

Supplementary information

Title:

Red algal extracts from *Plocamium lyngbyanum* and *Ceramium secundatum* stimulate osteogenic activities *in vitro* and bone growth in zebrafish larvae

Author list:

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Methods

hFOB cell culture and experimental design

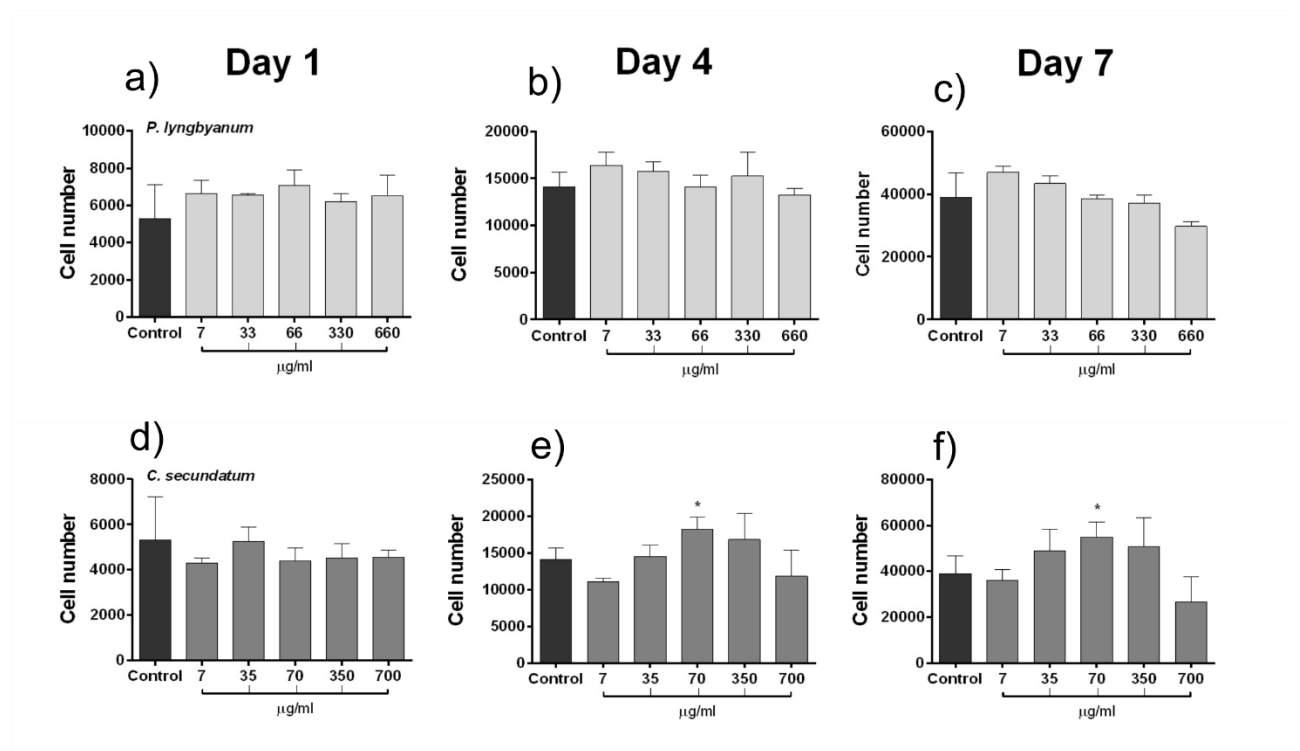
The hFOB 1.19 (ATCC® CRL-11372™) cell line was used during original screening of extracts. Cells were cultured in T75 flasks and expanded by subculturing (0.25% Trypsin EDTA, 1:4 slitting ratio). Culture media was DMEM/HAM F12 (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum, 2 mM L-glutamine and 0.3 mg/ml geneticin. Cells were used between passages 6 and 10.

Extracts were added to complete hFOB culture medium at concentrations of 7, 33, 66, 330 and 660 µg/ml for *P. lyngbyanum* and 7, 35, 70, 350 and 700 µg/ml for *C. secundatum*. hFOBs were plated in 96-well plates at a density of 1×10^4 cells/cm² and challenged by the extracts for 1, 4 or 7 days. After the stated treatment periods cell proliferation was assessed via crystal violet staining, in the method detailed previously.

Cytotoxicity (hBMSCs)

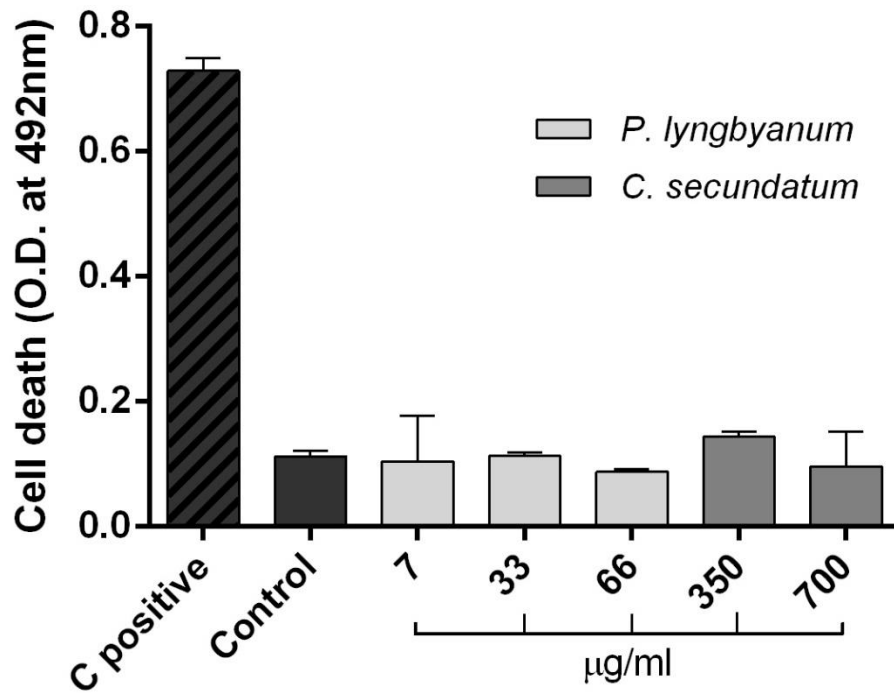
The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, UK) was used to quantify lactate dehydrogenase (LDH) level. This enzyme is normally constrained to the cytoplasm of healthy cells and therefore gives an indication of extract cytotoxicity. hBMSCs (2×10^4 cells/cm²) were given a 24 h attachment period and 24 h with extract treatments, before conditioned medium was removed and LDH level quantified according to the manufacturer's protocol.

Supplementary figure 1: hFOB screening – cell proliferation



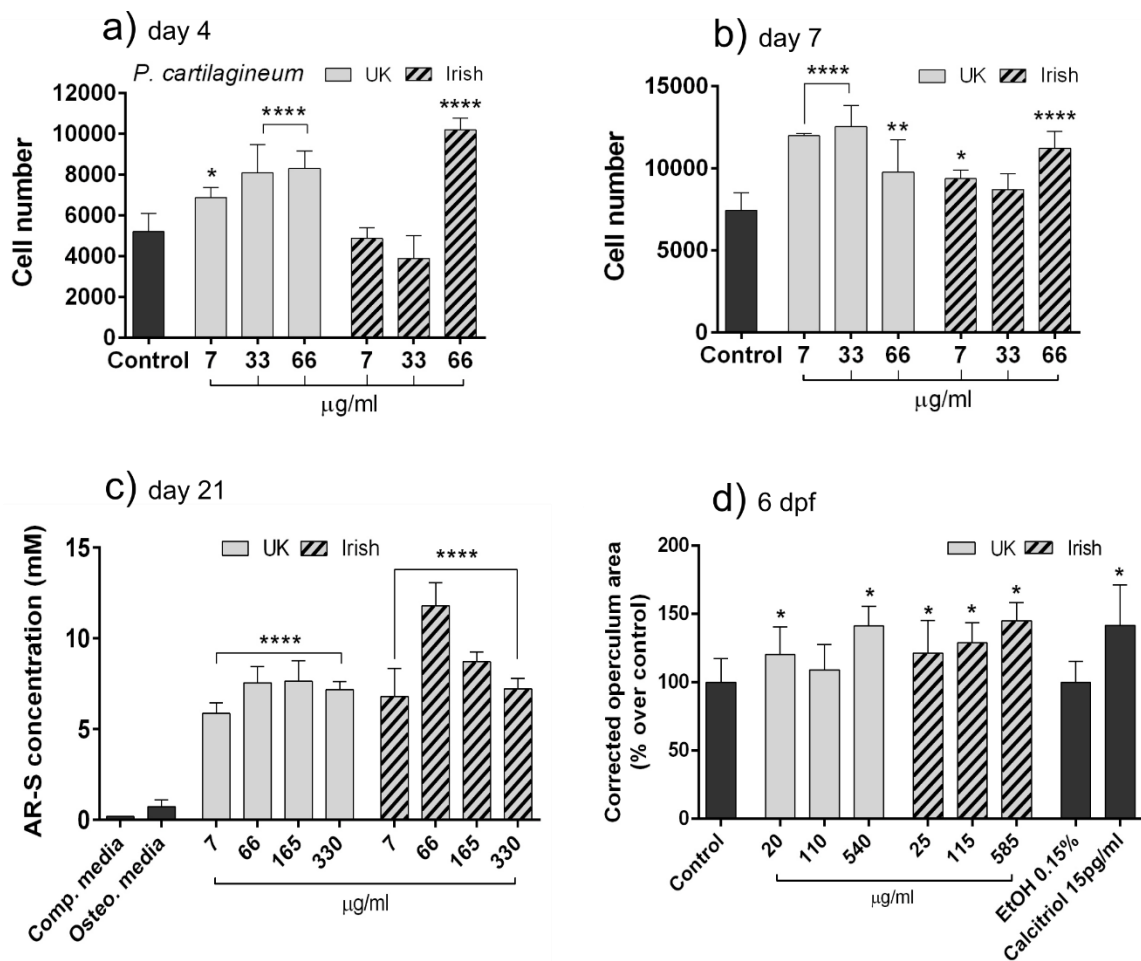
Proliferation day 1, 4 and 7 crystal violet assays conducted on hFOB. After a 24-hour attachment period cells were treated with either a control solution, *P. lyngbyanum* concentrations of 7, 33, 66, 330 and 660 µg/ml or *C. secundatum* concentrations of 7, 35, 70, 350 and 700 µg/ml. Results are presented as the mean cell number for each treatment, (n=3 or 4 (P3), +/- SD). * indicates a significant difference (p<0.05) between the treatment and control.

Supplementary figure 2: Cytotoxicity of extracts on hBMSCs



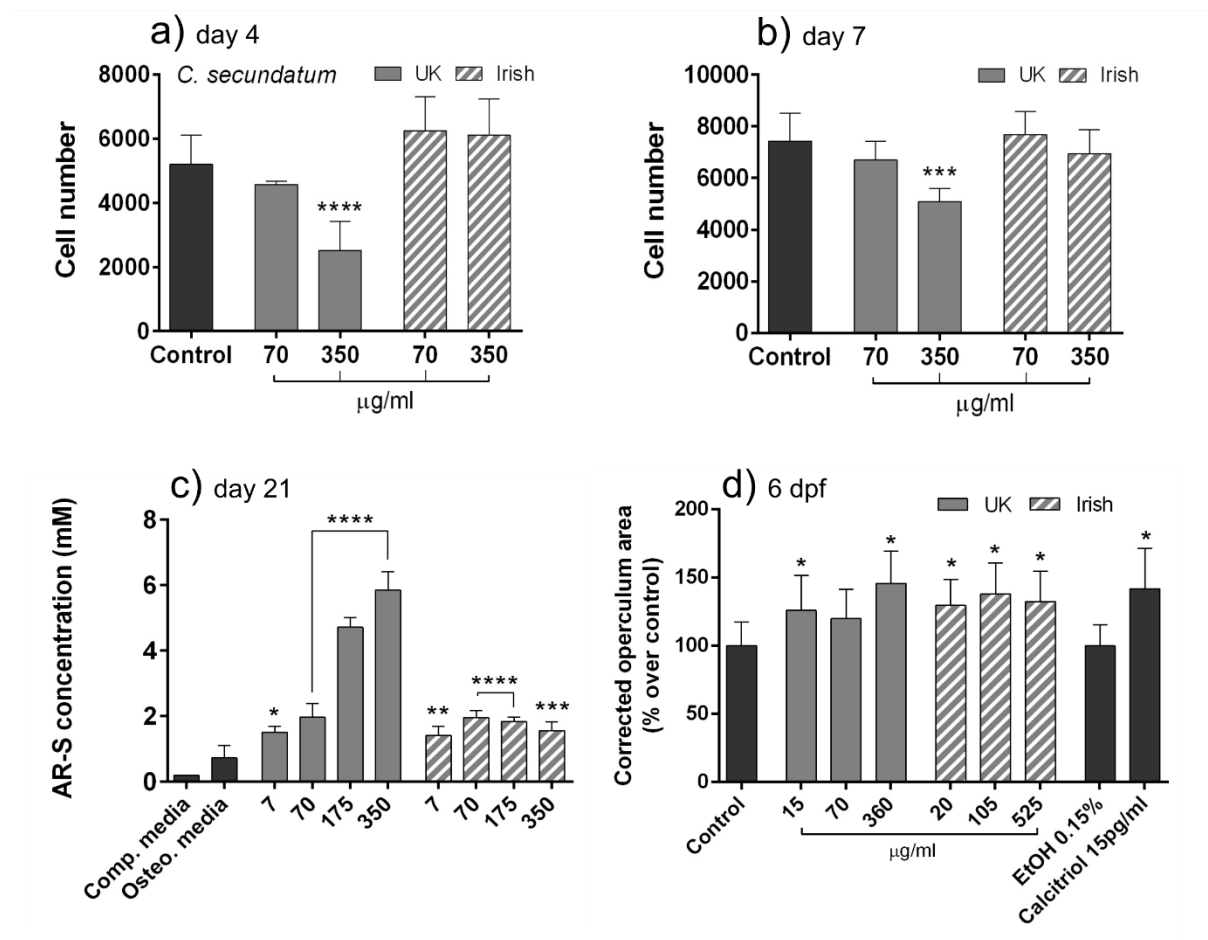
Lactate dehydrogenase assay results are presented as mean optical density reading $\pm$ SD (n=3, 1 exclusion in 7 µg/ml group). C positive shows the absorbance values for 100% cell death. All values are corrected for background absorbance by subtracting appropriate negative control values.

Supplementary figure 3: Osteogenic potential of closely related species *P. cartilagineum*



P. cartilagineum, extracted from UK and Irish coast raw material. **a) b)** Proliferation of hBMSCs at day 4 (a) and 7 (b) using crystal violet assay. Results are presented as the mean cell number $\pm$ SD (n=3 or 4 (P3)). **c)** *In vitro* mineralisation assay of hBMSCs at day 21. Controls were complete media and osteogenic media. Each reading is displayed as mean AR-S concentration $\pm$ SD (n=4). **d)** Operculum growth at 6 dpf for *P. cartilagineum* treatments at concentrations between 20 and 585 µg/ml. Extract control was system water with 10% saline solution. A 0.15% EtOH control corresponded to the positive calcitriol control, dissolved in EtOH at a 15pg/ml concentration. Results are presented as mean corrected operculum area as a percentage of the control $\pm$ SD (n=15) for all treatments - excluding small losses of 1-2 fish. * indicates a statistically significant difference ($p < 0.05$) compared to the relevant control.

Supplementary figure 4: Osteogenic potential of fresh and re-extracted *C. secundatum* sample



C. secundatum, re-extracted from UK and Irish coast raw material. **a) b)** Proliferation of hBMSCs at day 4 (a) and 7 (b) using crystal violet assay. Results are presented as the mean cell number $\pm$ SD (n=3 or 4 (P3)). **c)** *In vitro* mineralisation assay of hBMSCs at day 21. Controls were complete media and osteogenic media. Each reading is displayed as mean AR-S concentration $\pm$ SD (n=4). **d)** Operculum growth at 6 dpf for *C. secundatum* treatments at concentrations between 20 and 585 µg/ml. Extract control was system water with 10% saline solution. A 0.15% EtOH control corresponded to the positive calcitriol control, dissolved in EtOH at a 15pg/ml concentration. Results are presented as mean corrected operculum area as a percentage of the control $\pm$ SD (n=15) for all treatments - excluding small losses of 1-2 fish. * indicates a statistically significant difference ($p < 0.05$) compared to the relevant control.