Original Research Article

Anti-osteoporotic activity of isoflavones from *Iris germanica* targeting NF-kappaB

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Abstract

Background: Most of the available anti-osteoporotic drugs are costly and possess great threat of severe side effects. Thus, an alternative approach is required to develop new therapeutic drugs.

Materials and Methods: An attempt was made to isolate two novel compounds followed by its analog synthesis from *Iris germanica*. All the compounds were subjected for *in vitro* anti-osteoporotic activities; % stimulation on osteoblast-like cells, % inhibition on osteoclast-like multinucleated cells (RAW264.7), cytotoxicity against RAW264.7 cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra assay, % tartrate-resistant acid phosphatase (TRAP) activity on RAW264.7 cell lines, and docking study into the active site of NF-kappaB.

Results: Out of six compounds, the compounds 1a, 1c, and 2a exhibited significant % stimulation (90%–98%) compared to standard drug diazedine (100%) while % inhibition was found in the range of 142–165, when compared with standard drug elcitonin. Cytotoxic assay results revealed that compound 1c and 1a have showed pronounced activity with IC_{50} values 4.2 and 5.2 against preosteoclastic RAW 264.7 cell lines. All the compounds showed significant inhibition of TRAP in NF-kappaB ligand-induced osteoclastic RAW 264.7 cells, with values ranging from 14.39% ± 2.62% to 66.67% ± 2.76%. Furthermore, all the compounds were docked into the active site of NF-kappa B and 1a exhibited docking score (−7.98 kcal/mole).

Conclusion: *In vitro* screening of all compound were carried out for anti-osteoporotic activity using NF-kappa B as a target. Isolated isoflavones showed excellent interactions with NF-kappaB and established a noticeable correlation between *in silico* score and *in vitro* anti-osteoporotic study.

Keywords: Anti-osteoporotic activity, docking study, *Iris germanica*, tartrate-resistant acid phosphatase: NF-kappaB

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INTRODUCTION

Osteoporosis is a multifactorial progressive skeletal disorder characterized by reduced bone mass and deterioration of bone microarchitecture, predisposing it to increased fracture risk.[1] Osteoporosis is an important public health issue threatening a large part of the elderly population, leading to an increased risk of spontaneous and traumatic fractures.[2,3] Osteoporosis arises due to loss

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of bone integrity which mainly depends on the complexly coupled activity of osteoblastic bone formation and osteoclastic bone resorption.[4] Hence, osteoporotic drugs are designed targeting these two basic processes, especially postmenopausal osteoporosis. Postmenopausal bone loss is the most common cause of osteoporosis in females.[5] Estrogen replacement therapy can relieve the patient from few problems associated with postmenopausal syndrome. However, this therapy is not having any compliance as it poses a greater threat of breast and endometrial cancer.[6] In addition, other alternative therapies developed in the last few decades as pharmaceutical agents are not affordable for common man. Thus, an alternative approach is required to develop new therapeutic drugs, which are safer, cheaper, and easily available on global scale. Of all these alternative therapies, recently herbal medicine is gaining its importance because of its wider availability and cost-effectiveness. Importantly, some of the plant bioactive molecules such as isoflavones, coumestans, and lignans are directly associated with consumption these compounds (isoflavone/coumestans/lignans) and low risk of osteoporosis.[7] The estrogen-like compounds of plant origin (phytoestrogens), such as soy isoflavones, have been characterized as naturally occurring selective estrogen receptor modulators[8] with similar beneficial effects to raloxifene on bone. The relationships between soybean isoflavones and bone tissue have been studied for less than decades.[9] A possible role of the soy isoflavones in modifying human bone mass has been suggested by (Brzezinski and Debi).[10] The osteoclastogenesis has long been a principal subject in the field of bone cell biology, though the molecular determinants were only recently identi′ed.[11]

Receptor activator of nuclear factor-ligand (RANKL), a membrane-bound protein, is induced by osteoblasts and bone marrow stromal cells.[12-14] Mature osteoclasts can be formed *in vitro* from bone marrow cells in the presence of RANKL.[15-17] Further, the receptor activator of NF-kappaB (RANK), a Type I transmembrane protein,[18] was identified as the receptor for RANKL on osteoclasts.[19] In the present study, isoflavones were shown to be inhibitors of osteoclastogenesis competing with RANKL for RANK.[20] All the compounds have been studied for % stimulation, % inhibition, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra (MTT) assay, and % tartrate-resistant acid phosphatase (TRAP) activity, and selected compounds show promising *in vitro* antiosteoporotic activity. Furthermore, all test compounds are subjected to docking study, targeting NF-kappaB, therefore, effective treatment strategies against osteoporosis could have been explored.

MATERIALS AND METHODS

Plant material

The rhizomes of *Iris germanica* were collected from the district Solan (HP) and duly authenticated by Dr. R. Raina, Senior Scientist/Professor (Medicinal Plants), Department of Forest Products, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni 173230, Solan (HP) India, linked to UHF-Herbarium with Field Book No: 13879.

Isolation and analog synthesis

Synthesis of (1a)

After the usual process of separation, the isolated compounds were subjected to repeated column chromatography, thus yielded yellow rosette shape crystals, identified as new isoflavone (1b) 600 mg, melting point 245–2487°C, and analyzed for C17H14O6 (TOF MS ES +.[m/z]: 314.26). Rf value was 0.65 (chloroform:methanol – 9:1).

Synthesis of (1b)

The (1b) 500 mg was subjected to the usual process of acetylation in the presence of acetic anhydride (5–7 mL) and few drops[2-3] of concentrated H_2SO_4, kept for 4–5 h to yield (1c) 550 mg. Acetylation yielded colorless triacetate (1c), recrystallized from methanol to yield needle-shaped crystals, melting point 270–275°C, analyzed for C23H20O9 (TOF MS ES +.[m/z]: 441.20), Rf value was 0.83 (chloroform:methanol – 9:1)

Synthesis of (1c)

Compound (1b) was subjected to demethylation by HBr to yield (1g), contains a hydroxyl group at C-4'position. The structure was clearly identified and confirmed by Pigozzi *et al.*[1] hydrogen-nuclear magnetic resonance (H-NMR) ($CDCl_3$) at δ values 12.930 ppm singlet exchangeable with D_2O.

Synthesis of (2a)

The isolated glycoside 350 mg was subjected to usual method of acid hydrolysis in the presence of 5% HCl in methanol and refluxed for 4–5 h to yield yellow solid mass (2c) 275 mg and recrystallized from absolute. The melting point of (2c) was 175–180°C, analyzed for $C_{17}H_{14}O_5$ (TOF MS ES m/z 299.26) and Rf value was 0.62 (chloroform:methanol – 9:1).

Synthesis of (2b)

The isolated glycoside was subjected to the usual process of acetylation (acetic anhydride in the presence of 10% H_2SO_4) followed by acid hydrolysis in the presence of 5% HCl. Further, subjected to per methylation under the usual process (dimethyl sulphate in the presence of K_2CO_3 in dry acetone), yielded fine needles (2b), melting point

180–183°C, analyzed for $C_{20}H_{18}O_6$, (TOF MS ES m/z 355.29). Rf value was 0.56 (chloroform:methanol – 9:1).

Synthesis of (2c)

The compound (1b) 1 g was subjected to per methylation by usual method (dimethyl sulfate in the presence of K_2CO_3 in dry acetone). After 6 h of refluxing, the product was processed. The (2c) was isolated, purified by column chromatography, and recrystallized with methanol to yield fine colorless needle crystals (700 mg), melting point 234–135°C analyzed for $C_{19}H_{18}O_5$, (TOF MS ES m/z 327). Rf value was 0.66 (chloroform:methanol – 9:1).

Biological activity (*in vitro* study)

Cell stimulation: SaOS-2 and UMR 106.06 cell line culture

SaOS-2 and UMR 106.06 cell line was obtained from the National Center for Cell Sciences (NCCS), Pune, and cultured.[21] Briefly, the cell line was procured from the NCCS, Pune Maharastra, India. The cells were cultured in a humidified atmosphere (5% CO_2, 95% air) at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 1% antimicrobial and antifungal solution, supplemented with 15%. Upon reaching confluence, the cells were detached using accutase and loaded in 96-well plate for culturing for 96 h in 6 increasing concentration (5, 10, 20, 40, 80, and 160 μg/mL) of all six different isoflavones, while positive control cells received the same volume of medium. After 96 h, MTT assays were carried out to understand the effect of the six isoflavones on osteoblastic cells.

Stimulation of proliferation of osteoblast-like UMR106.6 and SaOS-2Cell Line

Osteoblast-like UMR106.6 and SaOS-2 cells were suspended in α-MEM and the suspension (200 μl) containing 8×10^3 cells was plated in 96-well plates. The cells were preincubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h in α-MEM containing 7% FBS to attach the cells and then for another 24 h in α-MEM without FBS. The cells were treated with test specimens (50 and 100 μg/ml) in α-MEM without FBS and incubated for 48 h under the same conditions. Then, MTT-zolium bromide was added to each well and the plates were incubated for 4 h. The amount of formazan formed was measured spectrophotometrically at 590 nm with a Bio-RAD ELIZA Model 3550 plate reader. Samples were dissolved in 5% dimethyl sulfoxide (DMSO) and then diluted with the medium. NaF (10^{-5} M) was used as a positive control. Proliferation rates were calculated from the mean values of the data from four wells.

Inhibition of formation of osteoclast-like multinucleated cells (RAW264.7. Mouse osteoclast precursor-like cells)

Cell culture − RAW 264.7 (macrophages [preosteoclasts] from BALB/c mouse) cells were cultured in 96-well plates (1×10^4 cells/mL) containing DMEM supplemented with 10% (v/v) FBS for 2 days. The medium was then replaced with test samples in a differentiation medium containing 50.0 ng/mL RANKL. The differentiation medium was changed every 2 days.

The cultured RAW 264.7 cell lines were flushed with α-MEM in the presence of 1 α 25(OH) $2D_3$ (10^{-7} M) and test samples at various concentrations (50 and 100 μg/ml). Elcitonin (2 U/ml) was used as a positive control. The medium was replaced with new medium containing test samples and 1 α 25(OH) $2D_3$ every 2 days. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After culture for 6 days, adherent cells were fixed with a solution of 10% formalin in phosphate-buffered saline (pH 7.2) for 10 min, dehydrated with ethanol–acetone (50:50, v/v) for 45 s and stained for TRAP for 12 min at room temperature. Cells possessing three or more nuclei were counted as osteoclast-like multinucleated cells under inverted microscope.

Cell cytotoxicity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra assay

RAW 264.7 cells were cultured in 24-well plates (2×10^4 cells/mL) containing DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics in a humidified atmosphere of 5% CO2 at 37°C for 5 days, washed with phosphate-buffered saline (PBS), and pretreated with different concentrations (1.0–20.0 μM) of samples to be tested. After 5 days' incubation, MTT reagent was added to each well, and the plate was incubated at 37°C for 1 h. The medium was removed and the plate was washed twice with PBS. The intracellular insoluble formazan was dissolved in DMSO. The absorbance of each cell was recorded in DMSO. The absorbance of each cell was then measured at 570 nm using an ELISA (Tecan, Salzburg, Austria) reader, and the percentage proliferation was calculated.[22]

Tartrate-resistant acid phosphatase staining

RAW 264.7 cells were seeded in 12-well plates (3×10^4 cells/well) containing DMEM medium plus 10% FBS, and the medium was replaced with test samples in differentiation medium containing 50.0 ng/mL RANKL. The differentiation medium was changed every 2 days. After 5 days, the medium was removed and the cell monolayer was gently washed twice using PBS. The cells were fixed in 3.5% formaldehyde for 10 min and washed with distilled water. The cells were incubated at 37°C in a humid- and

light-protected incubator for 1 h in the reaction mixture of a leukocyte acid phosphatase assay kit as directed by the manufacturer. The cells were washed three times with distilled water, and TRAP-positive multinucleated cells containing three or more nuclei were counted under a light microscope.[23]

Tartrate-resistant acid phosphatase activity

TRAP activity − After differentiating the RAW 264.7 cells into osteoclasts for 5 days, the medium was removed and the cell monolayer was gently washed twice using ice-cold PBS. The cells were fixed in 3.5% formaldehyde for 10 min and ethanol–acetone (1:1) for 1 min. Subsequently, the dried cells were incubated in 50 mM citrate buffer (pH 4.5) containing 10 Mm sodium tartrate and 6 mM PNPP. After 1 h incubation, the reaction mixtures were transferred to new well plates containing an equal volume of 0.1N NaOH. Absorbance was measured at 405 nm using an enzyme-linked immunoassay reader and TRAP activity was expressed as the percent of the control.[23]

Molecular docking into DNA binding site of NF-kappaB

All the six substituted isoflavones analogs were prepared using ChemDraw ultra 8.0. All ligands were prepared through AutoDock Tools. The three-dimensional crystal structure of the NF-kappaB was obtained from Protein Data Bank (PDB code: 1NFK).[24]

RESULTS

Isolation and characterization

All the compounds were characterized and identified by IR, ^{1}H-NMR, ^{13}Carbon NMR, and mass spectrometry.

5,7,8-trihydroxy-3-(4-methoxyphenyl)-2-methyl-4H-chromen-4-one (1a)

IR (KBr, cm^{-1}): 1518 (ArC = C), 1659 (C = O), 3080 (ArC-H), 1372 (C-O), 2825 (C-H Str), 3368 (Ar–OH). The IR data indicated the presence of carbonyl group, aromatic ring, and hydroxyl groups. (^{1}H-NMR 400 MHz, δ, DMSO, TMS = 0): 2.42 (3H, s, 2-H), 3.89 (3H, s, 4'-H), 6.46 (1H, s, 6-H) 6.85 (2H, d, 3',5'-H, *J* = 8.60 Hz), 7.37 (2H, d, 2',6'-H, *J* = 8.64 Hz), 8.15 (1H, s, 2-H), ^{13}C-NMR (400 MHz, δ, DMSO, TMS = 0): C-4, 180.50, C-4' 157.39, C-5 153.22 C-7 153.30, C-2 153.31, C-6 131.22, C-8a 121.92, C-8 132.47, C-3',5', 115.01, C-2',6' 129.91, C-1',104.89, C-3 121.14, C-4a 93.71, OCH_3-59.80, 2-CH_{3}-18.21. Mass spectrometry (1a): TOF MS ES^{+} 2.33e. 3 m/z.

5,7,8-acetoxy-3-(4-methoxyphenyl)-2-methyl-4H-chromen-4-one (1b)

IR (KBr, cm^{-1}): 1514 (ArC = C), 1760 (C = O), 3063 (ArC-H), 1320 (C-O), 2870 (C-H Str). 1190, (-COOR). ^{1}H NMR (400 MHz, δ, DMSO, TMS = 0): 2.06 (3H, s, 2-CH_3), 2.12 (3H, s, 2CH_3) 2.31,2.38,2.46 (3-(3H), s, 8, 7 and 5-H), 3.86 (3H, s, 4'-H), 7.19 (1H, s, 6-H), 7.15 (2H, d, 3',5'-H, *J* = 8.56 Hz), 7.49 (2H, d, 2',6'-H, *J* = 8.60 Hz), 7.87 (1H, s, 2-H).

^{13}C-NMR (400 MHz δ, DMSO, TMS = 0): (3>C = O,169.42,169.16,167.95), (C-8a 152.07), (C-7, 148.41), (C-5,142.96), (C-8, 142.43), (C-6,110.23), (3C-CH3,21.16,21.07, 20.71) (2-C,19.3). Mass spectrometry: TOF MS ES^{+} 2.98e. 3 m/z.

5,7,8-trihydroxy-3-(4-hydroxyphenyl)-2-methyl-4H-chromen-4-one (1c)

The structure was clearly identified and confirmed by ^{1}H-NMR ($CDCl_3$) at δ values 12.930 ppm singlet exchangeable with D_2O. 1H-NMR (400 MHz, δ, DMSO, TMS = 0): 1.98 (3H, s, 2-H) 6.970 (2H, d, 3',5'-H, *J* = 8.60 Hz), 7.37 (2H, d, 2',6'-H, *J* = 8.60 Hz), 8.15 (1H, s, 2-H), 10.70 (1H, s, 7-OH), 12.930 (1H, s, 4'-OH) Exchangeable with D_2O.

6,7,-dihydroxy-3-(4-methoxyphenyl)-2-methyl-4H-chromen-4-one (2a)

The compound (2a) was isolated after repeated column chromatography and characterized with various spectroscopic techniques, Viz, IR, UV, ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry.

IR (KBr, cm^{-1}): 1590 (Ar (C = C), 1659 (C = O), 3030 (Ar C-H)], 1340 (C-O), 3350 (Ar-OH).

^{1}H-NMR (400 MHz, δ, DMSO, TMS = 0): 2.36 (3H, s 2-H), 3.80 (3H, s, 4'-H), 6.46 (1H, s, 8-H), 6.85 (2H, dd, 3',5'-H, *J* = 8.60 Hz), 7.37 (2H, dd, 2',6'-H, *J* = 8.64 Hz), 8.15 (1H, s, 5-H), 8.24 (1H, s, 2-H), ^{13}C-NMR (400 MHz, δ, DMSO, TMS = 0): C-4, 180.00, C-5 117.48, C-4' 159.99, C-2 153.24, C-7 153.86, C-8a 151.32, C-6 142.97, C-2',6' 127.49, C-1'124.95, C-3 123.50, C-3',5' 114.22, C-8, 110.54 C-4a 118.27, 4'-OCH_3, 55.9. 2-CH_3-18.56. Mass spectrometry: (TOF MS ES m/z 299.26)

7-methoxy-3-(4-methoxyphenyl)-2-methyl-4-oxo-4H-chromen-6-yl acetate (2b)

IR (KBr, cm^{-1}): 1592 (Ar (C = C), 1666 (C = O), 1745 (C = O), 3040 (Ar C-H), 1342 (C-O), 3352 (Ar-OH). ^{1}H NMR (400 MHz, δ, DMSO, TMS = 0): 1.24 (3H, s 2-H), 2.55 (3H, s, 7-H), 3.90, 3.88 (6H, s, 6, 4'-OCH_3), 6.46 (1H, s, 8-H), 6.83 (2H, m, 3',5'-H), 7.35 (2H, m, 2'4'-H) 9.16 (1H, s, 5-H). ^{13}C-NMR (400 MHz, δ, DMSO, TMS = 0): C-4, 180.25, C-5 126.35, C-4' 159.29, C-2 153.28, C-7 159.66, C-8a 155.47, C-6 135.43, C-2',6' 128.17, C-1'124.25, C-3 123.53, C-3',5'114.04, C-8, 105.54

C-4a 117.12, C-4'-OCH_3, 55.9, C-6, OCH_3, 56.98, C-7, CH_3 23.56, C-7, C=O, 176.43. Mass spectrometry: TOF MS ES m/z 355.29).

6,7-dimethoxy-3-(4-methoxyphenyl)-2-methyl-4H-chromen-4-one (2c)

The compound (2c) was isolated and characterized by various spectroscopies.

IR (KBr cm^{-1}): 1560 (Ar (C=C), 1760, 1758 (C=O), 3060 (Ar C-H), 1340 (C-O), 1195-(OCH_3).

^{1}H NMR (400 MHz, δ, DMSO, TMS = 0): 2.38 (3H, s, 2-H), 3.91,3.86, 3.85 (6H, s, 6,7, 4'-OCH_3), 7.16 (2H, m, 3',5'), 7.26 (1H, s, 8-H), 7.43 (1H, m, 2',6'-H), 7.83 (1H, s, 5-H).

^{13}C-NMR (400 MHz, δ, DMSO, TMS = 0): C-4, 187.20, C-5 122.47, C-4' 159.99, C-2 153.24, C-7, 152.64, C-8a 154.54, C-6 140.43, C-2',6' 127.38, C-1'124.95, C-3 123.53, C-3',5' 114.02, C-8, 110.54 C-4a 121.12, 4'-OCH_3, 55.9, 6-OCH_3, 56.98, 7-OCH_3, 2-CH_3, 16.84. Mass spectrometry (TOF MS ES m/z 327).

Biological activity

Stimulation of proliferation of osteoblast-like UMR106.6 and SaOS-2cell line

The clonal osteoblast-like UMR 106 and SaOS-2 cell lines were derived from a rat osteogenic sarcoma, possesses many of the enzymatic properties of normal osteoblasts (including high alkaline phosphatase activity and parathyroid hormone-simulated adenyl cyclase activity). UMR106 and SaOS-2 cells were used for screening of the stimulation of six isoflavones from *I. germanica* of bone formation. The results were incorporated in Table 1.

% Inhibition of six compounds on formation of osteoclast-like cells (RAW264.7 cell lines

The percentage inhibition of all isolated compounds and its analogs were done on RAW 264.27 cell line. The results were summarized in Table 2.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra assay

To date, the anti-osteoporosis effects of compounds isolated from *I. germanica* have not been reported. Therefore, we assayed the anti-osteoporosis activity of six isoflavones on RAW 264.7 cells [Table 3]. To address this, the compounds (10–100 μg/mL) were tested for their cytotoxic activity on RAW 264.7 macrophage cells during a 5 day differentiation period.

Drug-receptor interaction study

The drug-receptor interaction study has been done to identify the ligands, which show lowest estimated free energy of binding, and thus, produce significant inhibition of NF-kappaB.

Table 1: Stimulation rates (% of control) at the doses of 100 μg/ml on proliferation of the osteoblast-like UMR 106 and SaOS-2 cell lines

Compounds	Concentration (μg/ml)	Stimulation (%)	
		UMR 106.6	SaOS-2
1a	100	98	97
1b	100	33	37
1c	100	92	94
2a	100	91	90
2b	100	26	15
2c	100	36	30
Diazedein (positive control)	100	100	100

Table 2: Percentage inhibition of six compounds on formation of osteoclast-like cells (RAW 264.7 cell lines)

Compounds	Concentration (μg/ml)	Inhibition (%) RAW 264.7 cell lines
1a	20	165.3
1b	20	58
1c	20	163
2a	20	142
2b	20	45
2c	20	30
Elcitonin (positive control)	2 U/ml	170.8

Table 3: IC_{50} of isolated compounds and its analogs toward RAW 264.7 cell lines by MTT assay method, after 48 h

Compounds	RAW 264.7 (IC_{50} value) μg/mL	Percentage TRAPE activity tested at 20 μg/mL for each compound (RAW 264.7)
1a	5.2	66.67±2.71
1b	44.4	28.98±3.07
1c	4.2	63.92±2.12
2a	6.3	57.32±2.46
2b	54.6	14.39±2.62
2c	34.5	16.98±1.05
Raloxifene	2.3	
Diazedine		70.34±1.87

TRAP activity was measured from cultures after 5 days of treatment with RANKL and test compounds (20.0 μg/mL). RANKL: Receptor activator of nuclear factor-ligand, TRAP: Tartrate-resistant acid phosphatase

DISCUSSION

All isolated compounds and its analogs were characterized and identified [Figure 1a and b].

Among the six compounds, the 1a, 1c and 2a showed strongest activity, with the stimulation rate of 90%–98% against both the cell lines [Table 1]. The high potential of stimulation rate of these compounds was attributed due to the presence of more numbers of hydroxyl groups on ring A and B. However, the others members (1b, 2b and 2c) of this series have not exhibited significant percentage stimulation (15%–37%) against cell lines presumably,

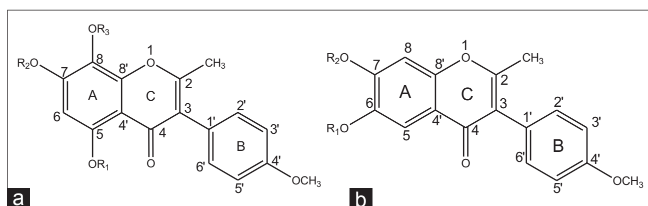

Figure 1: (a and b) Structure of isoflavones. (1A)-(1a) R1, R2, R3 = H; (1b) R1, R2, R3 = OCOCH3; (1c) R1, R2, R3 = H; OCH3 = OH. (1B)-(2a) R1, R2 = H, (2b) R1 = CH3; R2= – OCOCH3 (2c) R1 = CH3; R2 = CH3

because of the conversion of hydroxyl group to either acetoxo or methoxyl groups. The law stimulation rate appears to be associated with the adequate interactions between pharmacophores (acetoxy/methoxy) and receptor cavity [Figure 2]. These results suggested that the potential of the stimulation rate of the compounds were dependent on the location and number of hydroxyl and methoxy groups in isoflavonoids nucleus. The newer compounds (1a, 1c, and 2a) showed potent activity nearly identical to the positive control diazedine.[10]

Among the six isoflavones examined, 1a, 1c, and 2a showed high % inhibition rates, 165.3, 163 and 142% at the dose of 20 μg/ml [Table 2], respectively. It has been concluded from the above results that the novel molecule (1a and 1c) have shown inhibitory activity nearly identical to the standard molecule (Elcitonin). However, the compounds 1b, 2b, and 2c have shown weak inhibition effect at the tested concentration.

When compared percentage stimulation and inhibition, 1a, 1b, and 1c were found to have both bone formation and decreased bone resorption potential, hence the present study considered as dual beneficial approach as anti-osteoporotic agents. Hence, the compound must be further subjected for *in vivo* analysis followed by the preclinical study.

The cytotoxic activities of the isolated compounds (six) were measured using MTT assay.

The results showed that compounds 1a, 1c, and 2a exhibited significant cytotoxic activities, with IC_{50} values ranging from 4.2 to 6.3 μg/mL [Table 3]. However, no significant cytotoxic effects were observed for the compounds 1b, 2b, and 2c with IC_{50} values [Table 3]. The results suggest that cytotoxic potential of compounds were dependent on the substitution patterns of pharmacophores (hydroxyl, methoxy, acetoxy) of ring A and B in isoflavones nucleus. However, a significant cytotoxic effects were observed. Thus, these results suggested that 1a and 1c possess both anti-osteoclastogenic activities and cytotoxic effects.

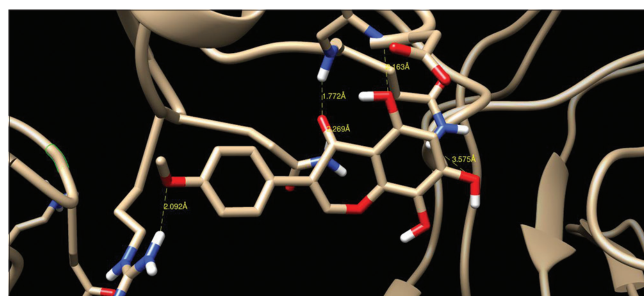

Figure 2: Stereoview of the complex formed by NF-kappaB and the docked compound (1b).The amino acids Gln 274, Gln 306, Arg 305, Lys 275, and Asp 276 were involved in interaction with compounds

The anti-osteoporotic activities of compounds (six) were evaluated based on the suppression of excessive bone breakdown by osteoclasts. The results showed that isoflavone derivatives 1a, 1b, 1c, 2a, 2b, and 2c suppressed osteoclast formation in a dose-dependent manner with TRA*P* values ranging from 14.39% ± 2.62% to 66.67% ± 2.71% at concentration of 20 μg/mL [Table 3]. Among them, compounds 1a and 1c showed the most significant when compared to daidzein used as a positive control, with values of 66.67% ± 2.71% and 63.92% ± 2.12%, respectively.

In addition, there is a correlation between TRAP activity and cytotoxic potential of tested 6 compounds. On the other hand, the significant anti-osteoporotic activities of isoflavonoid derivatives 1a, 1c, and 2a showing significant TRAP activities *in vitro* may be attributed to their strong ligand-receptor binding interactions.

Ligands were ranked according to docking score/estimated free energy of binding. The free energy of binding of ligands was in the range between −3.37 and −8.50. Kcal/mole [Table 4]. Top-ranked compound (1a) and (1c) with −7.98 and −7.84 Kcal/mole free energy of binding, respectively, were in correlation with wet lab experiments. The protein-ligand analysis also has shown its strong interactions with target protein and had five hydrogen bond interaction in (1a) and six hydrogen bond interaction in (1c) see Figure 1a and b. The excellent interactions of NF-kappaB with two top-ranked compounds (1a) and (1c) indicated a high degree of coherent relationship between *in silico* approach and *in vitro* studies. High anti-osteoporotic activity and excellent interaction profile of compounds (isoflavone and its analogs) demand further *in vivo* and clinical studies, and these compounds might find an important place in the new array of molecules targeting NF-kappaB-dependent biological functions as anti-osteoporotic agents.

Table 4: Estimated free energy of binding of isolated compounds in the target nuclear factor-kappaB as homodimer (p50-p50)

Compounds	Estimated free energy of binding (Kcal/mol)
1a	−7.98
1b	−4.16
1c	−7.84
2a	−6.29
2b	−3.95
2c	−3.37
Diazedine	−8.03

3D structures of NFK, p50–p50 homodimer (from 1NFK), was used for virtual screening. NFK: Nuclear factor-kappaB

CONCLUSION

Two novel isoflavones were isolated from *I. germanica* and further its analogs were synthesized and characterized. *In vitro* screening of isolated isoflavones and its analogs were carried out for anti-osteoporotic activity using NF-kappa B as a target. Isolated isoflavones and its analogs showed excellent interactions with NF-kappaB and established a noticeable correlation between *in silico* score and *in vitro* anti-osteoporotic study. Most of the compounds illustrated a fair *in vitro* anti-osteoporotic activity in different cell lines. Among them, the compounds (1a) and (1c) have shown marked dual activity, that is, both % stimulation on osteoblast cell lines (UMR 106.6 and SaOS-2) and significant % inhibition on osteoclast cell lines (RAW 264.7). The IC_{50} value and % TRAP activity were also in good tune with docking results. The isoflavones as phytoestrogens displayed significant broad-spectrum anti-osteoporotic profile, and thus, promising activity of these compounds (1a) and (1c) demands further *in vivo* and clinical studies.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Pigozzi F, Rizzo M, Giombini A, Parisi A, Fagnani F, Borrione P, *et al.* Bone mineral density and sport: Effect of physical activity. J Sports Med Phys Fitness 2009;49:177-83.
2. Khajuria DK, Razdan R, Mahapatra DR, Bhat MR. Osteoprotective effect of propranolol in ovariectomized rats: A comparison with zoledronic acid and alfacalcidol. J Orthop Sci 2013;18:832-42.
3. Cummings SR, Melton LJ. Epidemiology and outcomes of osteoporotic fractures. Lancet 2002;359:1761-7.
4. Riggs BL, Melton LJ 3rd. The prevention and treatment of osteoporosis. N Engl J Med 1992;327:620-7.
5. Bonnick SL, Harris ST, Kendler DL, McClung MR, Silverman SL. Management of osteoporosis in postmenopausal women: 2010 position statement of the North American Menopause Society. Menopause 2010;17:25-54.
6. Persson I, Weiderpass E, Bergkvist L, Bergström R, Schairer C. Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement. Cancer Causes Control 1999;10:253-60.
7. Bedell S, Nachtigall M, Naftolin F. The pros and cons of plant estrogens for menopause. J Steroid Biochem Mol Biol 2014;139:225-36.
8. Arjmandi BH, Smith BJ. Soy isoflavones' osteoprotective role in postmenopausal women: Mechanism of action. J Nutr Biochem 2002;13:130-7.
9. John BJ, Garner SC. The effects of phytoestrogens on bone. Nutr Res 1997;17:1617-32.
10. Brzezinski A, Debi A. Phytoestrogens: The "natural" selective estrogen receptor modulators? Eur J Obstet Gynecol Reprod Biol 1999;85:47-51.
11. Setchell KD, Cassidy A. Dietary isoflavones: Biological effects and relevance to human health. J Nutr 1999;129:758S-767S.
12. Jimi E, Nakamura I, Amano H, Taguchi Y, Tsurukai T, Tamura M, *et al.* Osteoclast function is activated by osteoblastic cells through a mechanism involving cell-to-cell contact. Endocrinology 1996;137:2187-90.
13. Udagawa N, Takahashi N, Jimi E, Matsuzaki K, Tsurukai T, Itoh K, *et al.* Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: Receptor activator of NF-kappa B ligand. Bone 1999;25:517-23.
14. Yasuda H. RANKL, a necessary chance for clinical application to osteoporosis and cancer-related bone diseases. World J Orthop 2013;4:207-17.
15. Baud'huin M, Lamoureux F, Duplomb L, Rédini F, Heymann D. RANKL, RANK, osteoprotegerin: Key partners of osteoimmunology and vascular diseases. Cell Mol Life Sci 2007;64:2334-50.
16. Wong BR, Josien R, Lee SY, Sauter B, Li HL, Steinman RM, *et al.* TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J Exp Med 1997;186:2075-80.
17. Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, *et al.* TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-jun N-terminal kinase in T cells. J Biol Chem 1997;272:25190-4.
18. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, *et al.* A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 1997;390:175-9.
19. Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, *et al.* Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci U S A 1999;96:3540-5.
20. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, *et al.* Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 1997;89:309-19.
21. Thangakumaran S, Sudarsan S, Arun KV, Talwar A, James JR. Osteoblast response (initial adhesion and alkaline phosphatase activity) following exposure to a barrier membrane/enamel matrix derivative combination. Indian J Dent Res 2009;20:7-12.
22. Ha H, Ho J, Shin S, Kim H, Koo S, Kim IH, *et al.* Effects of eucommiae cortex on osteoblast-like cell proliferation and osteoclast inhibition. Arch Pharm Res 2003;26:929-36.

23. Lee SH, Ding Y, Yan XT, Kim YH, Jang HD. Scopoletin and scopolin isolated from artemisia iwayomogi suppress differentiation of osteoclastic macrophage RAW 264.7 cells by scavenging reactive oxygen species. J Nat Prod 2013;76:615-20.
24. Alam A, Jaiswal V, Akhtar S, Jayashree BS, Dhar KL. Isolation of isoflavones from iris kashmiriana baker as potential anti proliferative agents targeting NF-kappaB. Phytochemistry 2017;136:70-80.