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Nano drug delivery system based- application of *Morinda officinalis* How. : preparation, characterization, and *in vitro* calcium mineralization activity

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***Abstract*—*Morinda officinalis* How. has long been known as a valuable source of medicinal herbs and has shown many pharmacological activities such as immunomodulatory, anti-inflammatory and analgesic, antioxidant, particularly in anti-osteoporosis. However, the low solubility in water of many active pharmaceutical substances in *M. officinalis* (MO) limits their therapeutic effects. Nano drug delivery system is an optimal solution to increase the effectiveness of poorly water-soluble active ingredients as well as optimize their pharmacological effects. In this work, we developed a herbal nanoparticle formulation (nano-MO) based on biologically active substances from MO. Hydrophobic compounds of MO ethanolic extract were encapsulated by natural polysaccharides in MO aqueous extract. Nano-MO had a uniform spherical shape with the obtained nanoparticle size in the range of 60 - 80 nm. Moreover, nano-MO had shown the ability to induce osteoblast differentiation and anti-osteoporosis at a statistically significant level ($p < 0.05$) when enhancing calcium mineralization activity. These superior *in vitro* data suggest that this nanoformulation may be an effective therapeutic modality for osteoporosis treatment.**

Keywords*—*Morinda officinalis; anthraquinone, calcium mineralization activity, nano drug delivery system

I. INTRODUCTION

Morinda officinalis How. is a herbaceous, perennial, climbing plant, which has been known as a valuable source of medicinal herbs for a long time. Modern biological and pharmacological studies have shown that *M. officinalis* (MO) has many important pharmacological effects. These profound effects including (1) protection of spermatozoa from harmful agents such as oxidative stress on human sperm [1]; cytoxan-induced defects in male rats [2]; (2) anti-fatigue and enhance physical strength [3]; (3) immunomodulatory effect by regulating the conversion of T and B lymphocytes as well as prolonging the lifespan of lymphocytes, and also increasing the thymus gland; (4) anti-depressant and anti-stress [4]; (5) central nervous system protection [5]; (6) anti-inflammatory and analgesic [6]; (7) hypoglycemic and antioxidant in a mouse model of diabetes mellitus [7]. Especially, in recent years, the supporting effect of osteoporosis prevention of the active ingredients in three sizes has been published in many studies both in vitro and in vivo. For example, an in vitro study on rat bone marrow cells showed that substances belonging to the anthraquinone group from three sizes were able to stimulate proliferation of osteoblasts [8]. Meanwhile, some other compounds in this group help reduce bone loss by inhibiting osteoclasts [9]. Specifically, eight anthraquinones isolated from *M. Officinalis* include Physcion (1); rubiadin - 1 - methyl ether (2); 2 -hydroxy - 1 - methoxy - anthraquinone (3); 1,2 - dihydroxy - 3 - methylanthraquinone (4); 1,3,8 - trihydroxy - 2 - methoxy - anthraquinone (5); 2 - hydroxymethyl - 3 - hydroxyanthraquinone (6); 2 - methoxyanthraquinone (7) and scopoletin (8). These substances all have the effect of inhibiting osteoclasts, anthraquinone 2,3 has the effect of stimulating the proliferation of osteoblasts, while substance 4, 5 has the effect of stimulating the activity of osteoblasts [10].

Furthurmore, polysaccharide present in *M. Officinalis* has the ability to stimulate the expression of genes involved in bone expression [10] and help reduce bone loss [11]. *In vivo* studies also confirmed that the compound monotropein extracted from three

cherries increased bone formation and prevented bone loss in mice [12].

However, the considerable disadvantage of anthraquinone is that it is difficult to dissolve in water, while iridoid small molecules are easily eliminated from the body. Therefore, it is necessary to have an appropriate drug delivery system that increases the solubility and absorption of the anti-osteoporotic anthraquinones and iridoids from *M. Officinalis.* Nanotechnology is a promising solution to this problem [13]. Nano drug delivery system is the most powerful application area in pharmaceutical technology, which is contributing to the creation of many preparations with superior absorption characteristics, expanding therapeutic efficacy, especially helping to develop drug forms that act at the target, effectively treat many diseases and reduce the side effects of drugs. Nanoparticle delivery systems consist of drugs combined with carriers to form nanoparticle structures. The carriers used can be polymers, micelles, dendrimers or metal-based, ceramics, proteins, viruses, and liposomal particles [14]. Drugs or therapeutic agents will be inserted into, chemically bonded or attached to the surface of these particles [15]. Nanoparticles (especially nanoparticles with pharmaceutical substances attached to carriers) easily pass through cells, penetrate into the blood, intracellular system, liver, bone marrow, intestinal membrane, mucosal layer, ... This is particularly important for drugs with poor biopharmaceutical properties such as poor cell epithelial permeability and poor water solubility [16].

In this work, we have applied nanotechnology and engineering to fabricate a nano drug delivery system carrying active ingredients/active groups in the roots of *M. Officinalis*. In this nanosystem, the anthraquinone active ingredients in the condensed ethanolic extract were encapsulated by the polysaccharides themselves extracted from the *M. Officinalis* aqueous extract. The use of these polysaccharides to create a nano-drug delivery system containing anthraquinones helps to fully exploit the medicinal resources and maximize the anti-osteoporotic effects of *M. Officinalis*.

II. MATERIALS AND METHOD

A. *Materials*

Morinda officinalis How. was harvested in Quang Ninh province, Vietnam. Rubiadin, Fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO) were purchased from Sigma. Ethanol, methanol, chloroform, magnesium acetate, glacial acetic acid, Trypsin - EDTA, hydrochloric acid, β-glycerophosphate, ascorbic acid, 3- (4,5- dimethylthiazol - 2- yl) - 2,5 - diphenyl tetrazolium bromid (MTT), n-butanol were purchased from Merck. Distilled water was used in all experiments. The mouse osteoblast cell line MC3T3-E1 was provided by the ATCC cell bank (Manassas, Virginia, USA).

B. *Experimental section*

Extraction of active ingredients of M. officinalis

After the core is removed, the roots of *M. officinalis* (MO) are dried and ground into a fine powder. 100 grams dry powder of MO was dispersed in 1L of ethanol, under 70^{o}C conditions for 24 hours. After that, the ethanol extract was evaporated under vacuum until became cataplasm (moisture < 20%), and dried at 60 - 70^{o}C. The medicinal residues, after being extracted with ethanol, were left to dry naturally to completely evaporate the ethanol, then put into a reflux extraction vessel, extracted 4 times with distilled water, filtered to obtain the aqueous extract. Remove the proteins by precipitating them with a mixture of n-butanol-chloroform that has a v/v ratio of 1:4, centrifugation at 8000 rpm for 15 minutes to remove the precipitate. Re-dissolve in distilled water, remove impurities by anion exchange chromatography. The obtained filtrate was precipitated overnight with cold ethanol, and dried to obtain an condensed aqueous extract containing polysaccharides and oligosaccharides.

Fabrication of Nano M. officinalis (Nano-MO)

The active ingredients in condensed ethanol extract were encapsulated into the nano-MO system by solvent-evaporation method. First, the aqueous extract from the roots of MO (containing polysaccharides and oligosaccharides) was dissolved in distilled water, in which the ratio of the aqueous extract to distilled water was 1:1 (w/v). Then, the anthraquinone-rich ethanol extract fraction was dissolved in absolute ethyl alcohol, in which the ethanol extraction : ethanol solvent ratio was 1: 1 (w/v). Next, slowly drop the obtained solution (at the rate of 0.5mL/min) into the reaction vessel containing the aqueous extract from the roots of MO at a ratio of 1: 1 (v/v). The mixture was kept stirring in a close flask at 600 rpm for 48 hours. Ethanol was evaporated under vacuum. The final transparent solution (nano-MO) was obtained by centrifugation at 6800 rpm to remove unencapsulated actives and then stored at 4 °C for further use.

C. *Characterization of Nano-MO system*

Molecular structure of synthesized copolymer was characterized by Fourier transform infrared spectroscopy (FTIR, SHIMADZU spectrophotometer) using KBr pellets in the wave number region of 400–4000 cm-1. Surface morphology of nano-MO particles was fi microscopy (FE-SEM) on a Hitachi S-4800 system. The average size and size distribution of nanosystem were measured by Dynamic Light Scattering (DLS).

Quantification of total anthraquinone by UV-VIS absorption spectroscopy. Anthraquinone quantification was carried out according to the principle that anthraquinone was chelated with a 0.5% solution of magnesium acetate in methanol. Determination of total anthraquinone concentration through chelating complex concentration by UV-VIS absorption spectroscopy.

Preparation of the test solution

The nano-MO system was extracted by Soxhlet with 150ml of 80% ethanol to exhaustion, evaporated, and collected. 10ml of a mixture of glacial acetic acid and 25% hydrochloric acid (in the ratio v/v is 10:21) added and sonicated for 5 minutes. Then add 10 ml of chloroform solution, reflux for 30 minutes, and cool. The resulting solution is decanted to get the chloroform fraction, and shaken 3 times with chloroform, each time 5ml. The extracts were then collected and the solvent was evaporated to collect a precipitate. The precipitate obtained was dissolved back into a 25 ml volumetric flask with 0.5% magnesium acetate solution in methanol just enough to obtain the test solution.

Preparation of standard solution

Rubiadine standard solution: 1 mg rubiadin was dissolved in 10 ml of methanol, sonicated until completely dissolved, obtaining a standard solution with a concentration of 0.1 mg/ml. From the 0.1 mg/ml rubiadine stock standard solution, a series of volumes including 0.2 ml; 0.4ml; 0.6ml; 0.8ml; 1ml is prepared into beakers, then evaporated. The precipitate obtained was then re-dissolved with 0.5% magnesium acetate solution in methanol and transferred to a 5 ml volumetric flask. Finally, a series of standard solutions are obtained by adding just enough 0.5% magnesium acetate solution in methanol. The absorbance of the rubiadine standard solution series at 501 nm was determined, with 0.5% magnesium acetate solution in methanol as the blank for the calibration of the calibration curve [17].

Determination of total anthraquinone content and encapsulation efficiency

The content of anthraquinone in the sample was quantified by measuring the UV-VIS absorption of the solution after dissolving the nano-MO system in 0.5% magnesium acetate solution in methanol at 501 nm and calculating the results based on the standard curve had been built. Each experiment was repeated 3 times. The encapsulation efficiency was calculated according to the following formulas:

$$\mathrm{EE}(\%) = \frac{\text{the weight of loaded anthraquinone}}{\text{the initial weight of total anthraquinone}} \times 100(\%) \quad (1)$$

D. Determination of the ability to in vitro stimulate mineralization

In vitro cell culture

MC3T3-E1 cells were cultured as monolayer in alpha-MEM culture medium supplemented with 10% Fetal bovine serum (FBS), 1% PS antibiotic at 37°C, and 5% CO_2. Cells were cultured with Trypsin - EDTA (0.05 %) after 2 days of culture. To determine the ability of MC3T3-E1 to differentiate into osteoblasts, cells, after covering 80% of the culture surface, were grown in differentiation medium supplemented with 10 mM β-glycerophosphate and 50 µg/mL ascorbic acid.

Biological test to determine the growth of cells under the influence of research samples

The MTT method was used to determine the growth of MC3T3-E1 cells under the influence of the studied sample. This method determines the cell growth through the formation of colored formazan products when MTT is introduced into the cell wells under the influence of enzymes in living cells. MC3T3-E1 cells were placed in a 96-well plate at a concentration of 1x104 cells/well. Cells in the dish were incubated with reagent (10 µl) mixed in 10% DMSO to a concentration of 100 µg/ml, 20 µg/ml; 4 µg/ml; 0.8 µg/ml. Cell wells with only sample diluent were used as negative controls. Then the plate of experimental cells was incubated in 37oC incubator, 5% CO2 for 3 days. Next, 20 µl MTT (5 mg/ml) was added to each well, incubated at 37°C for 4 h. The supernatant was removed, 100 l DMSO was added to each well to dissolve the formazan color formed, put on a plate shaker to shake gently for 10 min and used the ELISA Plate Reader (Bio-Rad) to read the results at wavelength 490. nm. The viability of cells in the presence of reagents will be determined through the following formula:

$$\%\, survival = \frac{OD_{sample}}{OD_{negative\ control}}\ x\ 100\ (\%) \quad (2)$$

Determining the ability to stimulate calcium mineralization

The ability to create minerals under the influence of nano-MO system was determined by the Anizarin red S staining method. After 15 days of incubation, cells were washed twice with PBS and fixed with 70% ethanol for 1 h. Cells were then stained with 40 mM alirazine Red S (pH 4.4) for 15 min and rinsed with deionized distilled water. The calcium-binding dye was dissolved with 10% cetylpyridium chloride and gently shaken for 15 min. Optical density was measured at 561 nm using a Microplate Reader (Biorad). The ability to stimulate mineralization under the influence of the experimental sample was compared with the negative control.

$$\%\, effect = \frac{OD_{sample}}{OD_{negative\ control}}\ x\ 100\ (\%) \quad (3)$$

The data are processed on Excel, presented as mean ± SD. Statistical algorithms Student's t-test, F'test and one way ANOVA method to test the significant difference compared with negative control, with P<0.05 considered a statistically significant difference.

III. RESULTS AND DISCUSSION

A. Structural and morphological characteristics

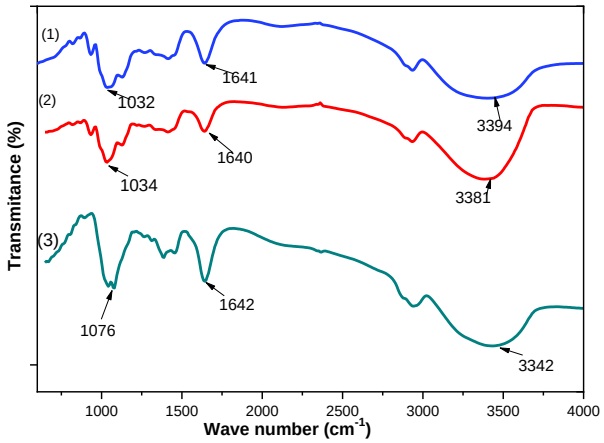

Fig 1. FT-IR spectra of (1) MO aqueos extract, (2) MO ethanolic extract and (3) Nano-MO system

Figure 1 illustrates the FT-IR spectrum of the aqueous extract, the ethanol extract from the roots of MO, and the nano-MO system. It can be seen that some characteristic absorption peaks of the materials (aqueous extract, ethanol extract of MO) were shifted when nanochemically applied in the product. Specifically, the characteristic oscillations of the O-H bonds of the aqueous extract (3394 cm^{-1}) and the ethanol extract (3381 cm^{-1}) were shifted in the spectrum of the products (3432 cm^{-1}). Similarly, the C=O double bond in the aqueous extract (1641 cm^{-1}) and the ethanol extract (1640 cm^{-1}) also changed the wave number when appearing in the spectrum of the nanoproduct (1642 cm^{-1}). In addition, the slight change of the C-O-C fluctuations of the starting substances (aqueous extract 1032 cm^{-1}, ethanol extract 1034 cm^{-1}) compared with that of the product (1076 cm^{-1}) also contributes to demonstrate the change in structure of composition in the product relative to the starting material. The shift of the peaks in the spectrum of the nanoproducts compared with the spectrum of the original compounds representing the nanochemistry of the active ingredients in the ethanol extract from the root of MO by the aqueous extract occurred.

The morphology and particle size of the nano-MO particles were evaluated by FESEM and shown in Fig. 2. The size of the nano-MO particles is quite small and uniform, spherical in shape and uniformly distributed with a diameter in the range of 60-80 nm.

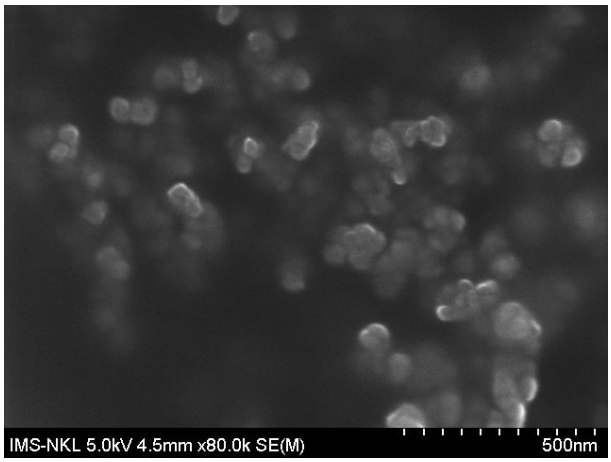

Fig. 2. FESEM image of nano-MO system

Thanks to the nano size, this nano-MO system has good solubility in biological fluids, absorbed through the cystic spaces in the gastrointestinal mucosa to enter the vascular system, protecting the drug safely when passing through the liver, at the same time, not being eliminated too quickly from the circulatory system. As a result, the therapeutic effect of the drug increases significantly, while the dose used is lower than usual.

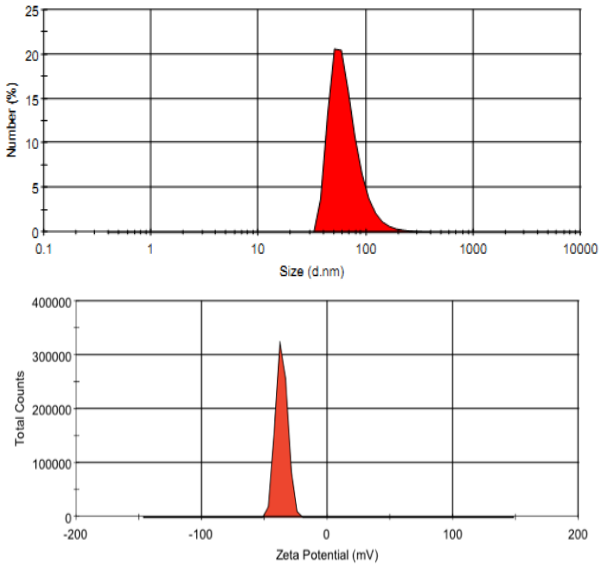

Fig. 3. Size distribution and zeta potential of nano-MO system

The size distribution results according to the DLS method (Figure 3) also confirm that the obtained nano-MO system has a narrow size distribution (PDI = 0.313), the average size was about 77 nm. The zeta potential is the sum of the charge that the particles are achieved in the dispersed environment. It characterizes repulsive force between particles. Particles with absolute value zeta value greater than 30 mV are considered a stable system. When the zeta value is between 20-30 mV, the system is considered to be relatively stable. In the case of zeta potential less than 10mV, the system is considered unsustainable. In which, the nano-MO system was evaluated to be quite

stable through the negative zeta potential value of -36.2 mV.

B. Determination of anthraquinone content and encapsulation efficiency

The total anthraquinone nano-MO system was quantified by UV-VIS absorption spectroscopy.

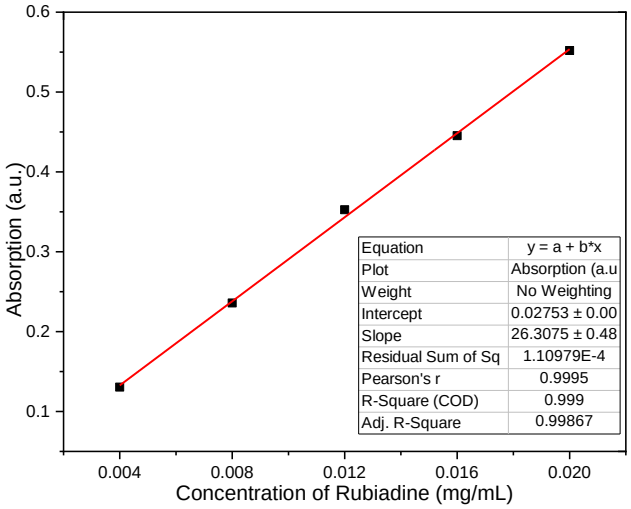

Fig. 4. Standard curve showing the relationship between concentration and absorbance of Rubiadine

The concentrations of Rubiadine in solutions were determined based on the equation obtained from the calibration curve (Y= 26.3075X + 0.02753, r2 = 0.99867). The results indicated that the total anthraquinone content in nano-MO system calculated according to Rubiadin is 177.481 ± 1,958 µg/g, with the corresponding encapsulation efficiency of 85.6% (calculated by equation (1)).

C. Determination of the ability to in vitro stimulate mineralization on MC3T3-E1 cells

MC3T3-E1 pro-osteoblasts are cells capable of differentiating into osteoblasts. During cell differentiation, MC3T3-E1 cells exhibit osteoblast-specific features such as production of alkaline phosphatase, enhanced synthesis of collagen, which is the extracellular matrix from procollagen and finally mineralization on the extracellular matrix [18]. Therefore, this cell line is widely used in the world to evaluate the anti-osteoporosis ability of research samples. In particular, the ability to enhance mineralization is a characteristic sign for the ability to fight osteoporosis [19].

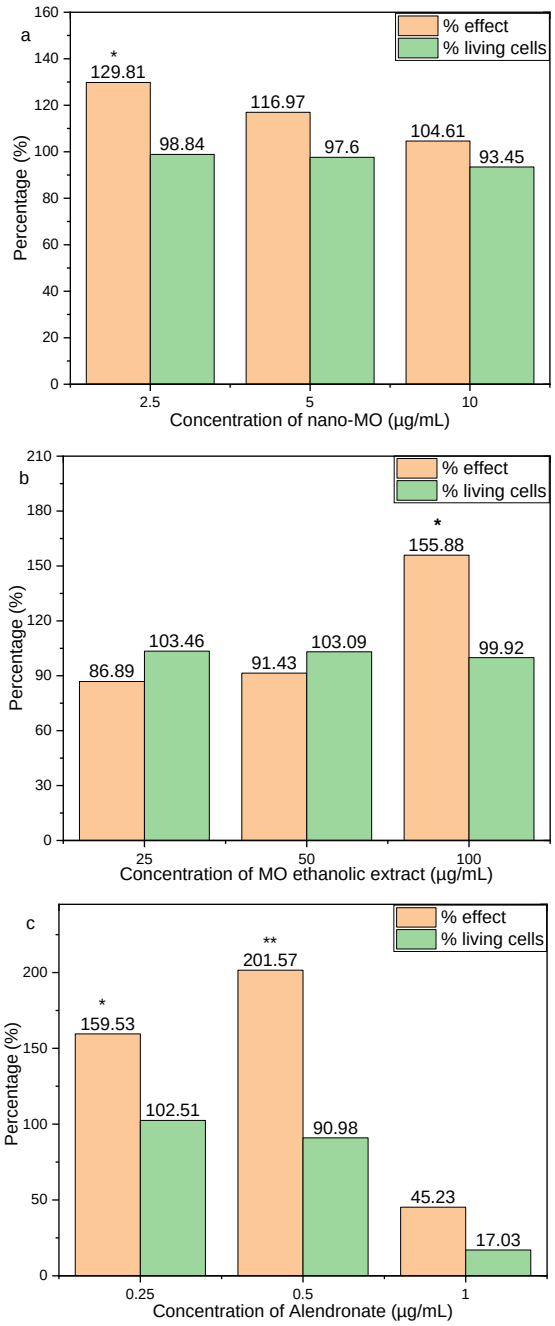

*Fig. 5. Effects of (a) nano-MO system, (b) MO ethanolic extract, and (c) Alendronate positive control on calcium mineralization (** is the difference at the level of statistical significance with P<0.01; *is the difference at the level of statistical significance with P<0.05)*

Calcium mineralization activity was enhanced at a statistically significant level under the influence of nano-MO system at a concentration of 2.5 µg/ml (P<0.05) with an activity level of 129.81%, and did not show statistically significant activity at the remaining studied concentrations (Fig.5a). Besides, Fig.5b shows that calcium mineralization activity was enhanced at a statistically significant level under the influence of MO ethanolic extract at a concentration of 100 µg/ml (P<0.05) with an activity level of 155.88%, and did not show activity at a statistically significant level at other concentrations studied. Compared with the nano-MO

system, MO ethanolic extract exhibited statistically significant calcium mineralization activity at concentrations 40 times higher. This demonstrates that the nano-MO system exhibits better calcium mineralization activity when the active substances in the roots of MO are encapsulated and nanoscaled. Additionally, our data suggest MC3T3-E1 pro-osteoblasts cells differentiate with alendronate treatment. For the positive control sample, the mineralization activity was enhanced at a statistically significant level under the influence of Alendronate at all studied concentrations ($P<0.05$) with an activity level of 109.14 - 116.74% (Fig. 5c). Our findings concur with those of a previous study, in which it was suggested alendronate potentiated calcitriol-stimulated mineralization of human osteoblastic cells at 10^{-8} – 10^{-7} M, but these effects were not observed at 10^{-6} and 10^{-5} M [20].

IV. CONCLUSION

In this study, we have successfully designed and fabricated a nano drug delivery system based on active compounds from the roots of MO. The nano-MO system had a spherical shape, uniform distribution, with size from 60 to 80 nm. The obtained nano-MO system exhibited better in vitro calcium mineralization activity than the ethanol extract when the active ingredients were not nanoscaled, at equivalent doses 40 times lower. However, our study only provides clues as to how this is achieved at in vitro assay. Therefore, additional investigations at in vivo model in odrer to stimulate calcium mineralization or inhibit osteoclast activity as well as its application in the treatment of osteoporosis.

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