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Osteogenic activity of constituents from *Butea monosperma* ☆

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ABSTRACT

Phytochemical investigation from the stem bark of *Butea monosperma*, led to the isolation and identification of three new compounds named buteaspermin A (**1**), buteaspermin B (**2**) and buteaspermanol (**3**), along with 19 known compounds. The structure of compounds **1**–**22** were established on the basis of their spectroscopic data. The isolated compounds **2**–**17** were evaluated using neonatal (1–3 day old) rat calvaria derived primary osteoblast cultures. Five of these compounds **7**, **10**–**13** showed promising osteogenic activity, attributed to increased osteoblast proliferation, differentiation and mineralization as evidenced by marked increase in expression of alkaline phosphatase, an early phase differentiation marker, and alizarin Red S staining of osteoblasts cultured for 48 h and von Kossa silver staining of nodules formed 15 days after culture with these compounds. Quantification of mineralization by optical density measurement of Alizarin Red S extracted from stained osteoblasts cultured for 7 days in presence of these compounds showed significant ($P < 0.05$, vs corresponding vehicle control group) increase in mineralization. On the basis of biological results, structure–activity relationships are discussed.

Butea monosperma (Lam.) Taub (Syn. *Butea frondosa*; Family Fabaceae) popularly known as Flame of the Forest, *Dhak*, *Palas* or 'Bastard teak' has proven to be a source of constitutive osteogenic agents belonging to isoflavonoid and pterocarpan groups. The genus *Butea* includes *Butea monosperma, Butea parviflora, Butea minor* and *Butea superba* widely distributed throughout India.[1] The roots of *B.* monosperma are useful in the treatment of night blindness and other eye diseases.[2] The stem bark of *B. monosperma* displays antifungal activity, which is due to the presence of an active constituent (−)-medicarpin.[3] The leaves of *B. monosperma* exhibit ocular anti-inflammatory activity in rabbits[2] and antimicrobial activities.[4] An extract from the flowers of this plant is used in India for the treatment of liver disorders and two antihepatotoxic flavonoids, isobutrin and butrin have been isolated.[5] It shows anticonvulsive activity, due to the presence of a triterpene.[6] Alcoholic extract of flowers of the title plant has also been reported to exhibit antiestrogenic[7,8] and antifertility[9] activities. Butin isolated from its flowers show both male and female contraceptive properties.[10] Previous phytochemical examination of this plant indicated the presence of flavones and flavanols,[11] chalcones,[12–14] isoflavones,[15,16] pterocarpans,[15,17,18] leucocyanidin tetramer,[19] as well as triterpens[15] and sterols.[20]

In the present study, we focus on bioassay-guided fractionation of ethanolic extract of stem bark of *B. monosperma*, which has been found to exhibit osteogenic activity in vitro. Its bioassay-directed fractionation studies have led to the isolation of ten isoflavones, four coumestans, two pterocarpans, one flavanonol, two triterpenes and three fatty esters. Remarkably, *B. monosperma* is clearly an abundant source of isoflavonoids.

Liquid–liquid partition of the ethanol (EtOH) extract using hexane, chloroform, butanol and water, yielded four fractions. Using alkaline phosphatase expression and mineralization of calvarial osteoblasts as parameters, potent osteogenic activity was observed in butanol soluble fraction of the extract, whereas low order of activity was seen in chloroform soluble fraction. Hexane and aqueous fractions were found to be inactive. Active chloroform and butanol soluble fractions were purified by repeated column chromatography over silica gel afforded pure compounds. These compounds were identified by detailed spectroscopic studies. The known compounds (**4**–**22**) were identified comparing their spectroscopic data with those previously reported in literature. Chloroform fraction yielded ten compounds: lupeonone (**18**),[15,21] lupeol (**19**),[22] flemmichapparin C (**4**),[23] 3-*O*-acetyl-8,9-methylenedioxycoumestan (**5**),[24] 3-methoxy-8,9-methylenedioxy pterocarp-6-ene (**6**),[17] medicarpin (**7**),[25] nonacosanoic acid 2′,3′-dihydroxy-propyl ester (**22**),[26] pentacosanoic acid 2,3-dihydroxy-propyl ester (**21**),[27] docosanoic acid (**20**),[28] buteaspermin A (**1**). While butanol soluble fraction furnished 12 compounds: 2-methyl, 7-hydroxy, 4′-methoxy isoflavone (**8**),[29] buteaspermin

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B (**2**), buteaspermanol (**3**), prunetin (**9**),[30] cajanin (**10**),[31] formonentin (**11**),[32,33] isoformonentin (**12**),[34] cladrin (**13**),[35] daidzein (**14**),[36] genistein (**15**),[37,38] 2′,4′,5,7-tetrahydroxy isoflavone (**16**),[39] ononin, (**17**).[40] Among these, compounds **6**, **7**, **9**, **18**–**20** have been reported from this plant, compounds **4**, **5**, **10**–**17**, **21**, **22** have been reported from the title plant for the first time and compound **8** is synthetically known but has been isolated for the first time from natural source. In the present communication, we describe the isolation and characterization of three new compounds named buteaspermin A (**1**), buteaspermin B (**2**) and buteaspermanol (**3**), and evaluation of osteogenic activity of compounds **2**–**17**.

The compound **1** was obtained as light yellow crystals, mp 211–213 °C. The FAB-mass spectrum of compound **1** showed a $[M+H]^+$ peak at m/z 365 consistent with the formula $C_{21}H_{16}O_5$, which was confirmed by ^1H and ^{13}C NMR and DEPT experiments. Compound **1** gave a blue coloration with $FeCl_3$. It showed UV pattern (λ_{max} 345, 244, 208 nm) characteristic of coumestan chromophore.[41] IR absorption band at ν_{max} 3352, 1719, 1601, 1429, 1261 cm^{-1} indicated the presence of hydroxyl group, unsaturated lactone ring and aromatic ring.

The ^1H and ^{13}C NMR spectra together with a DEPT experiment of **1** (Table 1) indicated the presence of a lactone carbonyl (δ 157.1), four isolated aromatic proton singlet at δ 7.18 (1H, s, H-1; δ_C 130.1), 6.93 (1H, s, H-4; δ_C 106.9), 7.50 (1H, s, H-7; δ_C 107.9), and 7.25 (1H, s, H-10; δ_C 99.5) and two proton singlet at δ 6.15 (δ_C 91.3) for methylene dioxy group. Further, ^1H and ^{13}C NMR spectra displayed signals of a prenyl group δ 1.75 (3H, s, $-CH_3$; δ_C 17.7), 1.78 (3H, s, $-CH_3$; δ_C 25.9), 3.29 (2H, d, J = 6.9 Hz, $-CH_2$-; δ_C 29.0) and 5.31 (1H, m, =CH–; δ_C 121.2). The placement of prenyl group was assigned to C-2 position from the HMBC spectrum of **1**, C-1′ proton (δ 3.29) exhibited correlations with the carbon resonating at δ 116.5 (C-2), 130.1 (C-1) and 164.1 (C-4). Therefore, the structure is represented by **1**, 3-hydroxy-2-preyl, 8,9-methylenedioxy benzo[4,5] furo [3,2-*c*] chromen-6-one, a new compound designated as buteaspermin A.

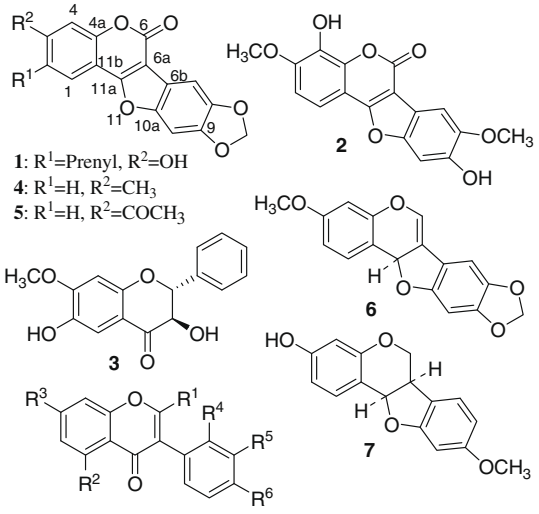
The compound **2** was obtained as off-white crystals, mp 310–311 °C and gave a blue coloration with $FeCl_3$. It showed UV pattern (λ_{max} 345, 244, 208 nm) characteristics of coumestan chromophore.[42] IR absorption band at 3350, 1716, 1608, 1427, 1253 cm^{-1} indicated the presence of hydroxyl group, unsaturated lactone ring, and aromatic ring. The FAB-MS furnished an $[M]^+$ peak at m/z 328 corresponding to molecular formula $C_{17}H_{12}O_7$ which was confirmed by elemental analysis.

Table 1
^1H and ^{13}C data of compound **1** in $CDCl_3$

Position	δ_H (J in Hz)	δ_C
1	7.18 s	130.1
2	–	116.5
3	–	164.1
4	6.93 s	106.9
4a	–	153.2
6	–	157.1
6a	–	121.0
6b	–	118.7
7	7.50 s	107.9
8	–	142.4
9	–	143.2
10	7.25 s	99.5
10a	–	150.0
11a	–	163.8
11b	–	121.1
$-OCH_2O-$	6.15 s	91.3
1′	3.29 d (6.9)	29.0
2′	5.31 m	121.2
3′	–	134.6
4′	1.75 s	17.7
5′	1.78 s	25.9

The ^1H and ^{13}C NMR spectra together with a DEPT experiment of **2** (Table 2) indicated the presence of a lactone carbonyl (δ 161.0), two *ortho*-coupled proton at δ 7.54 (1H, *d*, J = 8.6 Hz, H-1; δ_C 116.6) and δ 7.00 (1H, *d*, J=8.6 Hz, H-2; δ_C 114.1) assigned to ring A, two singlets at δ_H 7.42, δ_C 102.2 and 7.20, δ_C 99.4 corresponding to one proton each assigned at C-7 and C-10 positions, respectively, thus confirming the substitution of C-8 and C-9 positions. Further, NMR spectra showed two methoxyl signals at δ 4.02 (3H, *s*, δ_C 61.0) and 3.99 (3H, *s*) (δ_C 56.4) and two D_2O exchangeable hydroxyl groups at δ 9.07 (1H, *s*) and 9.96 (1H, *s*). The signals at δ_C 102.6, 105.5, 114.4, 135.4, 147.2, 147.1, 154.0, 157.0, 149.8 and 159.4 were quaternary carbons. Further, the quaternary signals at δ_C 147.2, 147.1 and 159.4, 149.8 revealed the presence of *ortho*-oxygen substituted carbons at C-8, C-9 and C-3, C-4, respectively.

Presence of two hydroxyl groups at C-4 (δ 9.07) and C-9 (δ 9.96), and that of two methoxyl groups at C-3 (δ 4.02) and C-8 (δ 3.99) was confirmed by HMBC and UV experiments. In HMBC (Heteronuclear Multiple Bond Connectivity) spectrum of **2**, methoxyl protons at C-3 (δ 4.02), C-8 (δ 3.99) exhibited correlation with carbon resonating at δ_C 61.0, δ_C 56.4, respectively. Similarly, the C-1 proton (δ 7.54) showed correlations with carbons resonating at δ 159.4 (C-3), 135.4 (C-4a) and 154.0 (C-11a). C-2 proton (δ 7.00) showed correlations with carbons resonating at δ 149.8 (C-4), and 102.6 (C-11b). Further, C-7 proton (δ 7.42) displayed interactions with carbons resonating at δ 105.5 (C-6a), 147.1 (C-9) and 157.0 (C-10a) while C-10 proton (δ 7.20) exhibited interactions with carbons resonating at δ 114.4 (C-6b) and 147.2 (C-8). UV spectrum of **2** showed no bathochromic shift on addition of shift reagents ($AlCl_3$, H_3BO_3), indicating absence of free *ortho*-dihydroxy system.



Thus, based on these observations, **2** was characterized as 4,9-dihydroxy 3,8-dimethoxy-benzo[4,5]furo[3,2-*c*]chrom-en-6-one. This is the new compound named buteaspermin B.

The compound **3** was obtained as greenish crystals, mp 210–211 °C, $[\alpha]_D^{22}$ +13.3° (c, 0.06, methanol). The FAB-mass spectrum

Table 2
NMR spectral data of compound **2**

Position	δ_H (J in Hz) in $CDCl_3$ + DMSO-d_6	δ_C in DMSO-d_6
1	7.54 d (8.6)	116.6
2	7.00 d (8.6)	114.1
3	—	159.4
4	—	149.8
4a	—	135.4
6	—	161.0
6a	—	105.5
6b	—	114.4
7	7.42 s	102.2
8	—	147.2
9	—	147.1
10	7.20 s	99.4
10a	—	157.0
11a	—	154.0
11b	—	102.6
4-OH	9.07 s	—
9-OH	9.96 s	—
3-OCH_3	4.02 s	61.0
8-OCH_3	3.99 s	56.4

of compound **3** shows a $[M+H]^+$ peak at m/z 287 consistent with the formula $C_{16}H_{14}O_5$ which was confirmed by ^1H and ^{13}C NMR and DEPT experiments. IR spectrum revealed hydroxyl group (3333 cm^{-1}), aromatic ring (1515 and 1471 cm^{-1}) and a carbonyl group (1653 cm^{-1}) present in the molecule. The UV spectrum of the compound **3** in methanol showed absorption maxima at λ_{max} 216, 235, 276 and 341 nm; no bathochromic shift was recorded on addition of sodium acetate.

The ^1H and ^{13}C NMR (Table 3) spectra suggested that **3** was a dihydroflavanol type of compound as H-2 and H-3 was observed at δ 5.14 (1H, *d*, J = 11.6 Hz; δ_C 72.9) and δ 4.53 (1H, *dd*, J = 3.7, 11.6 Hz; δ_C 84.0), respectively, and a hydroxyl signal exchangeable with D_2O was observed as a doublet at δ 5.68 (1H, J = 3.7 Hz, 3-OH). In the ^1H NMR spectrum, the presence of two singlets at δ 7.19 (1H, H-5; δ_C 107.6) and δ 6.43 (1H, H-8; δ_C 103.6) indicated that A-ring was disubstituted. To clarify the position of methoxy group δ 3.80 (3H, s, δ_C 56.2), HMBC NMR spectrum of **3** was recorded. The methoxy proton (δ 3.80) exhibited long-range correlation with δ 155.3 (C-7) suggesting that methoxyl group is located at C-7 position. Further, **3** showed positive cotton effect for $n \rightarrow \pi^*$ transition and negative for $\pi \rightarrow \pi^*$ transition, which was comparable with the known compounds (2*R*,3*R*)-3,7-dihydroxy,6-methoxy flavanone[43] and (2*R*,3*R*)-3,6,7-trihydroxyflavanone.[44] This suggested 2*R*,3*R*-configuration for compound **3**. Thus, based on these observations, **3** was characterized as 3,6-dihydroxy-7-methoxyflavanone, designated as buteaspermanol. This is a new compound isolated for the first time from plant source.

Table 3
^1H and ^{13}C spectral data of compound **3** in DMSO-d_6

Position	δ_H (J in Hz)	δ_C
2	5.14 d (11.6)	72.9
3	4.53 dd (3.7, 11.6)	84.0
4	—	192.5
5	7.19 s	107.6
6	—	137.9
7	—	155.3
8	6.43 s	103.6
9	—	157.5
10	—	111.0
1′	—	144.3
2′	7.55 m	128.8
3′	7.48–7.41 m	128.5
4′	7.48–7.41 m	128.3
5′	7.48–7.41 m	128.5
6′	7.55 m	128.5
3-OH	5.68 br d (3.7)	—
7-OCH_3	3.80 s	56.2

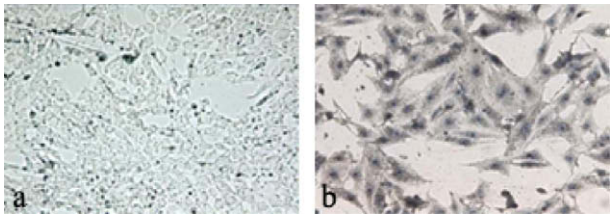


Figure 1. Primary osteoblast cells derived from calvariae of neonatal (1–3 day old) female Sprague–Dawley rats and cultured for 48 h in α-MEM. Osteoblasts arise from pluripotent stem cells of fibroblast, chondrocyte and adipocyte lineage, synthesize collagen, secrete calcium and form new bone (a) unstained, (b) hematoxylin stained osteoblasts.

In preliminary screening, EtOH extracts of leaves, twigs, flowers, seeds and stem bark of *B. monosperma* were evaluated for in vitro osteogenic activity using neonatal rat calvaria derived primary osteoblast cultures (Fig. 1). Of these, only the ethanolic extract of stem bark presented significant osteogenic activity. This was based on marked increase in expression of alkaline phosphatase, an early phase differentiation marker (Fig. 2a and b), and Alizarin Red S staining (Fig. 2c and d) of osteoblasts cultured for 48 h and von Kossa silver staining of nodules formed 15 days after culture (Fig. 2e and f) with these compounds. Quantification of mineralization by optical density measurement of Alizarin Red S extracted from stained cultures showed significant ($P < 0.05$, vs corresponding vehicle control group, Fig. 3) increase in mineralization of osteoblasts cultured for 7 days in presence of these compounds. Based on these findings, compound **7** was found to be most potent followed by compounds **10**–**13**. The remaining compounds were either very weakly active or inactive.

Structure–activity relationship studies reveal that the presence of a free hydroxyl group at C-7 and methoxyl group at C-4′ (**13**, **11**)

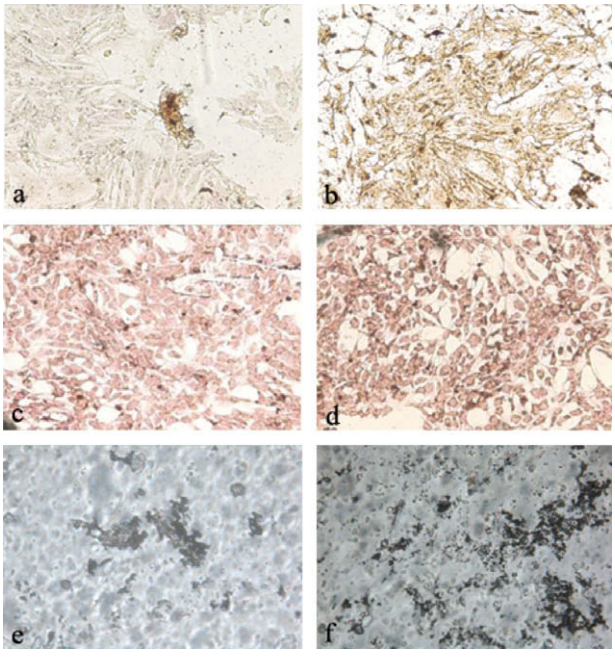


Figure 2. Representative photographs of alkaline phosphatase expression (a and b) and Alizarin Red S stained nascent calcium deposition (c and d) in neonatal rat calvarial osteoblasts cultured for 48 h and von Kossa stained nodule formation (e and f) by the osteoblasts cultured for 15 days. Increase in these parameters as seen in treated versus corresponding vehicle control groups demonstrate osteogenic potential of the test agent.

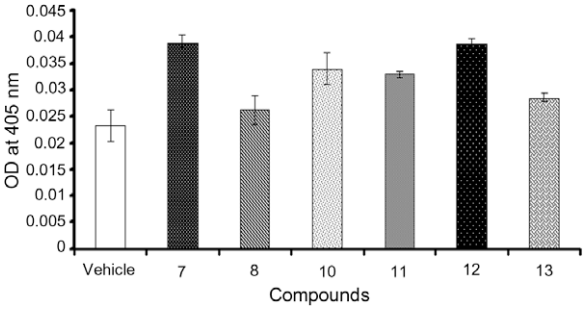


Figure 3. Quantification of mineralization (mean ± SEM) in osteoblasts after 7 days of culture as determined by acetic acid extraction of Alizarin Red S from stained cultures and measurement of optical density. *Note.* Significant increase in mineralization of osteoblasts cultured in presence of 5 of the 19 compounds evaluated in this study. Results are representative of three independent experiments.

or presence of free hydroxy group at C-4′ and methoxy group at C-7 (**10**, **12**) in isoflavones and free hydroxyl group at C-3 and methoxy group at C-9 in pterocapan (**7**) appear essential for in vitro osteogenic activity. It appears that the methoxyl substituent was an important unit for eliciting better osteogenic activity. The compounds **2**, **8** and **9** though possessing this moiety were either less active or inactive. This might be due to the presence of other substituents. In addition, presence of all the free hydroxy groups or all the substituted hydroxyl groups do not appear essential for this activity, as compounds (**14**, **15**, **16** and **17**) were found to be inactive. Presence of methylenedioxy moiety at C-8/C-9 position also seems non-essential for the activity since **4**, **5** and **6** were inactive compounds.

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Supplementary data

Experimental procedures, biological evaluation methods are provided. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.12.064.

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