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Research Article

Anti-Osteoporotic Activity of Bioactive Compounds from *Iris germanica* Targeting NF-kappaB

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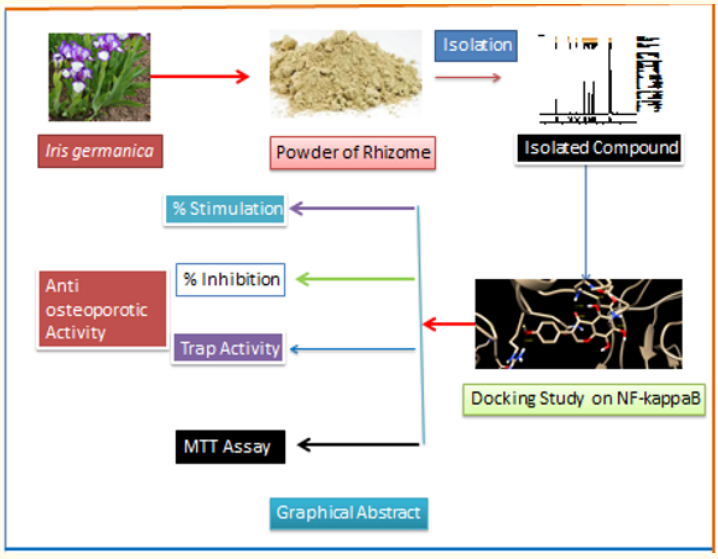

Figure

Abstract

Background: Almost all anti-osteoporotic drugs are costly and possess great the rate of severe side effects. Thus, there is a need of development of new drugs which should be cheaper, safer and widely accepted in health care.

Objective: The purpose of the research work was to develop new anti-osteoporotic agents from plant origin based on an *in silico* approach targeting NF kappa B.

Method: An attempt was made to isolate two novel compounds followed by its analogue synthesis from *Iriss germanica*. Isolated test compounds were characterized by various spectroscopic techniques such as UV, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. Further, compounds were analysed by *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 MTT assay, % 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 percent) while % inhibition was found in the range of 142 - 165, when compared with standard drug Elcitonin (170.8 percent). Cytotoxic assay results revealed that compound 1c and 1a have showed pronounced activity with IC_{50} values 4.2 and 5.2 against pre-osteoclastic RAW 264.7 cell lines. All the compounds showed significant inhibition of tartrate-resistant acid phosphatase 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 k cal/mole), a significant value compare to Diazedine (-8.03 k cal/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: *Iriss germanica; Anti-Osteoporotic Activity; Tartrate-Resistant Acid Phosphatase (TRAP); NF-kappaB; Docking Study*

Introduction

Osteoporosis is related to skeletal disruption predicted by decreased in bone density, derangement of bone articulate system, liable to enhance risk factor of bone fracture [1]. Osteoporosis is a prominent public health issue alarming a broad part of the elderly people, directed to enhance risk of indigenous.

Osteoporosis is an important public health issue alarming a broad part of the elderly people, directed to enhanced risk of indigenous and traumatic fractures [2]. The deformities caused by the incidence of osteoporosis have a massive impression on general healthcare, as they are always linked with high risk of morbidity, mortality and comparatively more expensive in the treatment regimen [3].

In osteoporosis, the accidental injury and bone fracture incidences are frequently observed resulting in further immobility leads to the enhancement of osteoporosis in the affected part of the bones. Generation and formation of osteoporosis is due to the imbalance between a complexly coupled activity of osteoblastic bone formation and osteoclastic bone resorption [4]. Consequently, osteoporotic drugs are developed to target these two basic pathways; particularly post-menopausal osteoporosis. In general, osteoporosis is frequently observed in females after the post-menopausal bone loss. Postmenopausal syndrome is mostly treated with estrogen replacement therapy. However, there is a great risk of incidence of breast and endometrial cancer [5,6]. Now-a-days many alternative therapies have come into the market in last few decades as pharmacological agents and are not economical for common man. Thus, an alternative approach is required to develop new therapeutic drugs, which are safer, cheaper and easily available on global scale. Among all these therapies, herbal products emerged as a potential class of drugs and receiving its importance because of its lower spectrum of side effect, widely distributed in nature and cost effectiveness. Importantly, some of the plant bioactive molecules such as flavone, isoflavones, coumestans and lignans are directly associated with consumption these compounds flavone (isoflavone/coumestans/lignans) and low risk of osteoporosis [7].

Phytoestrogen such as flavonoids and soy isoflavones may have shown a modulatory effect on selective estrogenic receptor [8], with similar pharmacological effects to raloxifene on bone tissue. The positive relationship has been thoroughly studied between soy isoflavone and bone masses for less than decades [9]. Beside that a direct association of intake of natural flavonoids and soy isoflavone and incidence of lower risk of osteoporosis has been well documented by Brzezinski and Debi [10]. They observed that soy product enriched in flavonoids and isoflavones drastically reduces bone loss in postmenopausal and perimenopausal women. Furthermore, the epidemiological study clearly indicated that women with more flavonoids and soy food diet have a lower risk of osteoporosis than who consume a typical western food habit [11].

Hence, regular consumption of flavonoids and soy isoflavone (Phytoestrogen) by the menopausal women leads to maintain their bone mass as they are devoid of all known side effects associated with steroidal therapy. The osteoclastogenesis has long been a principal subject in the field of bone cell biology, though the molecular determinants were only recently identified. 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, flavones were shown to be inhibitors of osteoclastogenesis competing with RANKL for RANK [20].

Anti-osteoporotic therapy should be coupled with both the bone formation and bone resorption process, in the present study, screening of the effect of active flavones against the formation of tartrate resistant acid phosphatase (TRAP)-positive osteoclast-like multinucleated cells have been explored. Based on the results of % stimulation, % inhibition, MTT assay and % TRAP activity some of the compounds have shown a promising *in vitro* anti-osteoporotic activity. Furthermore, all the test compounds and its analogues were subjected to docking study, targeting NF-kappaB, using Autodock 4.2 as a model of protein-ligand interaction experiments. Therefore, effective treatment strategies against osteoporosis could have been explored.

Material and Methods

Chemicals

Sodium β-glycerophosphate was procured from Sigma chemicals. Methanol and Triton X -100 were of domestic AR grade products. Accutase and FBS were purchased from High Media Dulbecco's modified Eagle's medium (DMEM), β-glycerophosphate, fetal bovine serum (FBS), Triton X-100, Hank's balanced salt solution (HBSS), RANKL, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium tartrate, phosphate-buffered saline (PBS, pH 7.4), p-nitrophenylphosphate (PNPP), and dimethylsulfoxide (DMSO) were procured from Sigma-Aldrich (St. Louis, MO, USA). RAW 264.7 SaOS-2cells and UMR106.6 were obtained NCCS Pune, Maharashtra.

Plant Material

The rhizomes of *Iriss germanica* were obtained from district Solan (HP) at an altitude of 1500 - 2000m above sea level in the month of April-May 2015 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.

Extraction and isolation

The rhizomes of *Iriss germanica* were collected, washed and dried in sun. The dried rhizomes were converted into powder. 1000g of powdered drug was subjected for extraction with petroleum ether (60 - 800C) using soxhlet apparatus (24h run) to remove fatty materials. The petroleum ether extract (gum and resign 5.13g) was separated and marc was again subjected for further extraction using soxhlet apparatus with methanol. The methanolic extract was collected and subjected for solvent successive fractionation with toluene, chloroform and ethyl acetate and n- butanol. Methanolic extract and their different fractionation were subjected for phytochemical screening, aglycone was substantially found in ethyl acetate fraction, hence selected for column chromatography. After considering TLC pattern, column chromatography was run keeping chloroform: methanol (9:1) as solvent system. Total 1 - 150 fractions were collected and TLC pattern was determined in UV chamber. Two different single spots were observes in the range of 60 - 72 and 90 - 115 test tubes respectively. The identical TLC pattern ones were pooled. The melting points of isolated compounds were determined and found to be un corrected. Phytochemical test were done for all fraction as and extract and following compounds were observed in ethyl acetate fraction after repeated column chromatography [21].

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 flavone **(1b)** 600 mg, melting point 245 - 248^{0}C and analyzed for $C_{17}H_{14}O_6$ (TOF MS ES$^+$. (m/z): 314.26). R_f value 0.65 (chloroform: methanol 9:1) Elemental Analysis Found (%): C; 64.00, H; 4.03, O; 31.97 and Required: C; 63.21, H; 4.91, O; 30.83. Further, the structure was confirmed by UV, IR, ^{1}HNMR, ^{13}CNMR, 2-DNMR and mass (TOF MS ES$^+$).

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 Conc. 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, m.p. 270 - 275^{0}C, analyzed for $C_{23}H_{20}O_9$ (TOF MS ES+. m/z: 441.20), R_f Value 0.83 (chloroform: methanol 9:1), Elemental Analysis Found (%): C: 61.97, H: 4.26, O: 33.77 and Required: C: 60.51, H: 4.92, O: 31.72. Further, the structure was confirmed by U.V. ^{1}HNMR, ^{13}CNMR 2-DNMR and mass (TOF MS ES$^+$) and 1HNMR.

Synthesis of (1c)

Compound **(1b)** was subjected to de methylation by HBr to yield (1g), contains a hydroxyl group at C-4'position the structure was clearly identified and confirmed by ^{1}H-NMR ($CDCl_3$) at δ values 12.930 ppm singlet exchangeable with D_2O. In the ^{1}H-NMR ($CDCl_3$) of **(1c)** all the signals remain unchanged except the up field displacement of the signal for the C-3'and C-5' proton from δ value at 7.155 - 6.970 ppm indicating the presence of one hydroxyl group at C-4' position in ring B.

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 - 5h to yield yellow solid mass **(2c)** 275 mg and recrystallized from absolute. The melting point of **(2c)** was 175 - 180^{0}C, analyzed for $C_{17}H_{14}O_5$, (TOF MS ES m/z 299.26), $R_f$value 0.62 (chloroform: methanol 9:1). Elemental Analysis Found (%): C: 67.60, H: 4.25, O: 28.14 and Required (%) C: 67.60, H: 4.25, O: 28.14.

Synthesis of (2a)

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. Which on subsequently subjected to per methylation under the usual process (method (di-methyl sulfate in the presence of K_2CO_3 in dry acetone) gave fine yellow needles (2b) melting point 180 - 183^{0}C analyzed for $C_{20}H_{18}O_6$ (TOF MS ES m/z 355.29), Rf value 0.56 (chloroform: methanol 9:1). Elemental Analysis Found %: C: 68.45, H: 4.73, O: 26.82, and Required %: C: 67.10, H: 5.02, O: 26.98.

Synthesis of (2c)

The compound **(1b)** 1g was subjected to per methylation by usual method (dimethyl sulfate in the presence of K_2CO_3 in dry acetone). After six hours 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^{0}C analyzed for $C_{19}H_{18}O_5$ (TOF MS ES m/z 327). R_f value 0.66 (chloroform: methanol 9:1). Elemental Analysis Found %: C: 62.02, H: 6.64, O: 31.34, and Required %: C: 61.90, H: 6.92, O: 30.98.

Structure of Isoflavones

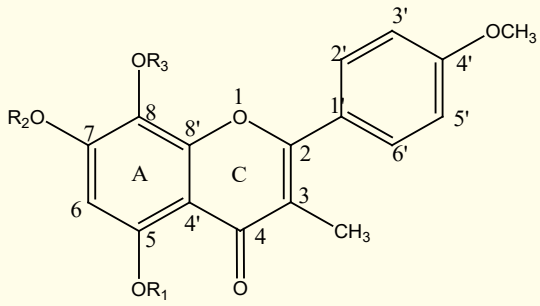

Figure 1A: *1a) R_1, R_2, R_3 = H; 1b) R_1, R_2, R_3 = $OCOCH_3$; 1c) R_1, R_2, R_3 = H; OCH_3 = OH,*

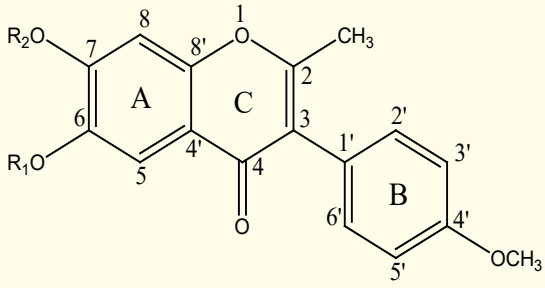

Figure 1B: *2a) R_1, R_2 = H, 2b) R_1 = CH_3; R_2 = -$OCOCH_3$; 2c) R_1 = CH3; R_2 = CH_3*

Figure 1: *(A) and (B) Structure of Isoflavones.*

Biological Activity (*In vitro* study)

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

SaOS-2 and UMR 106.06 cell line were obtained from NCCS, Pune and cultured [22]. The cells were cultured in a humidified atmosphere (5% CO2, 95% air) at 37ºC in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% anti-microbial and anti-fungal solution, supplemented with 15% FBS (High Media, Bombay). Upon reaching confluence, the cells were detached using Accutase (High Media, Bombay) and loaded in 96 well plate (Merck Scientific, Bombay) for culturing for 96 hours in different concentration of 5, 10, 20, 40, 80, 160 µg/mL of all six different flavones, while positive control cells received the same volume of medium. After 96 hours, MTT assays were carried out to understand the effect of the six flavones on osteoblastic cells.

Stimulation of proliferation of osteoblast-like UMR106.6 and SaSO-2 cell 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 placed in 96-well plates.

The cells were pre-incubated at 37°C in a humidified atmosphere containing 5% CO2 for 24h in α-MEM containing 7% FBS to attach the cells and then for another 24h 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 48h under the same conditions. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) was added to each well and the plates were incubated for 4h. 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% 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 (pre-osteoclasts) 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 two 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 minutes, dehydrated with ethanol-acetone (50:50, v/v) for 45s, and stained for TRAP for 12 minutes at room temperature. Cells possessing three or more nuclei were counted as osteoclast-like multinucleated cells under inverted microscope.

Cell cytotoxicity by MTT 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% CO_2 at 37°C for 5 days, washed with 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 1h. 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 [23].

TRAP 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 minutes and washed with distilled water. The cells were incubated at 37°C in a humid and light-protected incubator for 1h in the reaction mixture of a leukocyte acid phosphatase assay kit (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. 387), 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 [24].

TRAP Activity (Tartrate-Resistant Acid Phosphatase)

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 minutes and ethanol-acetone (1:1) for 1 minute. Subsequently, the dried cells were incubated in 50.0 mM citrate buffer (pH 4.5) containing 10.0 Mm sodium tartrate and 6.0 mM PNPP. After 1h 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 [24].

Molecular docking into DNA binding site of NF-kappaB

All the six substituted isoflavones analogues were prepared by using chemdraw ultra 8.0. All ligands were prepared through Auto Dock Tools. The 3D crystal structure of the NF-kappaB was obtained from Protein Data Bank (PDB code: 1NFK.The 3D structures of NF-kB, p50-p50 homo dimer (from 1NFK), was used for virtual screening. Docking parameters were set to default values on the basis of Lamarckian genetic algorithm principle. Autogrid program of AutoDock suit was used for generation of grid around binding pocket within target protein. DNA fragment was present in the binding pocket of NF kappa B which was removed and grid was generated on same position in the binding cavity [21].

Results

Isolation and characterization: All the compounds were characterized and identified by IR, 1H-NMR, 13CNMR 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*=8.64Hz), 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 (rel. int): 315.45 [M]$^+$(189.20) 301 [M-CH_3]$^+$ (45.94), 294 [M-6H]$^{+2}$(13.12), 285 [M-CH_3]+(36.79), 269 [M-CH_3-O]+(12.05), 253 [M-CH_3-O-O]+(63.63), 237 [M-CH_3-O-O-O]+(10.32), 208 [M-CH_3-O-O-CO_2]+(7.01), 201 [M-C_6H_4CO ring B]+(6.63), Ring B = m/z 100. 187 [M-CH_3-O-O-O-C_4H_2]+(61.82), 178 [M-C_6H_4CO-C=C-]+(28.26), 162 [M-C_6H_4CO-C=C-O]+(34.94), 146 [M-C_6H_4CO-C=C-O-O-]+(22.05), 136 [M-C_6H_4CO-C=C-O-C_2H_2]+(7.05), 117 [M-CH_3-3O-ring A] (4.91). Ring A = m/z116. 107 [M- C_6H_4CO-C=C-O-C_2H_2-CHO] (59.13). 85 [117-20] (100).

From IR, ^{1}H-NMR, ^{13}C- NMR and mass spectrometry data, the structure was assigned as (1a).

The structure was further confirmed by 2- D NMR (Homo and Hetero) details given in supplementary file.

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). The IR value 1760 cm^{-1} (C=O) also clearly indicates the presence of acetyl groups which is the characteristics of phenol acetate. As there is no any peak of hydroxyl group was found which also confirms that acetylation has been taken place. The ester bond was further confirmed by strong peak at 1182 cm^{-1} ^{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).

From ^{1}H-NMR data δ (ppm) value was found at 2.31, 2.38, 2.46 (3-(3H), s, 8,7, and 5-H), it has been clear the presence of 3- acetyl groups, which indicates each of the three hydroxyl groups were located on C-5, C-7 and C-8. The important fact that C-6 has shown very down field δ, value at 7.19 ppm (1H, s, 6-H), this again indicates the presence of glycosidic linkage was at C-7 position. ^{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-CH_3, 21.16, 21.07, 20.71). (2-C,19.3). Mass spectrometry: TOF MS ES$^+$ 2.98e.3 m/z (rel. int): 441[M]$^+$ (345.45), 427 [M$^+$-CH_3] (73.45), 429 [M{2D}+{Wallström, 2000 #3}]$^+$ (5.77), 425[M-2H]$^+$ (6.73), 385[M-2H-$COCH_3$]+ (100), 289[M-2H-$COCH_3$ -$C_6H_5OCH_3$]+(7.45), 269[M{2D}-2H-3×C_2H_3O]+(7.88), 273 [M-2H-$COCH_3$ -$C_6H_5OCH_3$ -CH_3]+(12.34), 244[M-2H-$COCH_3$ -$C_6H_5OCH_3$ -CH_3 -CO]+(4.78), 228[M-2H-$COCH_3$ -$C_6H_5OCH_3$ -CH_3 - CO-CH_3]+(3.04), 212[228-O]+(8.01), 181[212-CH_2O](8.01), 126[M-2H-3×C_2H_3O-ring A]+(5.84), Ring A= m/z142, 117[269-ringB-H]+(5.34). Ring B m/z151.

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. In the ^{1}H-NMR ($CDCl_3$) of **(1c)** all the signals remains unchanged except the up field displacement of the signal for the C-3'and C-5' proton from δ value at 7.155- 6.970 ppm indicating the presence of one hydroxyl group at C-4' position in ring B. ^{1}H-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*=8.60Hz), 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 SaSO-2Cell Line

The clonal osteoblast-like UMR 106 and SaSO-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). They produce bone-specific type I collagen and have similar resting membrane potentials to those of osteoblasts. Taking advantage of their higher purity and faster proliferation rate than those of osteoblasts, UMR106 and SaSO-2 cells were used for screening of the stimulation of six flavones from *Iriss germanica* of bone formation. The results were incorporated in table 1.

Compounds		Concentration (µg/ml)	Stimulation (%)	
			UMR 106.6	SaOS-2
1	1a	100	98	97
2	1b	100	33	37
3	1c	100	92	94
4	2a	100	91	90
5.	2b	100	26	15
6	2c	100	36	30
7.	Diazedein (Positive Control)	100	100	100

Table 1: *Stimulation Rates (% of Control) at the Doses of 100µ g/ml on Proliferation of the Osteoblast-Like UMR106 and SaSO-2 Cell Lines.*

% Inhibition of Six compounds on formation of Osteoclast-Like Cells (RAW264.7 Cell lines): The % inhibition of all isolated compounds and its analogues were done on RAW 264.27 cell line. The results were summarized in table 2.

Compounds		Concentration (µg/ml)	Inhibition (%) RAW264.7 Cell lines
1	1a	20	165.3
2	1b	20	58
3	1c	20	163
4.	2a	20	142
5	2b	20	45
6	2c	20	30
7.	Elcitonin (positive control)	2 U/ml	170.8

Table 2: *% Inhibition of Six compounds on formation of Osteoclast-Like Cells (RAW264.7 Cell lines).*

MTT Assay

To date, the anti-osteoporosis effects of compounds isolated from *Iriss germanica* have not been reported. Therefore, we assayed the anti-osteoporosis activity of six isoflavones on RAW 264.7 cells (See 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 five days differentiation period.

Sl. No	Compounds	RAW264.7 (IC_{50}Value) µg/mL	% TRAPE Activity tested at 20 µg/mL for each compound (RAW264.7)
1	1a	5.2	66.67 ± 2.71
2	1b	44.4	28.98 ± 3.07
3	1c	4.2	63.92 ± 2.12
4	2a	6.3	57.32 ± 2.46
5	2b	54.6	14.39 ± 2.62
6	2c	34.5	16.98 ± 1.05
7	Raloxifene	2.3	
8	Daidzein		70.34 ± 1.87

Table 3: *IC50 of isolated compounds and its analogues towards RAW 264.7 cell lines by MTT assay method, after 48h. TRAP activity was measured from cultures after 5 days of treatment with RANKL and test compounds (20.0 µg/mL).*

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

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
Diazdein	-8.03

Table 4: *Estimated free energy of binding of isolated compounds in the target NF-kappaB as homo dimer (p50-p50). 3D structures of NF-kappaB, p50-p50 homo dimer (from 1NFK), was used for virtual screening.*

Discussion

Isolation and Characterization

All isolated compounds and its analogues were characterized and identified (See Figure 1A and Figure 1B),

Biological Activity

Stimulation of Proliferation of Osteoblast-Like UMR106.6 and SaSO-2 Cell Line

The clonal osteoblast-like UMR 106 and SaSO-2 cell lines were used for the screening of the stimulation of six isolated compounds isolated from *Iriss germanica* of bone formation, derived from a rat osteogenic sarcoma, has many of the enzymatic properties of normal

osteoblasts (including high alkaline phosphatase activity and parathyroid hormone-simulated adenyl cyclase activity). They produce bone-specific type I collagen and have similar resting membrane potentials to those of osteoblasts.

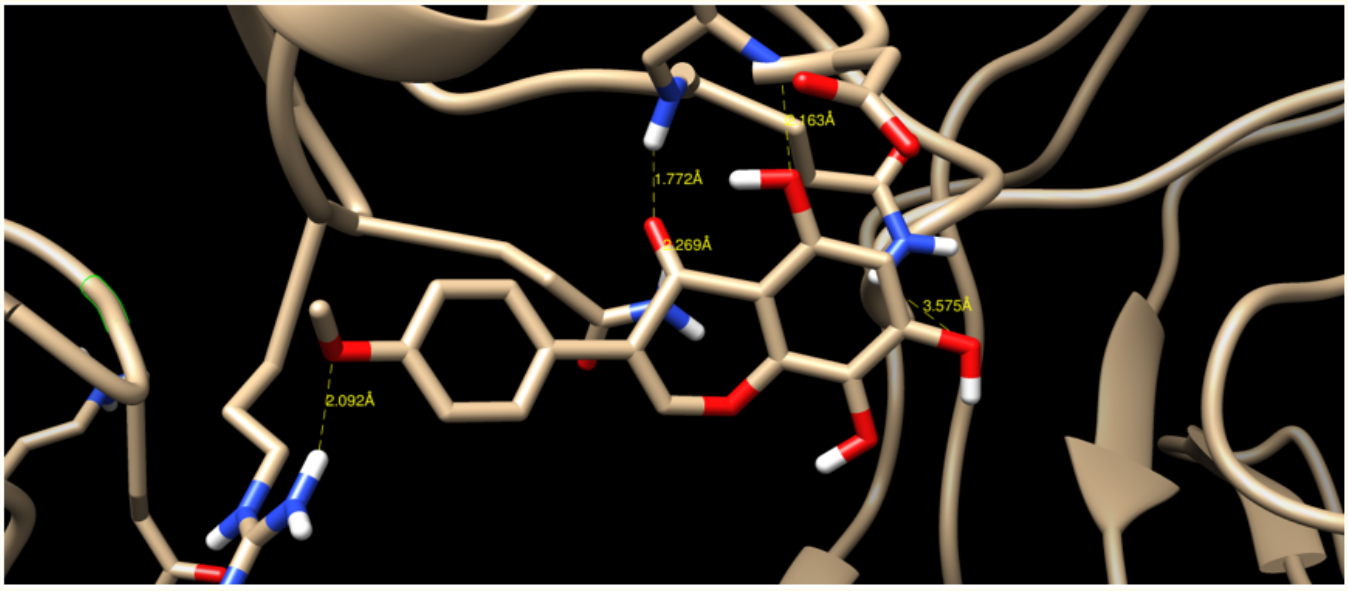

Figure 2: *Stereoview of the complex formed by NF-kappaB and the docked compound (1b). The amino acids Gln 274, Gln 306 , Arg 305, Lys 275 and Asp 276 were involved in interaction with compounds.*

Among the six compounds, the **1a, 1c** and **2a** showed strongest activity, with the stimulation rate of 90 - 98 % against both the cell lines (See Table 1). The high potential of stimulation rate of these compounds were 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 (UMR 106 and SaSO-2) presumably, because of the conversion of hydroxyl group to either acetoxo or methoxyl groups. The law stimulation rate appears to be associated with an adequate interactions between pharmacophores (acetoxy/ methoxy) and receptor cavity. These results suggest 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 than the positive control Diazedein, which is used to treat osteoporosis and was reported to stimulate proliferation of bone- forming cells [10].

% Inhibition of Six compounds on formation of Osteoclast-Like Cells (RAW264.7 Cell lines)

We determined the inhibitory activity of flavones and its analogues (six compounds) on the formation of osteoclast-like cells (RAW 263.7). Among the six flavones examined, the **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**, **1c**) have shown inhibitory activity

nearly identical to the standard molecule (Elcitonin). However, the compounds **1b**, **2b** and **2c** have showed weak inhibition effect at the tested concentration. The results were not sufficient for discussion regarding the structure-activity relationship of isolated compounds and its analogues. However, more studies may be required for the understanding of their selective potential of inhibition. 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-osteoprotic agents. So, the compound must be further subjected for *in vivo* analysis followed by pre-clinical study.

MTT Assay

The cytotoxic activities of the isolated compounds (six) were measured using MTT assay, which is commonly used for cytotoxic assessment and provides important information regarding the cytotoxic potential of biological samples. 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 (see Table 3), However, no significant cytotoxic effects were observed for the compounds **1b**, **2b** and **2c** with IC_{50} values ranging from 34.5 to 54.6 µg/mL (see Table 3) as compared to standard compound Raloxifene (IC_{50} value 2.3). 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 flavones nucleus. Moreover, the interaction of pharmacophore with active site of the receptors play an important role for the cytotoxic potential of the isolated compounds. However, a significant cytotoxic effect was observed. Thus, these results suggest that **1a** and **1c** possess both anti-osteoclastogenic activities and cytotoxic effects.

MTT Assay

The anti-osteoporotic activities of compounds (six) were evaluated based on the suppression of excessive bone breakdown by osteoclasts. The results showed that is flavone derivatives **1a**, **1b**,**1c**, **2a**, **2b** and **2c** suppressed osteoclast formation in a dose dependent manner with TRAP values ranging from 14.39 ± 2.62 to 66.67 ± 2.71% at concentration of 20 µg/mL (see 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. In other hand, the significant anti-osteoporotic activities of is flavonoid derivatives **1a**, **1c** and **2a** showing significant TRAP activities *in vitro* may be attributed to their strong ligand-receptor binding interactions. Therefore, more research may be needed to determine the significant potential of compound **1a**, **1c** and **2b** by docking study, *in vivo* and pre-clinical study.

Drug Receptor Interaction Study

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 to -8.50 Kcal/mole (see 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)**. The residues involved in hydrogen bond interaction were Gln 274, Gln 306, Arg 305, Lys 275 and Asp 276 in **(1a)** (Figure) and Ser 303, Ser 347, Thr 352, Thr 355, Asp 347 and Glu 488 in **(1c)** with the active site of NF-kappaB. Virtual screening of six isolated flavone and its analogues were done. Among all compounds top ranked compounds **(1a)** and **(1c)** have lowest estimated free energy of binding (-7.98 and -7.84 Kcal Kcal/mole respectively), also had promising anti-osteoprotic activity through inhibition of NF-kappaB. The excellent interactions of NF-kappaB with two top ranked compounds **(1a)** and indicated a high degree of coherent relationship between *in silico* approach and *in vitro* studies.

High anti-osteoprotic activity and excellent interaction profile of compounds (flavone and its analogs) demands 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-osteoprotic agents.

Conclusion

Two novel flavones were isolated from *Iriss germanica* and further its analogues were synthesized and characterized. *In-vitro* screening of isolated flavones and its analogues were carried out for anti-osteoporotic activity using NF-kappa B as a target. Isolated flavones and its analogues 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 i.e. 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 flavones 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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