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,

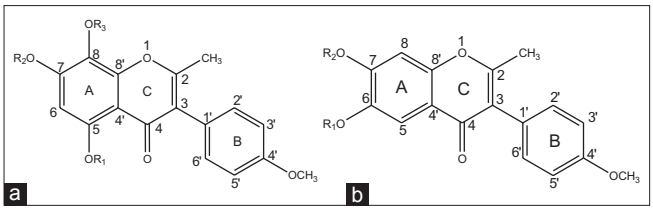

Figure 1: (a and b) Structure of isoflavones. (1A)-(1a) R1, R2, R3 = H; (1b) R1, R2, R3 = OCOCH3; (1c) R1, R2, R3 = H; OCH3 = OH. (1B)-(2a) R1, R2 = H, (2b) R1 = CH3; R2= – OCOCH3 (2c) R1 = CH3; R2 = CH3

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.

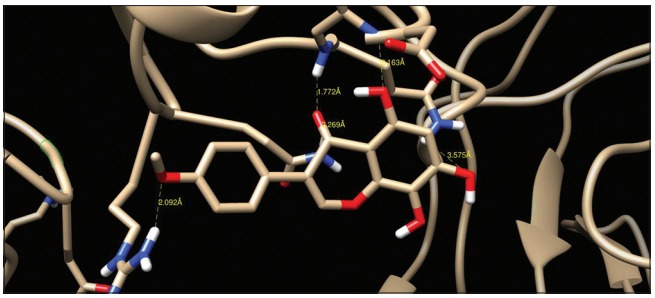

Figure 2: Stereoview of the complex formed by NF-kappaB and the docked compound (1b).The amino acids Gln 274, Gln 306, Arg 305, Lys 275, and Asp 276 were involved in interaction with compounds

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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Nil.

Conflicts of interest

There are no conflicts of interest.

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