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Combination treatment of bisphosphonate (pamidronate) and *Quercus infectoria* semi-purified fraction promotes proliferation and differentiation of osteoblast cell via expression of Osterix and Runx2 marker

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ABSTRACT

Objective: To understand the effects of combination treatment of pamidronate with isolated *Quercus infectoria* semi-purified fraction (*QI*sm-F) on human foetal osteoblast cell model (hFOB 1.19 cell line) through assessment of Runt related transcription fraction-2 (Runx2) and Osterix (Osx). **Methods:** The isolation and purification of *QI*sm-F were conducted by chromatographic technique. In order to assess relative efficacy of *QI*sm-F to the osteoblast model, the determination of half maximal effective concentration (EC_{50}) was performed by MTT assay. hFOB 1.19 cells were cultured in DMEM F-12 and supplemented with 10% fetal bovine serum along with 1% penicillin-streptomycin incubated in 5% CO_2 at 37 ℃. Expression of Runx2 and Osx was assessed through western blotting and confirmed with immunofluorescence staining. **Results:** Results of western blot analysis and immunofluorescence staining demonstrated that compared to hFOB 1.19 cells treated with single individual treatment of *QI*sm-F and control groups, levels of Runx2 and Osx were elevated with higher fluorescence intensity and more rapid proliferation in hFOB 1.19 cells treated with combined treatment of *QI*sm-F and pamidronate. **Conclusions:** The finding demonstrates the synergistic effect between osteoporotic drug pamidronate and established *QI*sm-F. The combination treatment helps increase the efficiency of pamidronate acting on osteoblast cells by stimulating osteoblast proliferation and differentiation via expression of Runx2 and Osx.

1. Introduction

Bisphosphonates (*e.g.*, pamidronate, etidronate, clodronate, alendronate, tiludronate, risedronate, and ibandronate) are established as effective treatments of osteoporosis for decades and shown to be effective in prevention of bone fracture[1]. Osteoporosis is a common type of bone disease characterised by reduced bone mass and increased in skeletal fragility[2]. Bisphosphonate acts by decreasing bone resorption, thereby slowing bone loss

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through reduce osteoclastic bone resorption[3,4]. It possesses unique pharmacological character in which they have the potential to continue to affect bone metabolism long after discontinuance of its intake[5,6]. Several research has reported possible side effects of bisphosphonate which can cause possible osteonecrosis of the jaw in oncology patients as well as atypical femoral fractures caused by over suppression of bone remodelling[7,8]. Meanwhile, orally administering bisphosphonates has a very low bioavailability and may induce gastrointestinal disturbances[1]. These agents have little ability to stimulate new bone synthesis. Therefore, investigations of alternative agents that can improve synthesis of new bone are fairly significant.

Bone is a dynamic tissue that undergoes constant remodelling through bone formation and resorption. The process of bone remodelling comprised of sequential events triggered initially from resorption of existing bone by osteoclast followed by bone formation by osteoblast[9]. Thus, osteoblast is the key component in bone multicellular unit. Osteoblasts are differentiated from multipotent mesenchymal cells that are regulated by several cytokines which include bone morphogenetic proteins-2 (BMP-2), transforming growth factor-β and so on[10,11]. The BMP-2 then faciliatates to differentiate mesenchymal cells with osteoblasts and induces bone formation by activating Smad signaling pathway and regulating transcription of osteoblast specific transcription factor such as Runt-related transcription factor-2 (Runx2)/Core-binding factor-1, Osterix (Osx), alkaline phosphatase, type Ⅰ collagen, osteocalcin, and bone sialoprotein 2[12,13].

Runx2 and Osx are osteoblast-specific transcription factors essential for the development of osteoblastic cells and bone formation[14,15]. The process of osteoblast differentiation is directed by Runx2 in which mesenchymal progenitor cells evolves to preosteoblast. Then, preosteoblastis directed to immature osteoblast by Runx2, Osx and β-catenin[16]. On the other hand, Osx is the second transcription factor that is essential for osteoblast differentiation whereby it is specifically expressed in all developing bones[16,17]. Given that these two transcription factors are essential for osteoblast differentiation from independent progenitor cells, the quantification of these factors could be used to determine the osteoblastogenic potential in discovery of new anabolic agent acting on bone[18-20]. Therefore, both Runx2 and Osx are important transcription factors for osteoblast differentiation and controlling the expressions of osteoblastogenic marker that are required for the terminal osteoblast differentiation and bone mineralisation[21].

Quercus infectoria (*Q. infectoria*) Olivier (Fagaceae) is a small tree or a shrub mainly presented in Greece, Asia Minor, Syria and Iran[22]. The galls of *Q. infectoria* have a great medicinal value and have pharmacologically been deciphered to be astringent, ant diabetic, antitremorine, local anaesthetic, antipyretic and anti-parkinsonian[23]. The main components of *Q. infectoria* comprise of 50% to 70% polyphenols, which is made up from abundance amount of tannins, gallic acid, syringic acid, ellagic acid, amentoflavone, hexamethyl ether, isocryptomerin, methyl betulate, methyl oleanate and hexagalloyl glucose[24]. Throughout the years, various study reported the effects of polyphenols on promoting the formation of new bone[25,26]. Recently, polyphenols derived from dried plum have been reported to enhance osteoblast activity and function by up-regulating Runx2, Osx and IGF-Ⅰ expression[27]. In addition, our preliminary study has found that the level of alkaline phosphates in hFOB 1.19 increases significantly after being treated with *Q. infectoria* galls extract,providing initial insight on the ability of *Q. infectoria* in modulating bone formation[28]. However, the effects of combining *Q. infectoria* with readily available osteoporotic agent such as biophosphonate have not been examined and understood.

In the present study, the paper aims to investigate and understand the effects of combining the readily available and commonly used osteoporotic drug (pamidronate) with *Q. infectoria* semi-purified fraction (*QI*sm-F) on human foetal osteoblast cell model (hFOB 1.19 cell line) by assessing the levels and expression of Osx and Runx2 important osteoblastogenic marker.

2. Materials and methods

2.1. Extraction of Q. infectoria extract

Q. infectoria galls were obtained from local market. The galls were identified based on its morphology parameters such as external colour, size, surface, texture, odour, taste and thickness[29]. Aqueous extraction of *Q. infectoria* was done by grinding the *Q. infectoria* galls to obtain powdered form followed by weighing 50 g of crude *Q. infectoria* extract in 100 mL of sterile distilled water and refluxing in water bath at 50 ℃ for 24 h.

2.2. Isolation and fractionation of QIsm-F

Flash column chromatography was performed in glass column (width: 40 mm; length: 500 mm) with silica gel 60, 0.063-0.200 mm (Merck Milipore, USA) using solvent mixture (ethyl acetate: methanol: acetonitrile: H_2O); ratio (1:1:7:1). Flash column was packed manually by using the slurry method. Aqueous *Q. infectoria* extract sample was prepared by dissolving in 95% ethanol. The solvent mix was continuously added as the final layer simultaneously with elution process until collections of fraction were accomplished. Collection of sample fractions was done in test tubes, graduated with the fraction volume, and visualised by spotting on thin layer chromatography plates using established solvent system (ethyl acetate: methanol: acetonitrile: H_2O); ratio (6:1.5:1.5:1). The plate was then visualized under UV lamp at wavelength 254 nm. The fraction with the same R_f values will be pooled together and each fraction will be subjected to second column chromatography by using different solvent mix ratio (ethyl acetate: methanol: acetonitrile: H_2O); ratio (5.5:1:2.5:1). The fraction with the same R_f value will again be pooled together

and considered as final semi-purified product[30]. The presence of polyphenols is confirmed by performing ferric chloride ($Fecl_3$) staining on thin layer chromatography plate and comparing to the standard.

2.3. Cell culture conditions

Human foetal osteoblast cell, hFOB 1.19 (CRL-11372) was purchased from American Type Cell Culture (Manassas, USA). The cells were revived in Dulbecco's Modified Eagle Medium F-12 (Gibco, USA) and supplemented with 10% fetal bovine serum; (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). The cells were incubated in 5% CO_2 incubator at 37 ℃. All cell culture work was maintained in a sterile condition by applying aseptic technique and being carried out in the Bio safety cabinet class 2 (ESCO, Singapore).

2.4. Cell viability assay/MTT assay

This study employs pamidronate as osteoporotic control drug and agent for treatment combination with *QI*sm-F. Meanwhile, tamoxifen, a type of selective estrogen receptor, on the other hand, was established as negative control drug due to its known negative effect on osteoblast cell[31,32] and untreated control was used as general control. hFOB 1.19 cells were plated in 96-well plate at a density 5×10^3 cells per well[33]. Twenty four hour after plating, the cells were exposed with different concentration of each treatment groups (pamidronate, *QI*sm-F and tamoxifen), ranging from 0.01 μg/mL to 99 μg/mL for 72 h. Each treatment was carried out in triplicate. At the end of the treatment, 20 μL of freshly prepared MTT (5 mg/mL in PBS) (Nacalai Tescue, Japan) was added into each of the 96-wells plate followed by incubation for 4 h in a CO_2 incubator at 37 ℃. Then, formazon salts were dissolved with 100 μL dimethyl-sulphoxide (NacalaiTesque, Japan) and the absorbance was determined at 570 nm in ELISA micro plate reader. The graph of the percentage of viable cell versus $\log_{10}$ concentration (μg/mL) of each fraction was plotted employing GraphPad Prism 6.0. The half maximal effective concentration (EC_{50}) was determined by the plotted graph.

2.5. Western blot

The total proteins were extracted at day 3 and day 7 of incubation with lysis buffer (Nacalai Tesque, Japan) for 20 min on ice. The protein samples were run on 10% SDS –PAGE gel electrophoresis at 100 V for 1 h for protein separation. The protein was then being transferred onto a polyvinylidene difluoride membrane using semi-dry transblot (BioRad, USA) at 15 V for 2 h. After transfer, the polyvinylidene difluoride membrane was blocked in 1% skim milk in tris-buffer saline with Tween-20 for 1 h followed by incubation with primary antibodies (against ß-actin 1:1 000, Runx2 1:400 and Osx 1:400 (Santa Cruz, USA) overnight at 4 ℃. The membrane were then matched with secondary antibody IgG HRP (1:1 000) (Santa Cruz, USA).

2.6. Immunocytochemistry

Immuno-fluorescence staining technique utilises the specificity of antibodies to their antigen to target fluorescent dyes to specific bio molecule targets within a cell. Each marker; Runx2 (Alexa Fluor 488) and Osx (FITC) were tagged with different fluorescence dyes. Cells were cultured and treated in 4-chamber slide with seeding density of 5×10^3. The cells have then been prepared for immune-fluorescence staining at day 3 and day 7 of incubation by fixing in 4% paraformaldehyde for 5 min at room temperature and then been permeabalized in 0.5% Triton X-100 (ThermoFisher Scientific, USA) in DPBS for 15 min. After being blocked in 3% bovine serum albumin in DPBS for 60 min, the cells were then incubated overnight at 4 ℃ with primary antibody against Runx2 (1:200) directly conjugated with Alexa Fluor 488 (Santa Cruz, USA) and Osx (1:200); (Santa Cruz, USA). The secondary antibodies, conjugating with fluorescein isothiocyanate were added at (1:500) dilution for cells incubated with Osx antibody and incubated for 1 h at 37 ℃ away from light. The cells were then mounted with Prolonged Diamond antifade mountant with DAPI (ThermoFisher Scientific, USA). Images were viewed under fluoresence microscrope (Olympus, Japan) and captured by employing image analyser (Olympus, Japan).

2.7. Statistical analysis

Data were expressed as the mean±SEM of n=3 independent experiments. Statistical analysis was conducted by applying SPSS (version 22.0 for windows). Data were tested for normality and homogeneity of variance. Statistical comparisons of the results were established using Repeated Measure ANOVA analysis. Differences among groups were conducted by using multiple comparison *post hoc* tests. Values of $P<0.05$ were considered to be statistically significant to the control.

3. Results

3.1. EC_{50} and effects of QIsm-F and combine treatment on cell viability

The EC_{50} values of each treatment were established by using MTT assay (Figure 1A) with pamidronate (15.270 μg/mL), *QI*sm-F (11.600 μg/mL) and tamoxifen (1.568 μg/mL), respectively. The concentration of *QI*sm-F (11.600 μg/mL) required to induce cell proliferation in hFOB1.19 cells was lower than required by pamidronate (15.270 μg/mL) which showed preliminary indicator of *QI*sm-F efficacy on osteoblast cell in comparison to pamidronate. Percentage of cell viability of hFOB 1.19 cells treated with respective

established EC_{50} values of each treatment were investigated and shown in Figure 1B. After treatment with pamidronate, *QI*sm-F, combined *QI*sm-F with pamidronate and tamoxifen after 72 h, there is a significant difference in hFOB1.19 cell treated with pamidronate, *QI*sm-F and tamoxifen when compared to control (Figure 1B).

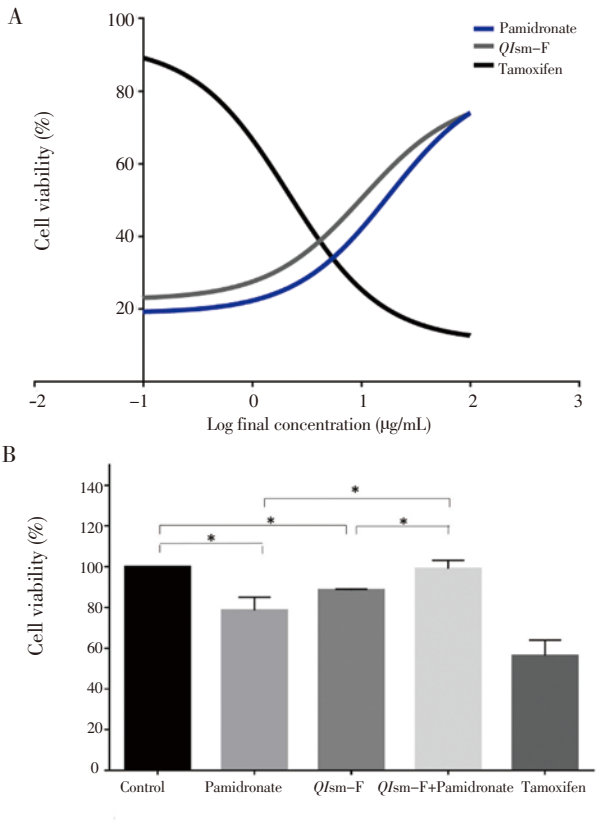

Figure 1. EC_{50} (A) and effect of pamidronate, *QI*sm-F and tamoxifen on hFOB1.19 cells after 72 h (B).

(A) Paired *t* test showed significant difference ($P<0.05$). EC_{50} values were calculated by using dose response stimulation curve in GraphPad Prism 6.0. (B) Column and bars represent mean±SEM of the results of 5 cultures in triplicate. Results are expressed as percentage of control. * indicates significant difference within each groups ($P<0.05$) by One way ANOVA test.

3.2. Effects of QIsm-F and combination of QIsm-F with pamidronate on Runx2 and Osx expression

Western blot analysis shows that the protein level of Runx2 (Figure 2A) and Osx (Figure 2B) increased significantly in a time dependant manner when treated with pamidronate, *QI*sm-F and combined *QI*sm-F with pamidonate. Meanwhile, treatment with negative control tamoxifen indicates decreased level of both Runx2 and Osx protein expression represented by fading blot on the top of each graph. Interestingly, both Runx2 and Osx expression were at peak in hFOB 1.19 cells treated with combined treatment of *QI*sm-F with pamidronate.

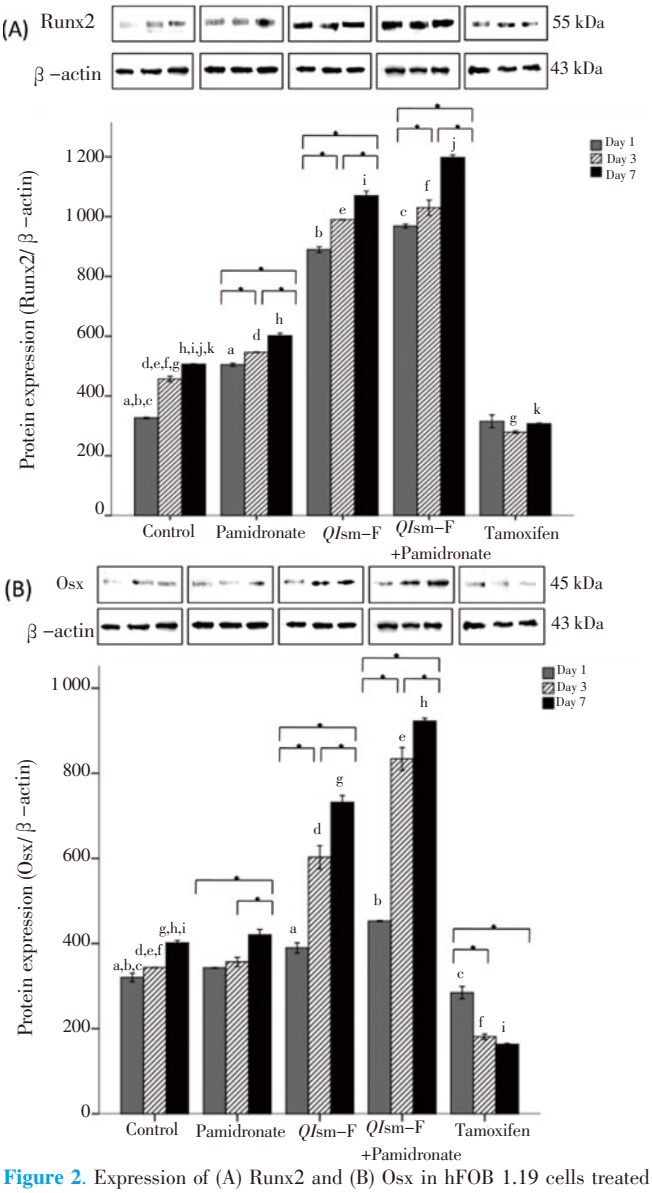

Figure 2. Expression of (A) Runx2 and (B) Osx in hFOB 1.19 cells treated with respective treatment groups at Day 1, 3 and 7 by western blotting.

The graph represents the quality density value based on ImageJ software with relevant β -actin as control. The blot at the top of each graph shows that Runx2 and Osx levels gradually increase with time in hFOB 1.19 cells treated with pamidronate, *QI*sm-F and combined *QI*sm-F with pamidronate. The blot at the bottom of each graph shows the expression of control protein β -actin. Data are shown as mean±SEM of three independent experiments. * indicates significant difference among three different days within the same group ($P<0.05$) whereas days that share similar letters show significant difference ($P<0.05$), with different groups and similar days by repeated measure ANOVA and *post hoc* test.

3.3. Up-regulated Runx2 and Osx during osteoblast differentiation of hFOB 1.19 cells

The image (Figure 3 & Figure 4) shows rapid proliferation and enhanced expression of Runx2 and Osx in hFOB 1.19 cells treated with combined *QI*sm-F with pamidronate at day 3 and day 7.

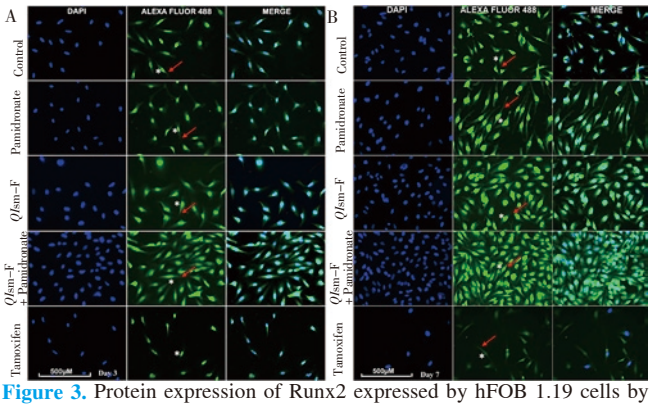

Figure 3. Protein expression of Runx2 expressed by hFOB 1.19 cells by respective treatment groups at (A) Day 3 and (B) Day 7 of treatment by immune-fluorescence staining (magnification 200 ×).

The nuclei were stained with DAPI (blue staining).

→indicates cytoplasmic spreading; *indicates nuclear expression.

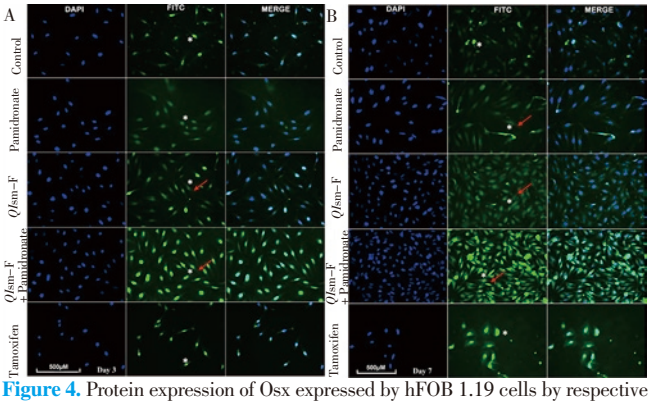

Figure 4. Protein expression of Osx expressed by hFOB 1.19 cells by respective treatment groups at (A) Day 3 and (B) Day 7 of treatment by immuno-fluorescence staining (magnification 200 ×).

The nuclei were stained with DAPI (blue staining).

→indicates cytoplasmic spreading; *indicates nuclear expression.

4. Discussion

As stated earlier, osteoporotic drug bisphosphonate mostly function as a resorptive inhibitor. The mechanism action of these drugs leads to decrease in important bone marker as well as exhibits negligible ability to enhance new synthesis of bone[34,35]. Hence, it is necessary to investigate other potential anabolic agent that can helps to repair the imbalance of the bone remodelling process. Natural anabolic agents from plant sources could be effective for use in the treatment of osteoporosis. Polyphenols, which is a major compound manifestation in *Q. infectoria* has been reported in the study's preliminary investigation to contribute to increasing in bone marker that plays a role in initial bone metabolism process[28]. This experiment demonstrated detailed effects of *QI*sm-F isolated from *Q. infectoria* as well as the synergistic effects of combining established *QI*sm-F with pamidronate acting on osteoblast model (hFOB1.19 cell) in comparison to individual treatments. The results shows a promising effect when combining *QI*sm-F with pamidronate whereby it enhanced osteoblast proliferation as well as expression of important bone osteoblastogenic marker that involves in osteoblast differentiation.

As mentioned earlier, there has been several reported research on the ability of different polyphenols from different types of natural plants products contributing to osteoblast differentiation and proliferation[25-27]. This study incorporates crucial solvents interaction from chromatographic technique in order to obtain desired *QI*sm-F. The presence of polyphenol in isolated *QI*sm-F was observed as black spot on thin layer chromatography plate as a results of $FeCl_3$ staining and similar R_f values when compared to standard polyphenols (gallic acid and tannins).

Cell viability assay is an essential tool to measure the number of viable cultured cells in order to determine the cytotoxicity and cell viability effects produced by the given treatments to cells[36]. the research utilises the usage of calorimetric MTT assay for determining EC_{50} of the treatments drugs and *QI*sm-F as well as measures the percentage of cell viability using the established EC_{50} concentration. Cell viability of hFOB1.19 treated with *QI*sm-F and combined *QI*sm-F with pamidronate is more than 80%; whereby, these exhibit the efficiency of *QI*sm-F and synergistic potential of combined treatment of *QI*sm-F with pamidronate as an anabolic agent that can contribute to the process of new bone formation. Combination treatment therapy between natural plants compounds with readily available drugs has been shown to exhibit synergistic effects in various diseases such as cancer; multiple myeloma[37] and prostate cancer[38]; viral infection[39], diabetic[40] and many more.

The process of bone remodelling has been illustrated and well documented to be directed by two classes of cells; bone removing cell (osteoclast) and bone forming cell (osteoblast). This study adapted osteoblast model so as to understand the mechanism of *QI*sm-F acting as well as combination treatment of readily available bisphosphonate with natural *QI*sm-F on bone forming cell for synthesis of new bone. As mentioned, osteoblast is the key component in bone multicellular unit whereby its optimal functions are influenced by many factors and pathway[41]. Important signalling pathway involves in osteoblast differentiation are the smad signalling pathway mediated by BMP-2 via differentiation mesenchymal cell to the osteoblast and the activation of osteoblastogenic transcripton factors which include Runx2, Osx, alkaline phosphatase, osteocalcin and many more[13,42]. The molecular actions of *Q. infectoria* on osteoblast cell has not been reported in any previous study. However, molecular action of polyphenols from other natural sources; as such by Bu *et al.*, stated that dried plum polyphenols enhance osteoblast activity and function by up-regulating Runx2 and Osx marker[27]; meanwhile Dai and colleagues, also reported up-regulation of the same marker in human bone marrow-derived mesenchymal stem cell model triggered by resveratrol; a type of naturally occuring phenols[41]. Our study, considers these two important osteoblastogenic marker (Runx2 and Osx) controlling osteoblast differentiation through smad signalling pathway which leads to formation of new bone forming cells. From the data indicated, the analysis of both Runx2 and Osx expression by western blot analysis shows elevated expression in all treatment

groups except tamoxifen in a time dependent manner. However, the highest expression of Runx2 and Osx marker is expressed in cells treated with combination of *QI*sm-F and pamidronate; particularly at day 7 of treatments. This study implies that the combination of the studied drugs and natural anabolic agent is a more efficient treatment on stimulating the proliferation and differentiation of hFOB1.19 cells when compares to each individual treatment. In addition, this study also points out the biological effects of polyphenols presence in *QI*sm-F on the hFOB 1.19 cell model. Thereby, it might provide a new insight in osteoporotic treatment discovery.

Immuno-fluorescence staining against similar osteoblastogenic marker Runx2 and Osx supported the finding of both protein expressions. Through immuno-fluorescence staining, the research could then observe the proliferation rate, cell morphology together with the expression of both markers. The images show that the rate of cells proliferation increases from day 3 to day 7 in all treatment groups except tamoxifen and are at peak in cells treated with the combination of *QI*sm-F with pamidronate treatment. Moreover, the cells supplied with combination treatment were observed to channel higher intensity of fluorescence staining expressed in the nuclei of the cells and spread to the cytoplasm; along with well attached character and elongated shape as well as appear to be overlapping on each other due to rapid cell proliferation which evidently shows heightened expression of Runx2 and Osx. Meanwhile, the rate of proliferation and expression of Runx2 and Osx in hFOB 1.19 cells treated with individual treatment; *QI*sm-F or pamidronate respectively has modest effect on hFOB1.19. Based on the data collected, the interaction of both compounds (*QI*sm-F and pamidronate) acts synergistically with each other and is proven to be a more effective treatment choice than individual treatment.

Throughout the years, the process of drug discovery utilizes the information obtained from natural product researches which are then being developed as well as incorporated towards discovering new drugs by combining the chemical structure with existing drug[43]. Hence, it tends to be authentic that the data obtained from this research could be used to develop and adapt for discovery of new improved osteoporotic drug with minimal risk of side effects to human.

The effect of *Q. infectoria* as astringent, antidiabetic, antitremorine and local anaesthetic are well documented, nevertheless its effect on bone cells and associated disease are relatively still scarce. This research shows conclusive evidence that combination of these two agents acts synergistically by up-regulating both Runx2 and Osx marker via smad signalling pathway. Overall, the paper concluded that combination treatment between *QI*sm-F and pamidronate is effective treatment candidate for bone associated disease particularly osteoporosis than individual treatments; at the same time helps to improve the efficiency of osteoporotic drug pamidronate acting on bone forming cell.

Conflicts of interest statement

The authors declare that they have no conflict of interests.

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