

Anti-osteoclastogenesis Potential Agents from Plants Naturalized in Vietnam

Thanh Huong Le, Thu Trang Duong¹, Phuong Thao Tran², Van Cuong Pham¹, Hai Dang Nguyen, Jeong-Hyung Lee²

University of Science and Technology of Hanoi, ¹Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam, ²Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon, Gangwon-Do 24341, Republic of Korea

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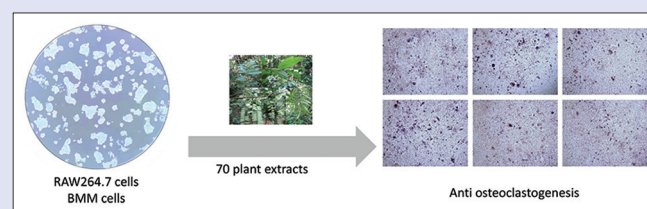
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ABSTRACT

Background: The balance between bone formation and bone resorption which is attributed to osteoblast and osteoclast is required to maintain skeleton homeostasis. Osteoclast differentiation is regulated by a tumor necrosis factor–receptor activator of nuclear factor NF- κ B ligand (RANKL). The dysregulation of bone-resorbing osteoclast differentiation can lead to osteoporosis. The adverse effects of the long-term use of bone resorption inhibitors are of concern and so the development of new osteoporosis therapy treatment is desirable. **Objective:** In this study, 67 plants (70 samples) were screened for osteoclastogenesis inhibitory activities on RAW264.7 mouse macrophages and bone marrow-derived macrophages (BMMs). **Materials and Methods:** The RAW264.7 cells and the BMMs isolated from male ICR mice were treated with various doses of plant extracts and TRAP histochemical staining of the cells was performed. TRAP-positive multinucleated cells were photographed under microscopy to observe the effects of the extracts on osteoclast differentiation. **Results:** Among 70 methanol extracts, we found that nine samples exhibited significant inhibitory effects in RANKL-induced osteoclast differentiation. They included *Aleurites moluccana* (S16 and S17), *Aporosa dioca* (S19), *Antidesma bunioides* (S21), *Cinnamomum balansae* (S32), *Macrosolen cochinchinensis* (S41), *Pinus kesiya* (S52), *Photinia benthamiana* (S54), and *Mischocarpus pentapetalus* (S59). **Conclusion:** In the present study, 70 plant extracts were screened for the osteoclastogenesis inhibitory effects in RAW264.7 murine macrophages and BMMs. Nine extracts have the potential as effective agents against osteoclastogenesis. This is the first report on the anti-osteoclastogenic activity of these plants. **Key words:** Bone marrow-derived macrophage, osteoclast differentiation, osteoclastogenesis, osteoporosis, plant extract

SUMMARY

• Sixty-seven plants (70 samples) from Vietnam were collected and screened for osteoclastogenesis inhibitory activities on RAW264.7 murine macrophages and bone marrow-derived macrophages isolated from mice. For the first time, nine extracts showed potential as effective inhibitors of osteoclastogenesis.



Abbreviations used: RANKL: Receptor activator of nuclear factor NF- κ B ligand; S: Stems; L: Leaves; SL: Stems + leaves; BL: Boughs + leaves; F: Flowers; C: Cortex; FtF: Flowers + fruits; R: Roots, BMM: Bone marrow-derived macrophages.

Correspondence:

Dr. Hai Dang Nguyen,
University of Science and Technology of
Hanoi, Vietnam Academy of Science and
Technology (VAST), 18 Hoang Quoc Viet, Cau Giay,
Hanoi, Vietnam.
E-mail: nguyen-hai.dang@usth.edu.vn
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INTRODUCTION

Bone development and homeostasis are dependent on the balance of bone formation and resorption process. The osteoblasts and osteoclasts are considered the major effectors' cells of these two processes. The imbalance between bone formation and resorption can cause abnormal bone conditions: too much bone formation (osteopetrosis) or too little bone (osteoporosis).^[1] While osteopetrotic diseases are rarely found in humans, osteoporotic diseases are one of the major bone health problems of today's society. Approximately 50% of women suffer from osteoporotic fractures throughout their lifetime.^[2]

Medicaments such as bisphosphonates are widely prescribed and highly effective at many disorders characterized by increased osteoclast-mediated bone resorption. Treatment with bisphosphonates may ameliorate the bone loss; however, there are many side effects are recorded. Short-term side effects of bisphosphonate therapy may include upper gastrointestinal effects, acute-phase reaction, severe musculoskeletal pain, hypocalcemia, risk of esophageal cancer, and ocular inflammation. Long term of using bisphosphonates associated with jaw osteonecrosis, atrial fibrillation, severe suppression of bone turnover, or subtrochanteric femoral fractures.^[3] For women

in menopause, estrogen therapy is preferred due to declining estrogen levels. The adverse effects resulting from estrogen therapy include endometrial cancer, hypertension, gallbladder disease, angina pectoris, and breast cancer.^[4] The side effects recorded due to the long-term use of these medicines raising the concern of the patients. Therefore, the development of new anti-osteoporotic drugs is recommendable.

In this study, 67 Vietnamese plants (70 methanol extracts) were collected and screened for the inhibitory activities of osteoclast differentiation in RAW264.7 murine macrophages and further confirmed on bone

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marrow-derived macrophages (BMMs) isolated from mice. These plants are distributed in the forests of Northern Vietnam.

MATERIALS AND METHODS

Plant materials

A total of 67 plants were collected in Cuc Phuong National Reservation Park, Ninh Binh, Vietnam in January 2019. The plant samples were identified by Dr. Nguyen the Cuong, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. Voucher of specimens was deposited at the Department of Life Sciences, University of Science and Technology of Hanoi

Preparation of extracts

The collected plants were classified into different parts: Stems (S), leaves (L), stems + leaves (SL); boughs + leaves (BL), flowers (F), cortex[®], flowers + fruits (FtF), and roots[®]. The samples were then dried and cut into pieces before extracting with MeOH for 20 min, three times 15 min in an ultrasonicator. The extracts were concentrated under the vacuum evaporator to yield the corresponding residues. The extracts were stored at -10°C until used.

Cell culture

RAW264.7 cells (murine macrophages) were obtained from the American Type Culture Collection (Manassas, VA, USA). Raw 264.7 culture, BMMs isolation and culture, as well as Assay for *in vitro* differentiation of osteoclasts and TRAP staining, were previously described.^[5]

Cell viability assay

Cell proliferation and cytotoxic effect of the extracts were assessed using MTT-based assay. At the end of incubation, 0.5 mg/ml MTT solution was added to each well and incubated for 3 h. After the removal of the culture supernatants, formazan crystals were dissolved in dimethyl sulfoxide. Absorbance was measured at 540 nm using a microplate reader.

Statistical analysis

Data are expressed as mean ± standard error. Statistical significance was assessed using Student's *t*-test. In all analyses, *P* < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In the present study, we screened 67 plant extracts divided into 70 samples [Table S1] for the inhibition of osteoclast differentiation in RAW264.7 murine macrophages. The RAW264.7 murine cell line has proven to be an important primary osteoclast precursor for *in vitro*

studies of osteoclast formation as it can differentiate into osteoclasts upon receptor activator of nuclear factor NF-κB ligand (RANKL) co-culture.^[6] Among the tested samples, 53 methanol extracts exhibited the negative effects on osteoclastogenesis inhibition and 8 methanol extracts revealed the toxicity effects on RAW264.7 cells at the concentration of 25 µg/ml. Nine extracts including *Aleurites moluccana* (L.) Willd. (S16 and S17), *Aporosa dioca* (Roxb.) Muell.-Arg. (S19), *Antidesma buniis* (L.) Spreng. (S21), *Cinnamomum balansae* Lecomte (S32), *Macrosolen cochinchinensis* (Lour.) van Tiegh. (S41), *Pinus kesiya* Royle ex Gordon (S52), *Photinia benthamiana* Hance var. benthamiana (S54) and *Mischocarpus pentapetalus* (Roxburgh) Radlkofer (S59) inhibited the osteoclast differentiation of RAW264.7 cells in dose-dependent manner [Figure S1].

Among the positive samples, the extracts at the concentration of 25 µg/ml, except S21, exhibited the strongest effect with the suppression of osteoclastogenesis by 75%–100% compared to the control [Table 1]. S21 displayed a moderate effect with <75% osteoclastogenesis inhibition. Treatment of cells with a higher concentration of extracts (50 µg/ml) was also performed; however, some samples S54 and S21 resulted in the toxicity to the tested cells as no osteoclast cells were founded (data not shown).

Next, based on the obtained results, nine positive samples were selected to further study in BMMs. As shown in Figure 1, all plant extracts dose-dependently inhibited RANKL-induced osteoclastogenesis in the BMMs. The extracts S21, S52, S54, and S59 showed stronger effects as they were almost completely inhibited RANKL-induced multinucleated mature osteoclasts at very low concentration [5 µg/ml, Figure S2]. Noteworthy, the sensitivity of the assays can be varied along with the difference between the origins of two cell types: BMMs are primary cells isolated from bone marrow whereas RAW264.7 cells are monocyte-lineage cell lines. RAW264.7 cell line does not always reflect the normal physiology of a real cell-like BMM. The signaling cascade of the cellular mechanism of RANKL-induced osteoclasts and the bone resorptive activity of RAW264.7 cells are somewhat different from RANKL-induced osteoclastogenesis in BMMs. For example, the proliferation and osteoclasts formation in RAW264.7 is independent from M-CSF.

Euphorbiaceae-a large family with many members have been used in folk medicines of many countries-stood out among the positive samples with 4 potential candidates. Some plants of Euphorbiaceae have been shown as the promising treatment of bone diseases related to excessive bone resorption.^[7,8]

The activities of the plants were mostly contributed by their active principles. Previous study showed that quercetin was one of the chief components of *M. cochinchinensis* (S41).^[9] This compound has been

Table 1: The positive effects of the plant extracts in the inhibition of osteoclastogenesis in RAW264.7 cells

Samples	Family	Name	Effects		
			5 (µg/ml)	10 (µg/ml)	25 (µg/ml)
S16	Euphorbiaceae	<i>A. moluccana</i> (L.) Willd.	*	**	***
S17	Euphorbiaceae	<i>A. moluccana</i> (L.) Willd.	*	*	***
S19	Euphorbiaceae	<i>A. dioca</i> (Roxb.) Muell.Arg.	*	***	***
S21	Euphorbiaceae	<i>A. buniis</i> (L.) Spreng.	*	*	**
S32	Lauraceae	<i>C. balansae</i> Lecomte	*	*	***
S41	Loranthaceae	<i>M. cochinchinensis</i> (Lour.) van Tiegh.	*	*	***
S52	Pinaceae	<i>P. kesiya</i> Royle ex Gordon	*	*	***
S54	Rosaceae	<i>P. benthamiana</i> Hance var. benthamiana	*	**	***
S59	Sapindaceae	<i>M. pentapetalus</i> (Roxburgh) Radlkofer	*	**	***

*, **, ***The plant extracts suppressed the osteoclastogenesis by 20%–50%; 50%–75%; 75%–100% compared to the control, respectively. Experiments were performed in triplicated. *A. moluccana*: *Aleurites moluccana*; *A. dioca*: *Aporosa dioca*; *A. buniis*: *Antidesma buniis*; *C. balansae*: *Cinnamomum balansae*; *M. cochinchinensis*: *Macrosolen cochinchinensis*; *P. kesiya*: *Pinus kesiya*; *P. benthamiana*: *Photinia benthamiana*; *M. pentapetalus*: *Mischocarpus pentapetalus*

shown to effectively reduce osteoclastogenesis and increase the new bone formation in combination in the collagen matrix.^[10,11] Further chemical studies may provide comprehensive data on the anti-osteoporotic effects of this plant. Figure 2 shows more details of the effect of S41 on RAW264.7 cells and BMMs. Data not shown for the other positive samples.

Phytochemical components of the leaves and the bark of *A. moluccana* (S16, S17) were investigated.^[12-14] However, no study about anti-osteoclastogenetic activity of these components was recorded. The studies on the extracts

of this plant suggested hypolipidemic activity and anti-inflammatory activity.^[15,16] The main components of leaf extracts from *C. balansae* were eugenol and eugenol derivatives^[17] which showed bone preserving efficacy and osteo-protective effects in alveolar bone tissues.^[18,19] The leaf essential oils of *P. kesiya* (S52) contain α -pinene, β -pinene as major constituents.^[20] Besides toxicity effects against mosquitos' larva stage, the monoterpenes are potent inhibitors of bone resorption.^[21] Chemical investigation of the stem barks of *A. dioca* (S19) revealed three compounds β -sitosterol, friedelan-3-one and 2-methyl-3-en-butyl-cyclohexyl phthalate. However,

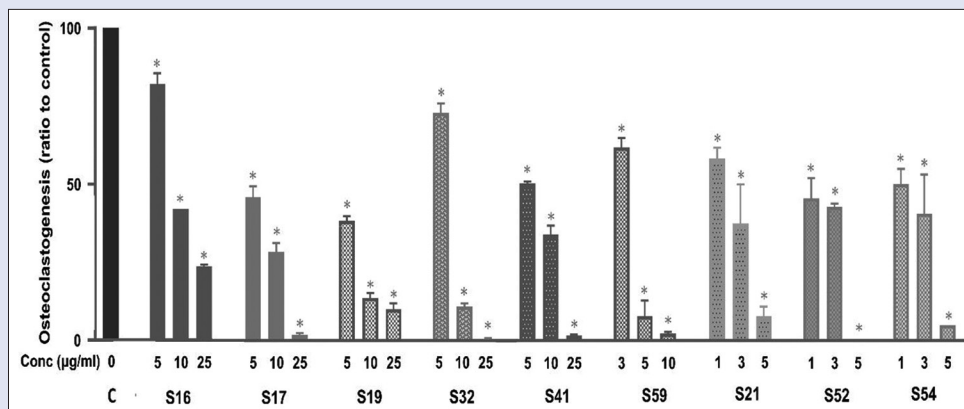


Figure 1: Plant extracts inhibit receptor activator of nuclear factor NF- κ B ligand-induced osteoclastogenesis in bone marrow-derived macrophages. Only the concentrations with significant changes in the inhibitory effects are shown

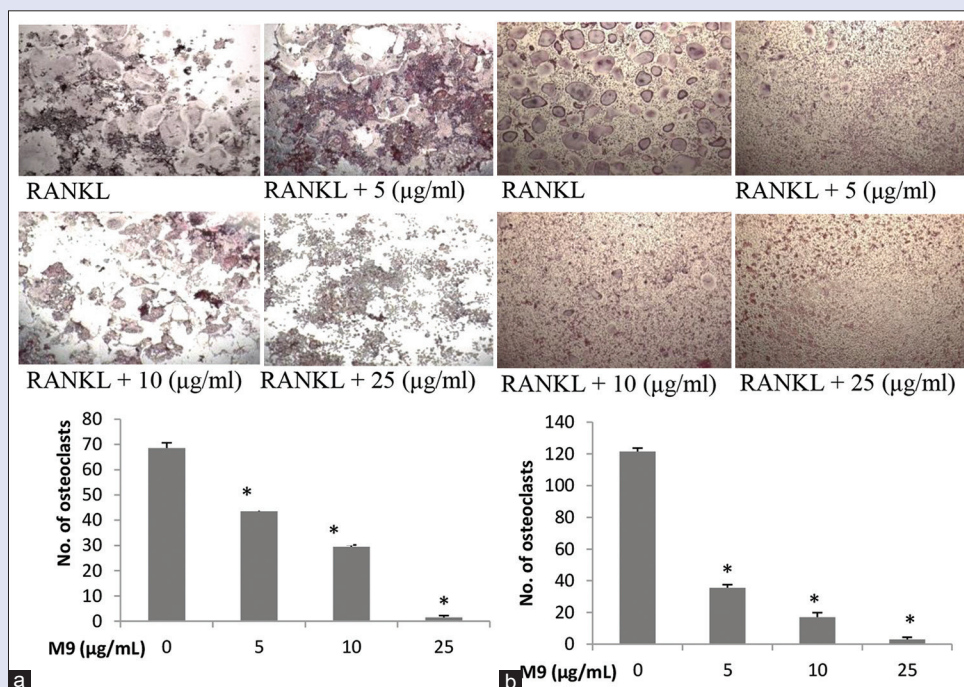


Figure 2: Extracts of sample S41 inhibits receptor activator of nuclear factor NF- κ B ligand-induced osteoclastogenesis in bone marrow-derived macrophages and RAW264.7 cells. (a) RAW264.7 cells were cultured into 96 well-plates under stimulation of receptor activator of nuclear factor NF- κ B ligand (100 ng/ml) with or without indicated concentrations of plant extract S41 for 4 days, then the cells were stained for TRAP. The quantities of TRAP-positive multinucleated (>3 nuclei) osteoclasts were determined following image capture ($\times 40$). Data are presented as the mean $\pm$ standard error (* $P < 0.01$, vs. vehicle-treated control; $n = 3$). (b) Effects of S41 on receptor activator of nuclear factor NF- κ B ligand-induced osteoclastogenesis in bone marrow-derived macrophages. Bone marrow-derived macrophages were seeded into 96 well-plates in the presence of M-CSF (30 ng/ml) and receptor activator of nuclear factor NF- κ B ligand (100 ng/ml) with or without indicated concentrations of the plant extract S41 and then the cells were stained for TRAP. The quantities of TRAP-positive multinucleated (>3 nuclei) osteoclasts were determined following image capture ($\times 40$). Data are presented as the mean $\pm$ SE (* $P < 0.01$, vs. vehicle-treated control; $n = 3$)

no anti-osteoclastogenetic activity was recorded. Traditionally, this plant has been used for the treatment of many diseases. The stem bark paste is used for curing rheumatism and it could be plastered on the bone fractured.^[22,23]

A. bunius (S21) has been widely used in traditional medicine for the treatment of widespread diseases. Leaves, fruits, roots, and barks of this plant contain many polyphenols namely luteolin, rutin, resveratrol, and quercetin^[24] which have been shown a potential for the prevention of bone loss or enhance bone cell proliferation.^[8,10,11,25,26] Based on our knowledge, there has been no information about the chemicals or the evaluation of effects on bone diseases related to *P. benthamiana* (S54) and *M. pentapetalus* (S59).

CONCLUSION

In the present study, 70 plant extracts were screened for the osteoclastogenesis inhibitory effects in RAW264.7 murine macrophages and BMMs. For the first time, nine extracts including *A. moluccana* (S16 and S17), *A. dioca* (S19), *A. bunius* (S21), *C. balansae* (S32), *M. cochinchinensis* (S41), *P. kesiya* (S52), *P. benthamiana* (S54) and *M. pentapetalus* (S59) have potential as effective inhibitors of osteoclastogenesis. In a future study, the phytochemical components of these plants should be analyzed and the active components of these plants should be evaluated, especially for *P. benthamiana* and *M. pentapetalus* – two plants exhibited high potential effect but rarely chemical investigation performed.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Lazner F, Gowen M, Pavasovic D, Kola I. Osteopetrosis and osteoporosis: Two sides of the same coin. *Hum Mol Genet* 1999;8:1839-46.
- Lems WF, Raterman HG. Critical issues and current challenges in osteoporosis and fracture prevention. An overview of unmet needs. *Ther Adv Musculoskelet Dis* 2017;9:299-316.
- Kennel KA, Drake MT. Adverse effects of bisphosphonates: Implications for osteoporosis management. *Mayo Clin Proc* 2009;84:632-7.
- Judd HL, Cleary RE, Creasman WT, Figge DC, Kase N, Rosenwaks Z, *et al.* Estrogen replacement therapy. *Obstet Gynecol* 1981;58:267-75.
- Tran PT, Dat NT, Dang NH, Van Cuong P, Lee S, Hwangbo C, *et al.* Ganomycin I from *Ganoderma lucidum* attenuates RANKL-mediated osteoclastogenesis by inhibiting MAPKs and NFATc1. *Phytomedicine* 2019;55:1-8.
- Vincent C, Kogawa M, Findlay DM, Atkins GJ. The generation of osteoclasts from RAW 264.7 precursors in defined, serum-free conditions. *J Bone Miner Metab* 2009;27:114-9.
- Kang JH, Lim H, Jeong JE, Yim M. Attenuation of RANKL-induced osteoclast formation via p38-mediated NFATc1 signaling pathways by extract of *Euphorbia Lathyris* L. *J Bone Metab* 2016;23:207-14.
- Abdel-Naim AB, Alghamdi AA, Algandaby MM, Al-Abbasi FA, Al-Abd AM, Eid BG, *et al.* Rutin isolated from *Chrozophora tinctoria* enhances bone cell proliferation and ossification markers. *Oxid Med Cell Longev* 2018;2018:5106469.
- Qing W, Lianagqiong L, Meirong L. Studies on the chemical constituents of qiaohuajisheng (*Macrosolen cochinchinensis*). *Chinese Tradit Herb drugs* 1996;27:518-21.
- Wong RW, Rabie AB. Effect of quercetin on bone formation. *J Orthop Res* 2008;26:1061-6.
- Forte L, Torricelli P, Boanini E, Gazzano M, Rubini K, Fini M, *et al.* Antioxidant and bone repair properties of quercetin-functionalized hydroxyapatite: An *in vitro* osteoblast-osteoclast-endothelial cell co-culture study. *Acta Biomater* 2016;32:298-308.
- Liu H, Li S, Zhao Y, Ni W, Hao X, Li J, *et al.* Four new podocarpane-type trinorditerpenes from *Aleurites moluccana*. *Helvetica Chim Actae* 2007;90:2017-23.
- Alimboyoguen AB, De Castro-Cruz KA, Shen CC, Li WT, Ragasa CY. Chemical constituents of the bark of *Aleurites moluccana* L. Willd. *J Chem Pharm Res* 2014;6:1318-20.
- Meyre-Silva C, Mora TC, Biavatti MW, Santos AR, Dal-Magro J, Yunes RA, *et al.* Preliminary phytochemical and pharmacological studies of *Aleurites moluccana* leaves [L.] Willd. *Phytomedicine* 1998;5:109-13.
- Pedrosa RC, Meyre-Silva C, Cechinel-Filho V, Benassi JC, Oliveira LF, Zancanaro V, *et al.* Hypolipidaemic activity of methanol extract of *Aleurites moluccana*. *Phytother Res* 2002;16:765-8.
- Mac Donald R, Camargo SS, Meyre-Silva C, Quintão NL, Filho VC, Bresolin TM, *et al.* Development of an oral suspension containing dry extract of *Aleurites moluccanus* with anti-inflammatory activity. *Braz J Pharmacogn* 2016;26:68-76.
- Cuong NM, Taylor WC, Sung VT. A new cyclobutane lignan from *Cinnamomum balansae*. *Nat Prod Lett* 2001;15:331-8.
- Abuhashish HM, Khairy DA, Abdelsalam MM, Alsayyah A, Ahmed MM, Al-Rejaie SS. *In-vivo* assessment of the osteo-protective effects of eugenol in alveolar bone tissues. *Biomed Pharmacother* 2018;97:1303-10.
- Karmakar S, Choudhury M, Das AS, Maiti A, Majumdar S, Mitra C. Clove (*Syzygium aromaticum* Linn) extract rich in eugenol and eugenol derivatives shows bone-preserving efficacy. *Nat Prod Res* 2012;26:500-9.
- Govindarajan M, Rajeswary M, Benelli G. Chemical composition, toxicity and non-target effects of *Pinus kesiya* essential oil: An eco-friendly and novel larvicide against malaria, dengue and lymphatic filariasis mosquito vectors. *Ecotoxicol Environ Saf* 2016;129:85-90.
- Maurya R, Singh G, Yadav PP. Antiosteoporotic agents from natural sources. *Stud Nat Prod Chem* 2008;35:517-48.
- Sahu BP, Gouda P, Patnaik C. *Aporosa octandra*, a less studied plant species with potential drug activities-I: Identification of a new compound from aqueous ethanolic extract of its stem bark. *Int J Adv Res Sci Eng Technol* 2016;3:2219-24.
- Begum M, Ahmed Y, Sohrab H, Islam J, Hasan CM. Chemical investigation of the stem barks of *Aporosa roxburghii*. *Dhaka Univ J Sci* 2011;59:179-80.
- Shariful Islam M, Sharif Ahammed M, Islam Sukorno F, Ferdowsy Koly S, Morad Biswas M, Hossain S. A review on phytochemical and pharmacological potentials of *Antidesma bunius*. *J Anal Pharm Res* 2018;7:6023-604.
- Kim TH, Jung JW, Ha BG, Hong JM, Park EK, Kim HJ, *et al.* The effects of luteolin on osteoclast differentiation, function *in vitro* and ovariectomy-induced bone loss. *J Nutr Biochem* 2011;22:8-15.
- Tou JC. Resveratrol supplementation affects bone acquisition and osteoporosis: Pre-clinical evidence toward translational diet therapy. *Biochim Biophys Acta* 2015;1852:1186-94.