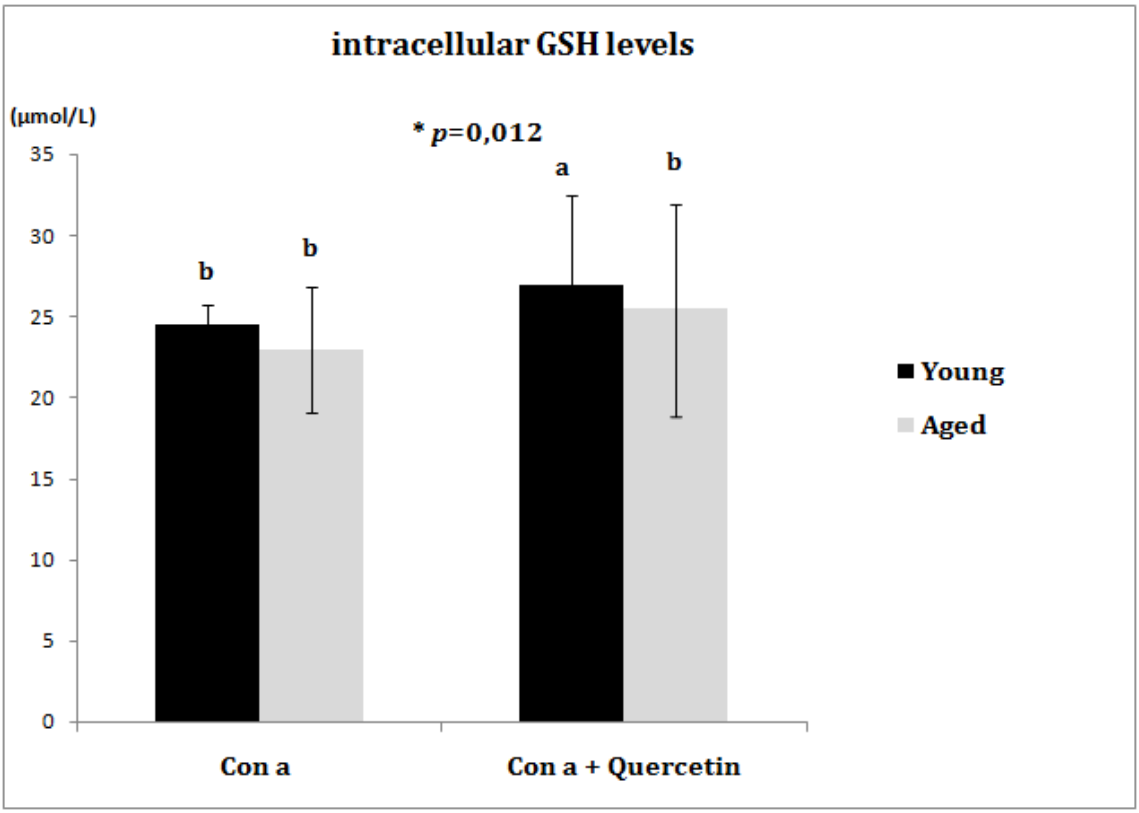

Fig 4: Intracellular GSH contents in PBMCs

Each value represents the mean ± standard deviation. PBMCs: Peripheral Blood Mononuclear Cells, Con A: concanavalin a lectin. Significant differences between groups are indicated by small letters (a,b,c,d) , with *P*- One-way ANOVA less than 0.05.