KYMASIN UP PHYTOTHERAPY PRODUCT MAINTAINS THE CORRECT BONE REMODELLING IN EXPERIMENTAL MODELS MIMICKING AGE-RELATED OSTEOPOROSIS

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BONE REMODELLING is the process at the basis of bone health maintenance. It depends on the coordinated activity of bone-reforming cells called osteoblasts (OBs), and bone-resorbing cells called osteoclasts (OCs) [1,2] (Fig. 1). The excessive differentiation/activity of the OCs is responsible for unbalanced bone resorption causing **OSTEOPOROSIS**, which is characterized by loss of bone mass and density [1-3]. Osteoporosis is considered a major global unresolved metabolic disorder affecting millions of people all over the world, especially the aging population and postmenopausal women, leading to poor quality of life, increased risk of fractures, chronic pain, loss of independence, and generating enormous direct healthcare costs [4,5]. Several factors, including chronic use of drugs, hormones, immobilization, unbalanced diet, and inflammation, predispose to osteoporosis by affecting the physiological turnover in bone. **RANKL** (receptor activator of nuclear factor kappa-B ligand) is the principal cytokine promoting OC differentiation and activation, while OPG (osteoprotegerin, a soluble decoy receptor for RANKL) reduces osteoclastogenesis [6]. Indeed, high values of the RANKL/OPG ratio are considered predictive of osteoporosis, indicating an excess of bone resorption [7].

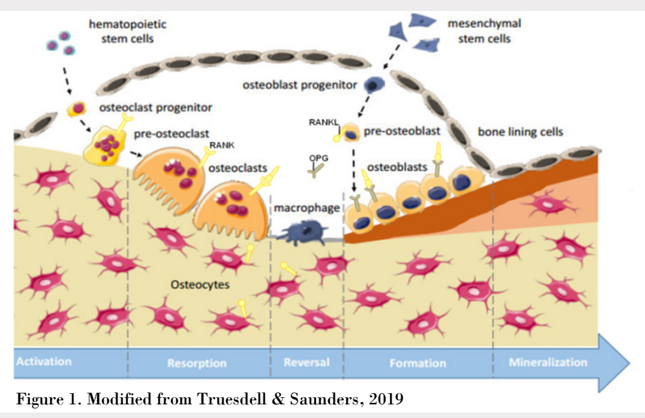
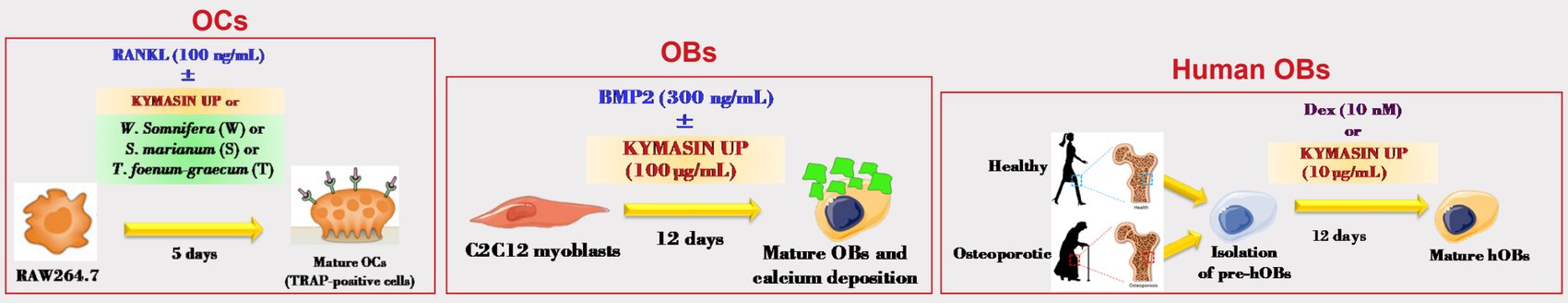


Figure 1. Modified from Truesdell & Saunders, 2019

AIM

We assessed the effects of KYMASIN UP, a novel dietary product containing *Withania somnifera*, *Silybum marianum* and *Trigonella foenum-graecum* (WST) developed based on our findings [8], in bone remodeling. We used established *in vitro* models mimicking the differentiation process of OCs and Obs, and consisting of RAW 264.7 monocyte cell line conditioned with RANKL [9], and C2C12 cells treated with BMP2 (bone morphogenetic protein 2) [10], respectively. The molecular mechanisms underlying the effects of KYMASIN UP and its efficacy in human primary OBs were also investigated.

METHODS



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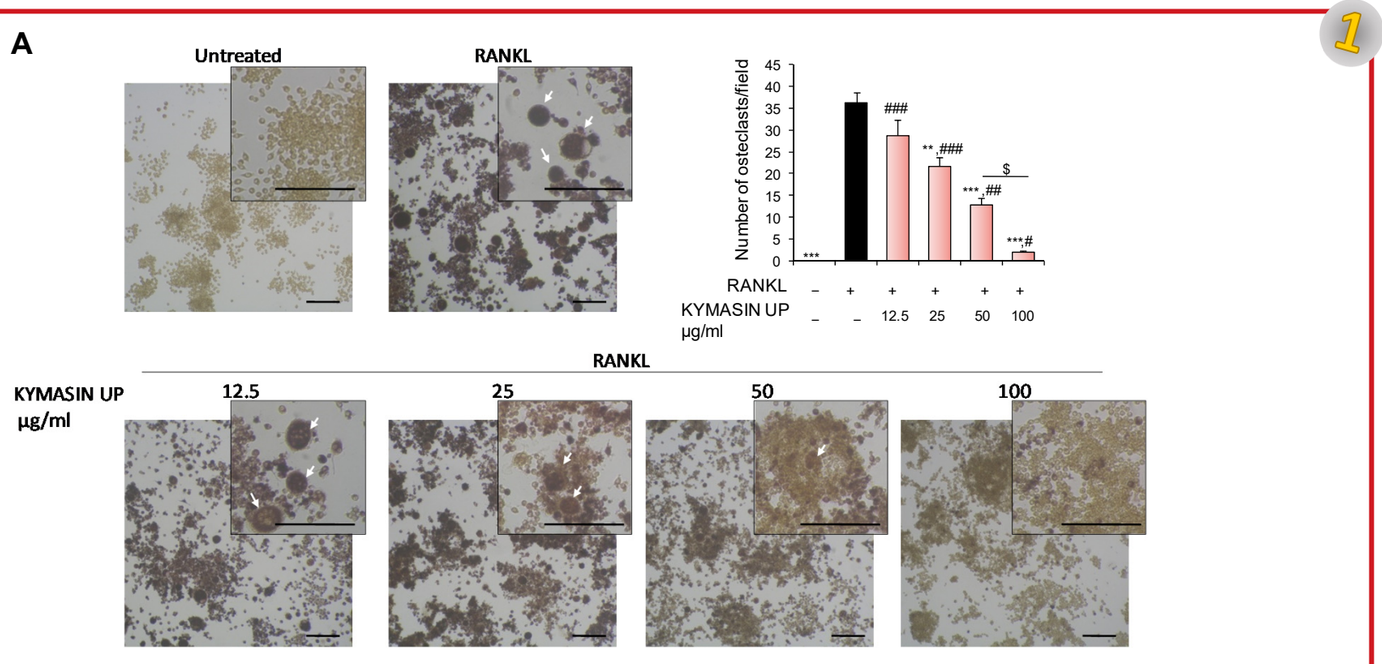

KYMASIN UP Inhibits RANKL-Induced Osteoclastogenesis and Restores the RANKL/OPG Ratio

(A) TRAP (tartrate-resistant acid phosphatase) staining was performed and TRAP-positive OCs (≥3 nuclei) were counted. Representative images and high-magnification insets are reported (arrows mark OCs). (B) The supernatants of cells in A were collected, and TRAP activity was measured by ELISA and reported as fold change *vs* RANKL treatment (black bar). (C) Real-time PCR analysis of OCs markers, *Acp5* (acid phosphatase 5, tartrate resistant), *Calcr* (calcitonin receptor), *Mmp9* (matrix metallopeptidase 9), and *Ctsk* (cathepsin K) was performed. Gene expressions were normalized to *Gapdh*. (D) Conditioned media were collected, trichloroacetic-acid precipitated and subjected to Western blotting for detection of released OPG. GAPDH amount in the cells was used as a loading control. Results are means ±Standard deviation (SD) (A-C). Statistical analysis was conducted using the two-tailed *t*-test (A-C). $^{*}p< 0.05$, $^{**}p< 0.01$, and $^{***}p< 0.001$ significantly different from RANKL. $^{\#}p< 0.05$, $^{\#\#}p< 0.01$, and $^{\#\#\#}p< 0.001$, significantly different from untreated control. $^{\$}p< 0.05$, significantly different. Scale bars (A), 100 µm.

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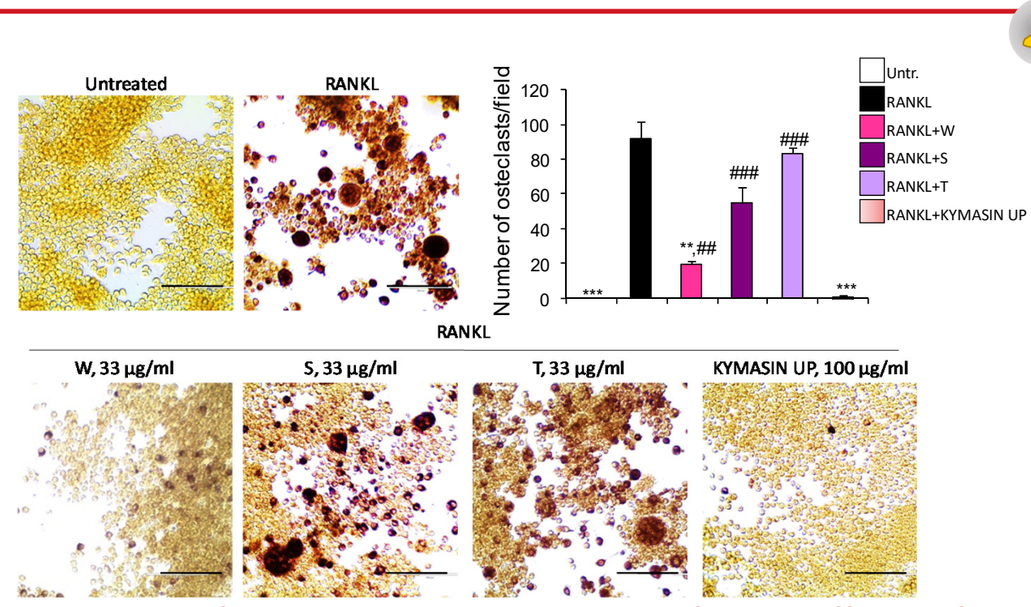

W. somnifera Is the Main Responsible for the Effects of KYMASIN UP on Osteoclasts

TRAP staining was performed and representative images are reported. The numbers of TRAP positive OCs (3 nuclei) were determined. Results are means ±SD. Statistical analysis was conducted using the two-tailed *t*-test. $^{**}p< 0.01$, and $^{***}p< 0.001$, significantly different from RANKL. $^{\#\#}p< 0.01$, and $^{\#\#\#}p< 0.001$, significantly different from untreated control (Untr). Scale bars, 100 µm.

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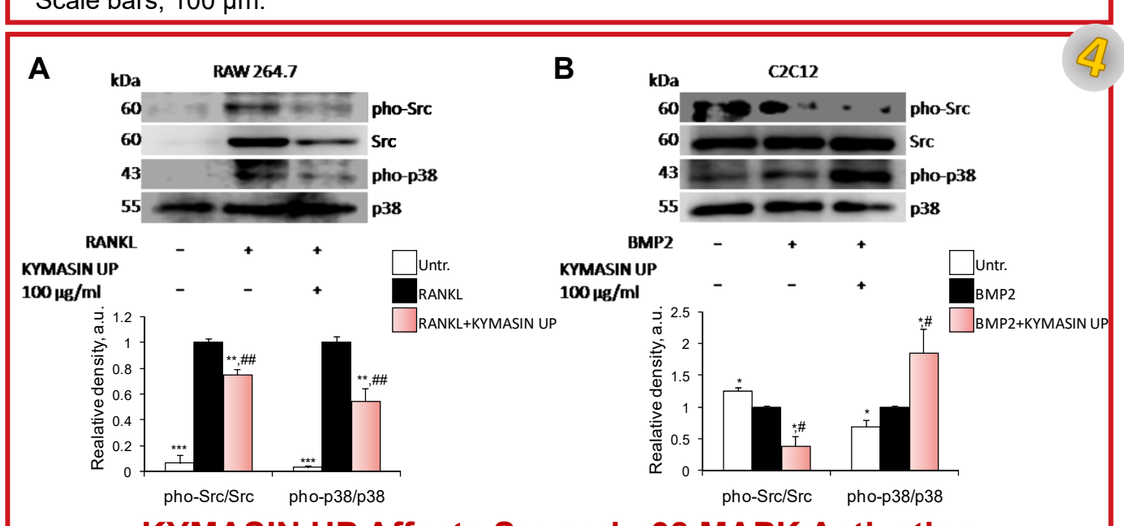
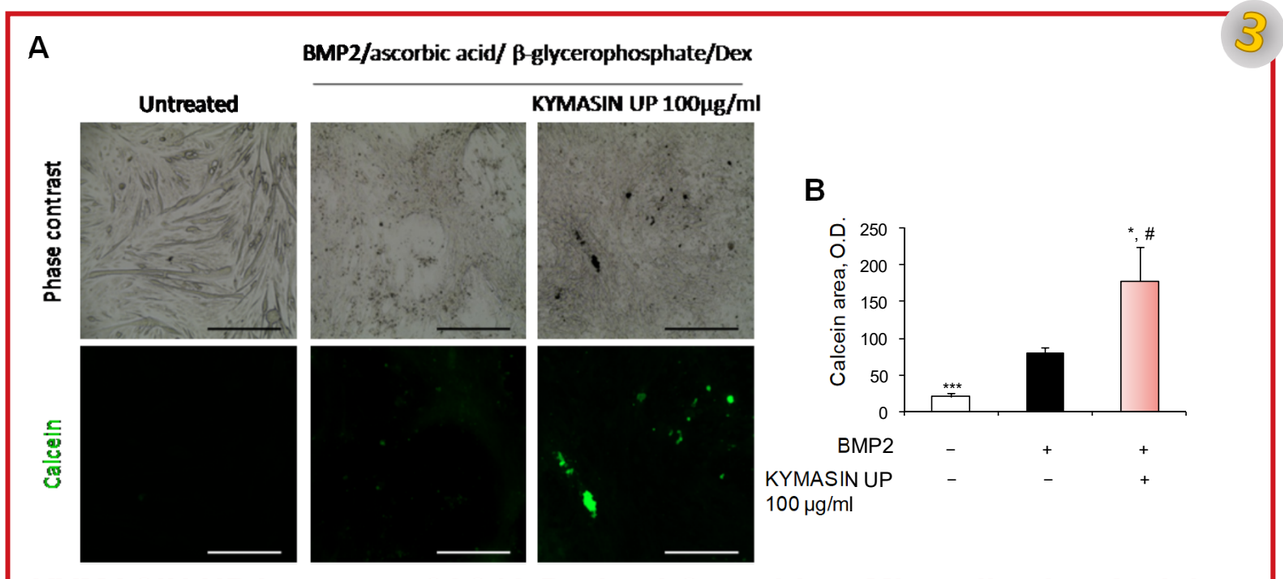


KYMASIN UP Affects Src and p38 MAPK Activation

(A,B) The expressions of phosphorylated (p) Src (non-receptor tyrosine kinase) and p38 MAPK (p38 mitogen-activated protein kinase), which are involved in osteoclastogenesis and OB activity, were analyzed by Western blotting in RANKL-treated RAW 264.7 (A) or in BMP2-treated C2C12 cells (B), in the absence or presence of KYMASIN UP (100 µg/mL). RANKL-dependent Src activation induces OC differentiation and activation of osteoclastogenic gene expression [11], while Src inhibits OB activity [10,12]. On the other hand, p38 MAPK plays a positive role in OC proliferation, differentiation, survival, and activity [13] and in BMP2-induced C2C12 trans-differentiation [14]. Representative images and the relative densities with respect to the total forms of Src and p38 are reported. Results are means ±Standard error of the mean (SEM). Statistical analysis was conducted using the two-tailed *t*-test. $^{*}p< 0.05$, $^{**}p< 0.01$, and $^{***}p< 0.001$, significantly different from RANKL (A) or BMP2 (B). $^{\#}p< 0.05$, and $^{\#\#}p< 0.01$, significantly different from untreated control (Untr).

3



KYMASIN UP Improves C2C12-Derived Osteoblast Mineralization Activity

(A,B) To induce transdifferentiation and deposition of calcium nodules, C2C12 cells were cultured for 12 days in the presence of BMP2, ascorbic acid, β-glycerophosphate and dexamethasone (Dex). (A) Phase contrast images were reported. Calcium nodule deposition was observed in fluorescence after calcein staining, (*green*). (B) The quantification of calcein-positive nodules was performed and reported as calcein optical density (O.D.). Results are means ±SD. Statistical analysis was conducted using the two-tailed *t*-test. $^{*}p<0.05$, and $^{***}p<0.001$, significantly different from BMP2. $^{\#}p<0.05$, significantly different from untreated control. Reported are representative images. Scale bars (A), 400 µm.

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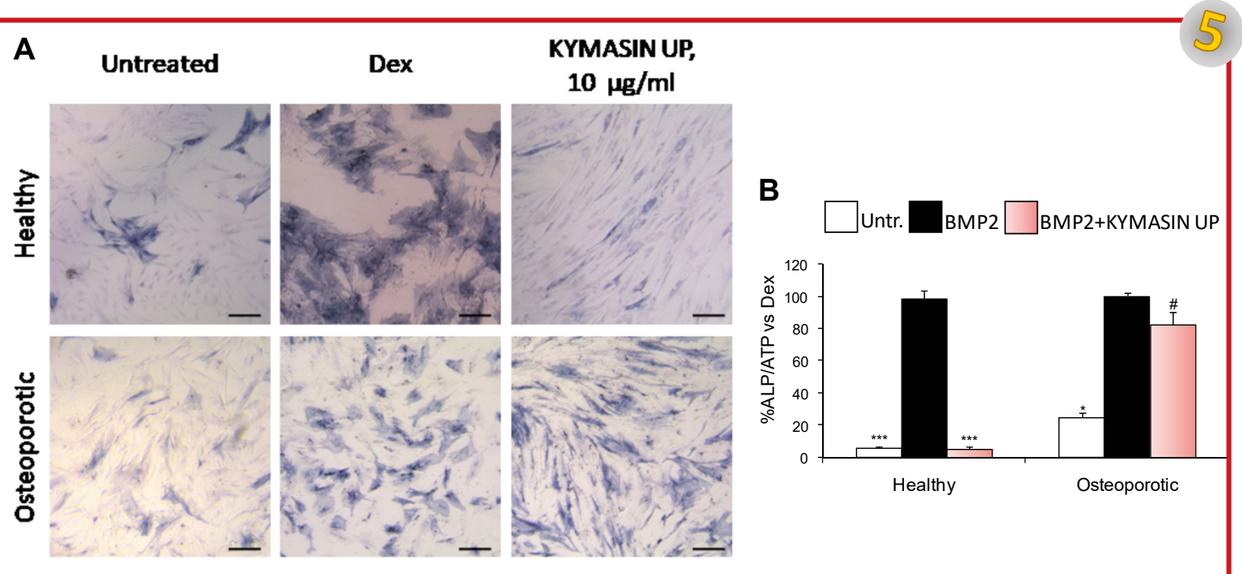
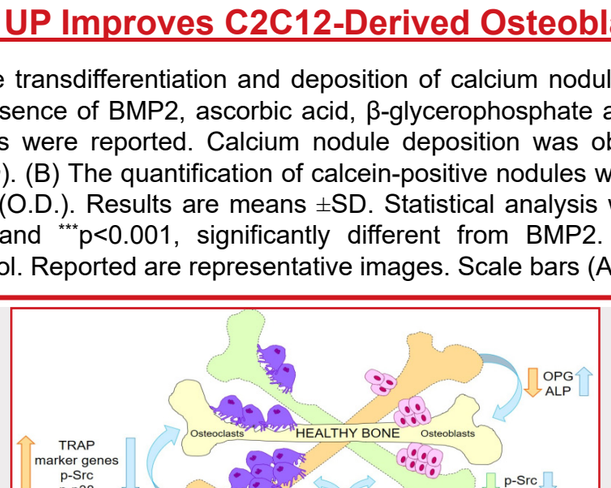


KYMASIN UP Induces Human Osteoblast (hOB) Differentiation in Osteoporotic Conditions

(A) Representative images of ALP (alkaline phosphatase) staining are reported. (B) ALP activity was measured and normalized to ATP (adenosine triphosphate) as an index of cell numbers and reported as percentages with respect to Dex treatment. Results are means ±SEM. Statistical analysis was conducted using the two-tailed *t*-test. $^{*}p< 0.05$, and $^{***}p< 0.001$, significantly different from Dex. $^{\#}p< 0.05$, significantly different from untreated control (Untr). Scale bars (A), 200 µm.



CONCLUSIONS

KYMASIN UP results as a promising candidate for the **treatment** of **osteoporosis**, **limiting** excessive **osteoclastogenesis** and **promoting OB maturation** in aging conditions, thus concurring in improving the elderly quality of life and reducing social and health-care costs.

References: **1.** Song *et al.*, *Pharmacol. Ther.* 2022; 237: 108168. **2.** Corrado *et al.*, *Int. J. Mol. Sci.* 2020;21: 3679. **3.** Noh *et al.*, *Int. J. Mol. Sci.* 2020; 21: 7623. **4.** Raisz, *J. Clin. Investig.* 2005; 115: 3318–3325. **5.** Choi *et al.*, *PLoS ONE* 2021; 16: e0248020. **6.** Boyce *et al.*, *Biochem. Biophys.* 2008, 473: 139–46. **7.** Azizieh *et al.*, *Biomark. Insights.* 2019; 14: 1177271919843825. **8.** Salvadori *et al.*, *Nutrients* 2020; 13: 49. **9.** Kim *et al.*, *J. Microbiol. Biotechnol.* 2019; 29: 11–20. **10.** Katagiri *et al.*, *J. Cell Biol.* 1994; 127: 1755–176. **11.** Zhi *et al.*, *J. Cell. Mol. Med.* 2020; 24: 5122–34. **12.** Hidaka *et al.*, *Pharmaceutics* 2020; 12: 218. **13.** Cong *et al.*, *Sci. Rep.* 2017; 7: 45964.**14.** Wu *et al.*, *Bone Res.* 2016; 4: 16009

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