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Research paper

Anti-ageing properties of Khelma Longevity™: treatment of human fibroblasts increases proteasome levels and decreases the levels of oxidized proteins

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ABSTRACT

We have determined the putative anti-ageing properties of Khelma Longevity™, a formula based on various natural compounds from the Mediterranean area. Human primary fibroblast cultures were treated with a wide range of concentrations of Khelma Longevity™ for 1 day or 3 consecutive days. Following these treatments, two major and complementary biomarkers of ageing were measured, namely, the proteasome and the amount of oxidized proteins. It was observed that 24 h of treatment with Khelma Longevity™ resulted in a maximum increase of about 41% of the total protein levels of 20S proteasome. Levels of oxidized proteins were reduced by almost 6.5-fold following longer treatments. Specifically we have observed a maximum decrease of protein carbonyls to 84.7% in comparison with nontreated control cells following 3 days of continuous treatment with Khelma Longevity™. These results support the notion that formulas rich in natural compounds from the Mediterranean area possess anti-ageing properties.

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Introduction

Ageing is a natural and inevitable biological process accompanied by a progressive decline in cellular and organismal homeostasis. Cellular damage is caused mainly by reactive oxygen species (ROS) [1], which are continually generated as by-products of a number of cellular processes, as well as when cells are exposed to various environmental cytotoxic factors. In turn, these unstable and reactive derivatives can cause damage to biological macromolecules such as lipids, proteins and nucleic acids [2].

In vitro studies, mainly on fibroblast cultures, have shown that these cells have a limited replicative capacity and, after serial passage, they enter a state of irreversible growth arrest termed replicative senescence [3]. Senescence is accompanied by various features including impaired function of the proteasome and increased levels of damaged proteins [4]. The proteasome is the major cellular non-lysosomal threonine protease implicated in the removal of normal as well as abnormal, denatured or otherwise damaged proteins [5]. The core particle, the 20S proteasome, is a barrel-shaped complex composed of 28 subunits, 7 different α - and 7 different β -subunits, arranged in an α 1-7 β 1-7 β 1-7 α 1-7 structure. The 20S proteasome is also central to the ATP/ubiquitin

dependent intracellular protein degradation pathway where it represents the proteolytic core of the 26S complex [6].

Many studies have shown a general decline in proteasome activities in different aged tissues [7]. We have demonstrated proteasome activation by overexpressing the β 5 subunit in different human cell lines. The proteasome-activated cell lines exhibited increased rates of proteolysis and cell survival following treatment with various cytotoxic agents [8,9]. Importantly, overexpression of the β 5 subunit significantly extended lifespan in human primary cultures [9]. Following these genetic studies, we also showed that proteasome activation is possible through treatment with several compounds, either through promotion of conformational changes in proteasome structure [10] or through the activation of the transcription factor Nrf2 [11]. In accordance with the genetic activation of the proteasome, use of these compounds also results in cellular lifespan extension [11,12]. These *in vitro* studies indicated that proteasome activation is feasible and results in significant delay of senescence in human cells [13].

Apart from the above work on proteasome activation, several other natural compounds possess putative anti-ageing properties. Work on them was initiated thanks to the great efforts of the late Prof Brian Clark. The compounds include resveratrol [14], ginger [15], coumarin [16] and various other plant polyphenols [17]. Given these findings, we sought to investigate the anti-ageing properties of a potent, natural anti-oxidant supplement on human

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primary fibroblast cultures evaluating the 20S proteasome content and the amount of oxidized proteins.

Materials and methods

Reagents

Khelma Longevity™ Mediterranean Formula contains: *Olea europea* dry extract (leaf) standard (std), to 6% oleuropein. *Punica granatum* dry extract (fruit) std. to 40% punicosides. *Vitis vinifera* dry extract (fruit) std. to 92% total polyphenols, 15% proanthocyanidins, 2% anthocyanidins, trans-resveratrol > 100 parts per million (ppm). *Polygonum cuspidatum* dry extract (root) std. to 98% trans-resveratrol. This material was diluted in dimethyl-sulfoxide (DMSO) (Sigma–Aldrich) to a concentration of 100 mg/ml.

Cell lines and culture conditions

Human embryonic fibroblast (HFL-1) human embryonic fibroblasts were obtained from the European Collection of Cell Cultures and were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 1% (v/v) non-essential amino acids (Complete medium). Cells were subcultured when 70–80% confluent at 37°C, 5% CO₂ and 95% humidity.

Cell treatment with Khelma Longevity™

2 × 10⁵ middle age (~2 population doublings per 1 week) HFL-1 cells were seeded in 100 mm petri dishes and were treated the following day with 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 50 µg/ml Khelma Longevity™ or solvent (0.1%, v/v DMSO) in the presence of complete medium, continuously for up to 3 days. Fresh solution was added each day to exclude the possibility of its inactivation at 37°C.

Protein extraction

For quantification of 20S proteasome, attached cells were washed with 1 × PBS and then harvested and lysed in 1 × lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% (v/v) NP-40) at a concentration of approximately 10⁷ cells/ml, 24 and 72 h post-treatment. For the quantification of oxidized proteins, attached cells were washed with phosphate buffer saline (PBS) and harvested in 100 µl of PBS, 24 and 72 h post-treatment. Protein content of the supernatants was determined by the Bradford method (Bio-Rad Laboratories).

Determination of 20S proteasome concentration

20S proteasome levels were measured by 20S/26S Proteasome enzyme-linked immunosorbent assay (ELISA) kit (Biomol International L.P., Exeter, UK). Cell lysate samples were diluted 1:200 in ELISA buffer and measurements were performed in duplicates.

Determination of protein carbonyls (oxidized proteins)

Protein carbonyls were measured by OxiSelect Protein Carbonyl ELISA kit (Cell Biolabs, Inc., San Diego, CA). Measurements were performed in duplicates and cell lysate samples were diluted ~100-fold to 10 µg/ml protein with PBS prior to adsorption onto ELISA plates.

Statistical analysis

Statistical analysis was performed using statistical package for the social sciences (SPSS) Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA). Significance was taken at the 0.05 level. Levels of 20S proteasome and protein carbonyls between control (non-treated HFL-1 cells) and test samples (treated HFL-1 cells) at each Khelma Longevity™ concentration were analyzed by 2-tailed Student's *t* test.

Results and discussion

Khelma Longevity™ promotes increased formation of 20S proteasome

First we determined the effects of Khelma Longevity™ on proteasome. Middle age HFL-1 cells were continuously treated with a range of Khelma Longevity™ concentrations for 24 and 72 h

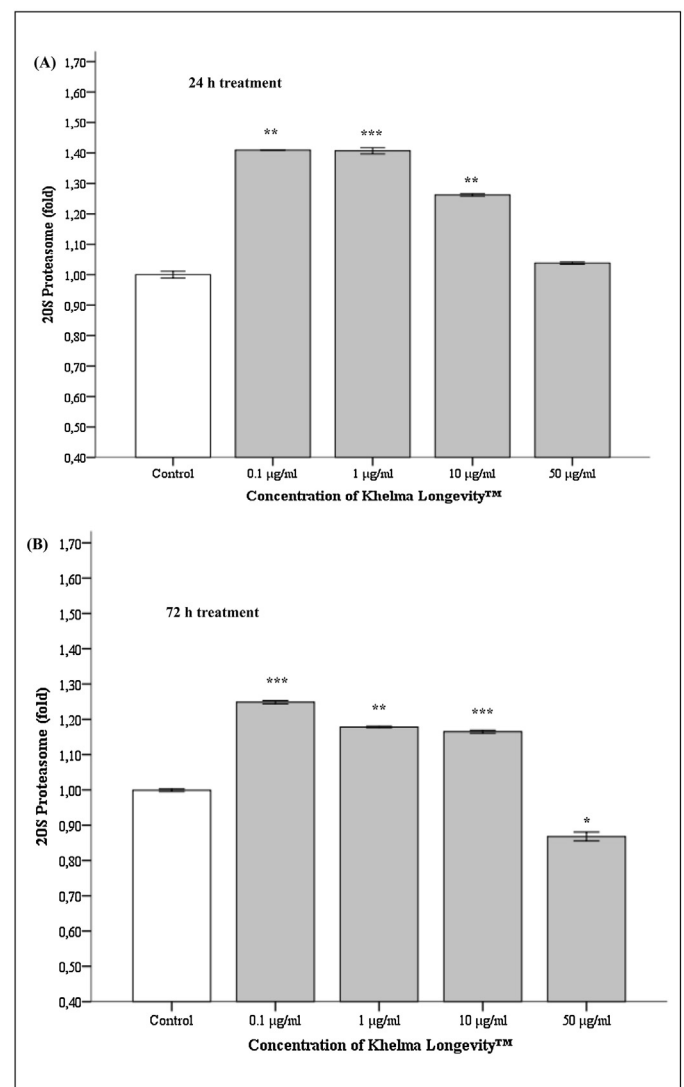


Figure 1. Khelma Longevity™ promotes increased formation of 20S proteasome. Manifold differences of 20S proteasome content in middle age HFL-1 cells treated with different concentrations of Khelma Longevity™ for (a) 24 h and (b) 72 h. 20S proteasome levels in control non-treated cells were arbitrarily set to 1. All values are reported as mean of two independent experiments ± standard error of the mean (S.E.M.). Single asterisk (*), double asterisk (**), and triple asterisk (***) denote significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

and their 20S proteasome content was measured. As shown in Fig. 1, the total protein levels of 20S proteasome increased approximately 1.4-fold (40.96%) following treatment with 0.1 $\mu\text{g}/\text{ml}$ Khelma LongevityTM for 24 h. The beneficial effects of Khelma LongevityTM were recorded over a wide range of concentrations (from 0.1 $\mu\text{g}/\text{ml}$ to 10 $\mu\text{g}/\text{ml}$) following both 24 and 72 h treatments. Thus treatment of human fibroblasts with Khelma LongevityTM increases the formation of 20S proteasome.

Khelma LongevityTM decreases the content of protein carbonyls

We next examined whether Khelma LongevityTM reduces the oxidative load in HFL-1 cells. As shown in Fig. 2, protein carbonyls were significantly decreased even after 24 h of treatment. The results were more profound following the 72 h treatment, where a maximum decrease of approximately 6.5-fold in protein carbonyls was observed at higher Khelma LongevityTM concentrations (from 1 $\mu\text{g}/\text{ml}$ to 50 $\mu\text{g}/\text{ml}$).

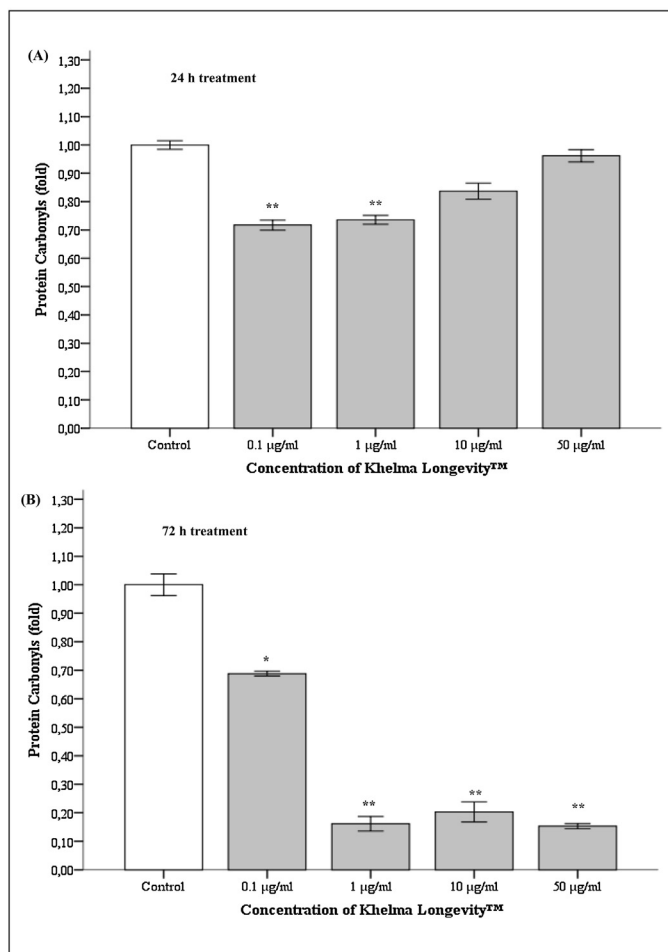


Figure 2. Khelma LongevityTM decreases the content of protein carbonyls. Manifest differences of protein carbonyls in middle-aged HFL-1 cells treated with different concentrations of Khelma LongevityTM for (a) 24 h and (b) 72 h. Protein carbonyl levels in control non-treated cells were arbitrarily set to 1. All values are reported as means of two independent experiments \pm S.E.M. Single asterisk (*) and double asterisk (**) denote significant differences at $p < 0.05$ and $p < 0.01$ respectively.

Conclusions

This *in vitro* pilot study indicates that Khelma LongevityTM is a potent proteasome activator. This formula contains a variety of flavonoids (or bioflavonoids), such as polyphenols, punicosides and proanthocyanidins, with known anti-oxidant properties. Ongoing studies focus on other Khelma Longevity formulas with emphasis on the action of *Linum usitatissimum*. Flavonoids constitute a class of naturally occurring polyphenolic compounds that act as anti-oxidants due to their scavenging activity [18]. Furthermore, they are used as nutraceutical ingredients with a wide range of beneficial properties including anti-inflammatory [19], antimicrobial [20] and anti-cancer [21] actions. This study highlights that apart from their well-documented anti-oxidant properties they also act as “secondary anti-oxidants” by activating the proteasome. The reported synergistic activation of more than one anti-oxidant mechanism opens up new directions for developing innovative anti-ageing therapies. Extensive clinical trials are essential for accomplishing these purposes.

Acknowledgments

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