Cycloartane and Stigmastane Type Triterpenoids from *Pothos scandens* Inhibit Estradiol (E_2) Induced Proliferations in Breast Cancer Cells

Md. Abdul Muhit[1], Kaoru Umehara[2], Nahid Sharmin[3] and Hiroshi Noguchi[2]

[1] Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka Dhaka 1000, Bangladesh
[2] School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan
[3] Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Dhaka Dhaka 1000, Bangladesh

(Received: April 07, 2019; Accepted: May 20, 2019; Published (Web): June 30, 2019)

ABSTRACT: Four cycloartane type triterpenoids and three stigmastane type steroids were isolated from the methanolic extract of stem and root part of *Pothos scandens* L. (Araceae), a Bangladeshi medicinal plant by high performance liquid chromatographic technique. The compounds were characterized as 24-methylenecycloartanol (**1**), 24-methylenecycloartenone (**2**), 24-en-cycloartenone (**3**), 24-methylenecycloartanyl ferulate (**4**), stigmast-4-en-3-one (**5**), stigmast-4,22-diene-3-one (**6**) and *β*-sitosterol glucoside (**7**) through extensive 1D and 2D NMR spectroscopic studies. All the isolates were evaluated for their estrogenic/antiestrogenic activity using the estrogen-responsive breast cancer cell lines, MCF-7 and T47D. The results showed that all the compounds possess mild to strong antiestrogenic activity in both cell lines which was compared to positive control tamoxifen. 24-Methylenecycloartanol (**1**), which contains a hydroxyl group in its C-3 position, inhibited 90% of estradiol (E_2)-induced cell proliferation in MCF-7 and T47D cell lines at a concentration of 0.01 µM only. 24-Methylenecycloartanyl ferulate (**4**) and stigmast-4,22-diene-3-one (**6**) showed 90% of estradiol (E_2)-induced cell proliferation in T47D cell only at a concentration of 0.01 µM whereas 10.0 µM was required for 24-methylenecycloartanyl ferulate (**4**) for the same activity in MCF-7 cells. This is the first report of isolation of these compounds from the plant along with their antiestrogenic property.

Key words: *Pothos scandens*, Araceae, cycloartane, stigmastane, antiestrogenic activity, Batilata

INTRODUCTION

17*β*-Estradiol (E_2), one of the primary circulating ovarian steroids, is predominantly responsible for the development and regulations of reproductive organs and secondary sex characteristics in female. The development of breast epithelium is mainly regulated by E_2 but excessive level of it may cause genesis of breast cancer.[1] During the menopause, the level of E_2 is markedly reduced. In response, aromatization of circulating androgens is facilitated causing excessive E_2 production, which increases the risk of breast cancers.[2] The incidence of breast cancer is increasing in developing countries like Bangladesh, due to the lack of proper information, inappropriate sexual life, poor government funding in maternity health, poor birth control, improper lifestyles and lack of facilities for early detection.[3] Previous clinical study suggested that breast cancer and uteri-cervical cancers are the most prevalent for last five years among the women in Bangladesh.[4]

E_2 has also physiological role in lactating mother. Generally, the level of E_2 is significantly decreased during breastfeeding while the level of prolactin hormone is increased for stimulating the mammary glands for milk secretion. High level of

Correspondence to: Md. Abdul Muhit
Phone: +88-01733-982854
E-mail: muhit@du.ac.bd

Dhaka Univ. J. Pharm. Sci. **18**(1): 93-102, 2019 (June)
DOI: https://doi.org/10.3329/dujps.v18i1.41896

estrogen inhibits the milk production in lactating mother.[5] In search of potential leads, several initiatives have been taken worldwide and some of the isolates like khainaoside A, terminalosides, pothobanosides, syringerasinol, principin showed antiestrogenic activity whereas biochanin, tectorigenin, genistein, dalparvins B, C etc. stimulated cell proliferation of estrogen-responsive breast cancer cells.[6-11]

As the majority of the Bangladeshi people are living under the poverty line, many of them tend to afford traditional medicines because of their easy availability, cheaper price, and little or no side effects. Among the 6000 plant species which are enlisted in national encyclopedia of Bangladesh, near about 1000 plant species are medicinally useful and have been documented.[12,13] So far, few attempts have been taken for the scientific evidence of Bangladeshi medicinal plants which have estrogenic and/or antiestrogenic properties.[14]

Pothos scandens L., belonging to a well-diversified family of Araceae, is a medicinal aroid commonly known as 'Batilata' in the tribal people of hill tracts region of Bangladesh. The whole plant is used in treating skin disorders, asthma, snake bite, diarrhoea, cancer, small pox, sprains, epilepsy, convulsions and wound.[14-17] The leaves of the plant is used to induce conception in women in certain part of India such as Tamilnadu.[18-19] Previous phytochemical investigations resulted the isolation of pothobanoside A-C, dodecanoic acid, tetradecanoic acid, phytol, markhamiosides, flavone-di-glycosides, methyl pothoscandensate, *N*-trans-cinnamoyltyramine, *N*-trans-feruloyltyramine, serotobenine and syringeresinol etc. [10]

The present study deals with the extraction and isolation of bioactive secondary metabolites followed by structure elucidations using spectroscopic methods. Estrogenic and/or antiestrogenic properties were also investigated using two different cell lines, MCF-7 and T47D.

MATERIALS AND METHODS

General experimental procedures. ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and 2D NMR spectra were recorded on a JEOL ECX-500 instrument. Chemical shifts are presented in δ (ppm) using tetramethylsilane (TMS) as internal standard and coupling constants (*J*) are expressed in hertz. Powdered Silica gel (Keiselgel 60, 230-400 mesh, Merck KGaA, Dermstadt, Germany) and styrene-divinylbenzene (Diaion HP-20, 250-800 μm particle size, Mitshubishi Chemical Co., Ltd.) were used for column chromatography. Precoated glass plates of silica gel (Keiselgel 60, F_{254}, Merck Co., Ltd., Japan) and RP-18 (F_{254}S, Merck KGaA) were used for TLC analysis. The TLC spots were confirmed under UV light at 254 nm and also by spraying with dil. H_2SO_4 followed by heating. HPLC was carried out mainly with a JASCO model 887-PU pump and an 875-UV variable-wavelength detector. For preparative separation, reversed-phase column (Tosoh TSK gel ODS-80Ts, 12-20 μm, 5.5 × 60 × 2 cm, Nomura Chemical Co. Ltd., flow rate at 45 ml/min with detection at 205 nm) was used whereas Inertsil C8-3, 5 μm, 2 × 25 cm, GL science Co. Ltd., flow rate at 9 ml/min with detection at 205 nm was used for semi-preparative HPLC separations. Further purification was done by semi-preparative column YMC-Pack R&D ODS, 5 μm, 2 × 25 cm, YMC Co. Ltd.

Chemicals. Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Eagle's minimum essential medium (EMEM) and Roswell park memorial institute medium (RPMI-1640) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Penicillin and streptomycin were purchased from Meiji Seika Kaisha Ltd. (Tokyo, Japan). L-glutamine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 17*β*-estradiol and dextran-coated-charcoal (DCC) were procured from Sigma Chemicals (St. Louis, MO).

Plant material. The whole plant was collected from the National Botanical Garden, Dhaka, Bangladesh in the month of September, 2013. The stems and roots were separated immediately from

other parts. The plant was identified by Mr. Sardar Nasir Uddin, Senior Scientific Officer, National Herbarium, Dhaka and a voucher specimen (DACB accession no. 38578) was deposited in the herbarium for future references.

Extraction and isolation. The dried powdered of stem and root parts (2.0 kg approx.) were extracted three times with hot methanol (3 × 15 L) by refluxing for 3 hrs. The extracts were then combined and the solvent was evaporated under reduced pressure at 45^0C to yield a viscous mass of 146 g. The concentrated extract was suspended in water (1.5 L) and partitioned with EtOAc (3 × 1.5 L) to yield dried EtOAc fraction (33 gm) and H_2O-soluble fraction (78 g).

The EtOAc soluble fraction (16.1 g) was subjected to silica gel column chromatography using glass column (6 × 50 cm) and fractionated (150 mL for each fraction) using a hexane: $CHCl_3$-MeOH (95:5) gradient solvent system [4:1, 2:1, 1:1, 0:1, $CHCl_3$-MeOH (9:1, 1:1), 3 L each]. All fractions were collected and pooled by TLC analysis to afford 14 combined fractions.

Among these fractions, fraction F [1.1 g: eluted with hexane: $CHCl_3$-MeOH (95:5) gradient solvent system (2:1)] was subjected to preparative HPLC on Tosoh TSK gel ODS-80Ts column (6 × 60 × 2 cm) using MeCN-H_2O (95:5) as mobile phase with flow rate at 45 mL/min to afford 19 fractions. Fraction F-19 (270 mg; Methanolic wash part) was subjected to semi-preparative HPLC with Inertsil C8-3 column (five times injected) using MeCN-H_2O (97.5:2.5) as mobile phase to afford 7 fractions. From these sub-fractions, fraction F-19-5 (27.5 mg; t_R 46 min) was again subjected to semi-preparative HPLC with YMC ODS column using MeCN-H_2O (97.5:2.5) as mobile phase to afford 24-methylenecycloartenone (**2**) (10.1 mg; t_R 145 min) and 24-methylenecycloartanol (**1**) (1.5 mg; t_R 178 min). Similarly, fraction F-19-4 (44.8 mg; t_R 37 min) was subjected to semi-preparative HPLC with YMC ODS column using MeCN-H_2O (97.5:2.5) as mobile phase to provide 24-en-cycloartenone (**3**) (3.8 mg; t_R 128 min), stigmast-4,22-dien-3-one (**6**) (1.8 mg; t_R 140 min) and stigmast-4-en-3-one (**5**) (20.1 mg; t_R 155 min). Fraction F-19-3 (19.6 mg; t_R 28 min) and F-19-6 (28.8 mg; t_R 52 min) were again subjected to semi-preparative HPLC to afford stigmast-4,22-dien-3-one (**6**) [5.5 mg; t_R 142 min, YMC ODS column, MeCN-H_2O (95: 5), flow rate 9 mL/min] and 24-methylenecycloartanyl ferulate (**4**) [3.5 mg; t_R 188 min, YMC ODS column, MeCN-H_2O (97.5:2.5), flow rate 9 ml/min].

From the combined fractions, fraction K (0.7 g), eluted with chloroform-MeOH (9:1) gradient solvent system, was dissolved in MeOH. This led to precipitation of *β*-sitosterol glucoside (**71**) (43.3 mg; white crystals) which was further purified through recrystallization.

Cell proliferation assay. MCF-7 and T47D human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured. The estrogenic activity (cell proliferation assay) was performed by the protocol described in the previous report.[10] Alamar blue reagent was used to determine the cell concentrations and fluorescence were measured at 590 nm with excitation at 530 nm using a Wallac 1420 ARVOsx multilabel counter (Perkin-Elmer Inc., Wellesley, MA).

Antiestrogenic assay. The antiestrogenic assay was performed according to the procedure described previously.[6] MCF-7 and T47D cells were seeded at a density of (1.0-1.2) x 10^4 cells/well in 96-well plates in 90 µL of 5% DCC-treated, FBS-supplemented RPMI phenol red-free medium. After 3 h incubation, 5 µL of each test compounds at four different concentrations of ranging 0.01 to 10 µM was added to each well together with 5 µL of estradiol (E_2) at a concentration of 20 nM to make final volume 100 µL in each well. Finally, the plates were incubated in a CO_2 incubator for 96 h. Then 5 µL of serially diluted tamoxifen at concentrations ranging from 0.01 to 10 µM, was used as positive control. The result was calculated from the cell populations, and iEqE values of each sample ($iEqE_{50}$, $iEqE_{10}$ and $iEqE_1$) were determined for the required concentrations to inhibit the E_2 effect ($iEqE_{50}$; $iEqE_{10}$; and $iEqE_1$, the

concentration suppressing the E_2 effect to the equivalent level of 50; 10; and 1 pM, respectively). When samples suppressed E_2 activity to the level less than 10 or 50 pM through the concentrations tested, they were categorized as strong (s) or mild (M), respectively.

RESULTS AND DISCUSSION

Isolation and structure identification. The crude methanolic extract of stem and root part of *P. scandens* was subjected to partitioning process between EtOAc and H_2O to get solid mass. Preliminary estrogenic activity screenings with EtOAc soluble fractionate showed moderate inhibitory estrogenic effects in different cell lines at a concentration of 0.2 µg/mL. In continuation with the results, EtOAc soluble fractionate was subjected to silica gel column chromatography and purified by HPLC to yield 7 nonpolar compounds.

Compound **1** was obtained as white amorphous powder. The ^{1}H NMR spectrum (Table 1) showed seven methyl groups, among which three appeared as doublet [δ_H 0.81 (3H, s, Me-30), 0.91 (3H, s, Me-28), 0.97 (6H, s, Me-18 and 29), 0.90 (3H, d, J = 5.5 Hz,

Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) NMR spectral data of compound 1-3 in $CDCl_3$).

Position	1		2		3	
	δ_H (J in Hz)	δ_C[a]	δ_H (J in Hz)	δ_C[a]	δ_H (J in Hz)	δ_C[a]
1	1.62, m	32.1	1.86, td (14.0, 5.5)	33.5	1.87, m	33.6
	1.29, m		1.54, m		1.53, m	
2	2.32, m	30.5	2.71, td (14.0, 5.5)	37.5	2.71, td (14.0, 5.5)	37.7
	2.24, m		2.31, m		2.31, m	
3	3.29, dd (12.0, 4.5)	78.9		216.6		216.6
4		40.6		50.3		50.5
5	1.55, m	47.2	1.72, dd (12.0, 4.0)	48.5	1.71, m	48.7
6	1.51, m	21.2	1.55, m	21.6	1.54, m	21.7
7	1.92, m	26.1	1.92, m	28.2	1.90, m	28.4
	1.31, m		1.31, m		1.33, m	
8	1.59, m	48.0	1.59, m	47.9	1.60, m	48.1
9		20.1		21.2		21.4
10		26.2		26.9		26.3
11	1.40, m	26.6	1.40, m	25.9	1.39, m	26.1
	1.10, m		1.10, m		1.10, m	
12	1.32, m	33.0	1.32, m	32.9	1.32, m	33.1
	1.32, m		1.32, m		1.32, m	
13		45.4		45.5		45.6
14		48.9		48.8		49.0
15	2.00, m	35.7	1.67, m	35.7	1.67, m	35.8
	1.67, m		1.67, m		1.67, m	
16	2.01, m	28.2	2.05, m	26.1	2.05, m	27.0
	1.14, m		1.14, m		1.14, m	
17	1.64, m	52.4	1.64, m	52.4	1.70, m	52.6
18	0.97, s	18.1	1.00, s	18.4	0.99, s	19.5
19	0.57, d (4.0)	29.9	0.79, d (4.5)	29.6	0.79, d (4.5)	29.7
	0.33, d (4.0)		0.57, d (4.5)		0.56, d (4.5)	
20	1.55, m	36.2	1.41, m	36.2	1.67, m	36.1
21	0.90, d (5.5)	18.4	0.90, d (6.0)	18.1	0.88, d (6.0)	18.3
22	1.28, m	35.1	1.13, m	35.1	1.13, m	36.6
	1.28, m		1.13, m		1.13, m	
23	2.09, m	31.5	2.13, m	31.4	2.13, m	25.2
	1.89, m		1.89, m		1.89, m	
24		157.0		156.9	5.10, td (5.5, 1.0)	125.5
25	2.25, m	33.9	2.24, m	33.9		131.1
26	1.02, d (5.0)	22.1	1.02, d (5.0)	22.1	0.90, s	17.8
27	1.03, d (5.0)	21.9	1.03, d (5.0)	21.9	0.92, s	25.9
28	0.91, s	25.5	0.91, s	19.4	0.91, s	18.5
29	0.97, s	14.2	1.05, s	22.9	1.05, s	21.0
30	0.81, s	19.4	1.10, s	20.8	1.10, s	22.4
31	4.73, br. s	106.0	4.72, br. s	106.1		
	4.68, br. s		4.67, br. s			

[a] Assignments are based on HMQC and HMBC experiments.

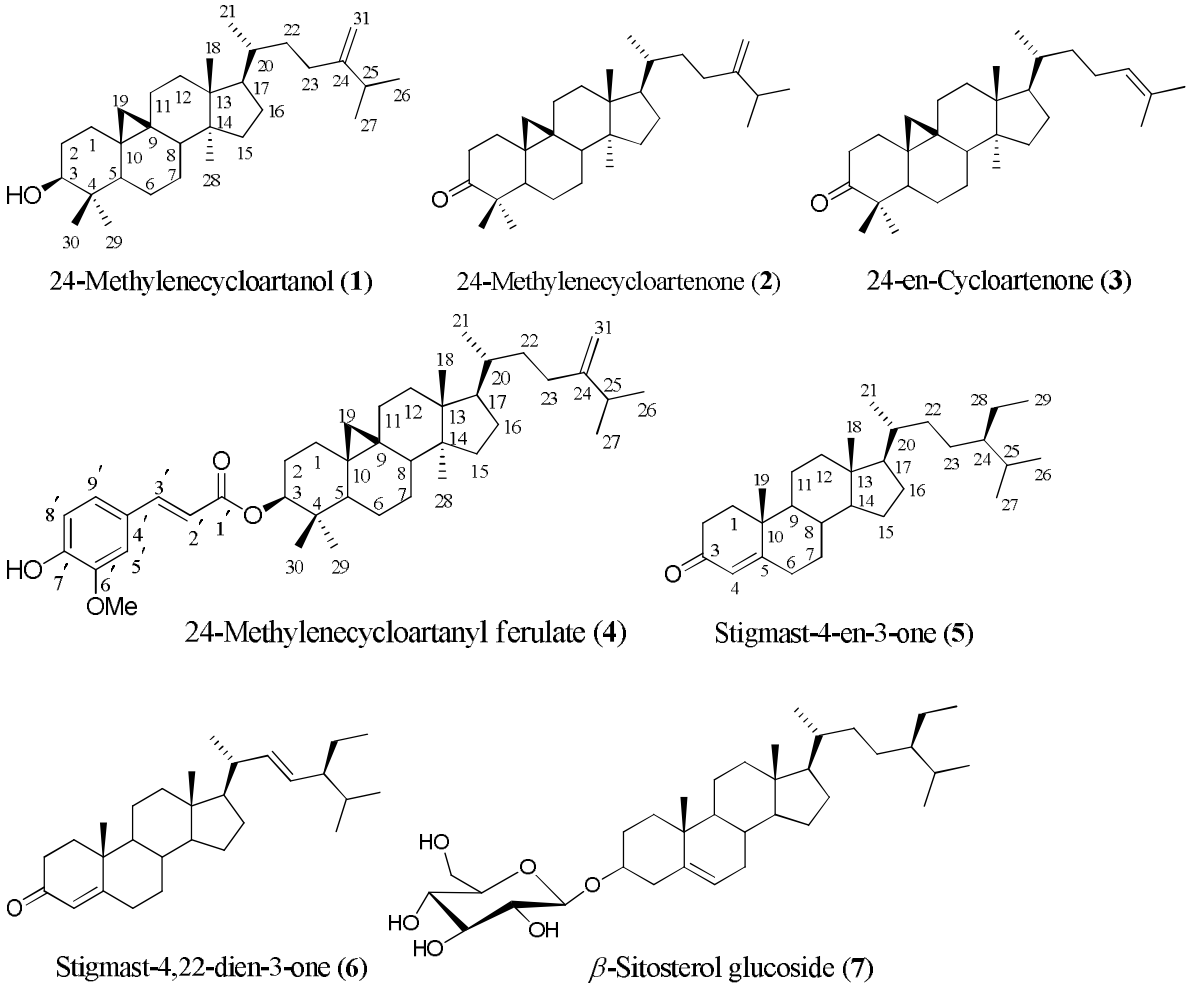

Figure 1. Cycloartane and stigmastane type compounds (**1**-**7**) from *Pothos scandens*.

Me-21), 1.02 (3H, d, *J* = 5.0 Hz, Me-26), 1.03 (3H, d, *J* = 5.0 Hz, Me-27)] while the ^{13}C NMR spectrum (Table 1) displayed 31 carbon resonances. The ^{1}H NMR spectrum also displayed the characteristic cycloartane type methylene signals [δ_H 0.57 (1H, d, *J* = 4.0 Hz, H-19) and 0.33 (1H, d, *J* = 4.0 Hz, H-19)] and two olefinic methlene protons at δ 4.73 and 4.68 in addition to an oxymethine proton signal at δ 3.29 (H-3). Considering the ^{1}H-^{1}H COSY and HMBC spectra, **1** was found to be 24-methylenecycloartanol which was confirmed by comparison with published values.[20]

Compound **2**, obtained as a white amorphous powder, showed very much similar NMR spectra (Table 1) to that of compound **1**. However, instead of an oxymethine signal at C-3 position in **1**, a carbonyl group resonance at δ_C 216.6 appeared in the ^{13}C NMR spectrum. Thus, the structure of **2** was identified as 24-methylenecycloartenone by comparing with published values.[21,22]

Compound **3** was also obtained as a white amorphous powder. The NMR spectra (Table 1) showed very much similarity with those of **2**. Instead of exomethylene protons signals in C-24 positions in the compound **2**, an olefinic proton signal [δ 5.10 (1H, td, *J* = 5.5, 1.0 Hz, H-24) appeared in **3**, suggesting that the difference was occurred in position C-24. In accordance with the published literatures, **3** was identified as 24-en-cycloartenone.[23]

Compound **4** was obtained as white amorphous powder and showed characteristic 24-methylenecycloartane type triterpenoid resonances in its NMR spectra (Table 3). However, a ferulate moiety was observed in **4**, which was configured on the basis of an ABX-type aromatic protons signal [δ_H 6.98 (1H, d, J = 2.0 Hz, H-5′), 6.86 (1H, d, J = 8.0 Hz, H-8′) and 7.02 (1H, dd, J = 8.0, 2.0 Hz, H-9′)], a pair of olefinic sp^2-hybridized proton [δ_H 6.24 (1H, d, J = 16.0 Hz, H-2′) and 7.54 (1H, d, J = 16.0 Hz, H-3′)] and an esterified carbonyl group at δ_C 167.1. Hence, the structure of **4** was identified as 24-methylenecycloartanyl ferulate.[24]

Table 2. **^{1}H (500 MHz) and ^{13}C NMR (125 MHz) NMR spectral data of compound 5 and 6 in $CDCl_3$).**

Position	**5**		**6**	
	δ_H (J in Hz)	δ_C[a]	δ_H (J in Hz)	δ_C[a]
1	2.02, m	35.9	2.02, m	36.0
	1.70, m		1.70, m	
2	2.41, m	34.2	2.41, m	34.2
	1.30, m		1.30, m	
3		199.7		199.7
4	5.72, s	123.9	5.72, s	124.0
5		171.8		171.7
6	2.25, tq (5.0)	33.2	2.25, tq (5.0)	33.2
7	1.84, m	32.3	1.84, m	32.3
	1.10, m		1.10, m	
8	1.51, m	35.9	1.51, m	36.0
9	0.91, m	54.1	0.91, m	54.1
10		38.8		38.9
11	1.53, m	21.3	1.53, m	21.3
	1.43, m		1.43, m	
12	2.35, m	39.9	2.35, m	39.8
	1.15, m		1.15, m	
13		42.7		42.5
14	1.01, m	56.2	1.01, m	56.2
15	1.66, m	24.4	1.66, m	24.5
	1.61, m		1.61, m	
16	1.85, m	28.4	1.85, m	29.0
	1.25, m		1.25, m	
17	1.13, m	56.3	1.13, m	56.3
18	0.71, s	12.2	0.73, s	12.4
19	1.18, s	17.6	1.19, s	17.6
20	1.20, m	36.3	2.04, m	40.6
21	0.92, d (7.5)	18.9	0.83, d (7.5)	19.2
22	1.37, m	34.2	5.16, dd (15.5, 8.0)	138.3
	1.30, m			
23	1.15, m	26.4	5.02, dd (15.5, 8.0)	129.8
	1.15, m			
24	0.94, m	46.1	1.57, m	51.5
25	1.68, m	29.3	1.70, m	29.0
26	0.85, d (7.0)	20.0	1.01, d (7.5)	21.4
27	0.83, d (7.0)	19.3	0.80, d (7.5)	21.3
28	1.23, m	23.3	1.55, m	25.6
	1.23, m		1.10, m	
29	0.84, d (7.5)	12.2	0.85, d (7.5)	12.3

[a,] Assignments are based on HMQC and HMBC experiments.

Table 3. **^{1}H (500 MHz) and ^{13}C NMR (125 MHz) NMR spectral data of compound 4 and 7 in $CDCl_3$).**

Position	**4**		Position	**7**	
	δ_H (*J* in Hz)	δ_C[a]		δ_H (*J* in Hz)	δ_C[a]
1	1.66, m & 1.28, m	31.8	1	1.79, m & 0.98, m	36.8
2	1.84, m & 1.68, m	27.1	2	1.83, m & 1.46, m	29.2
3	4.66, dd (12.0, 4.5)	80.7	3	3.46, m	76.9
4		39.9	4	2.36, m & 2.12, m	38.3
5	1.40, dd (12.0, 4.0)	47.4	5		140.4
6	1.58, m & 0.77, m	21.1	6	5.32, d (5.0)	121.1
7	1.90, m & 1.20, m	28.3	7	1.93, m & 1.50, m	31.3
8	1.50, dd (12.0, 4.0)	48.0	8	1.40, m	31.4
9		20.3	9	0.88, m	49.6
10		26.2	10		36.2
11	1.28, m & 1.04, m	25.9	11	1.47, m & 1.0, m	20.5
12	1.26, m	35.7	12	1.96, m & 1.14, m	39.1
13		45.5	13		41.8
14		49.0	14	0.98, m	56.1
15	1.56, m	33.0	15	1.54, m & 1.03, m	23.8
16	1.94, m & 1.11, m	26.7	16	1.80, m & 1.25, m	27.7
17	1.59, m	52.4	17	1.09, m	55.4
18	0.92, s	18.1	18	0.64, s	11.6
19	0.56, d (4.0) & 0.31, d (4.0)	29.9	19	0.95, s	19.0
20	1.26, m	36.3	20	1.34, m	35.4
21	0.85, d (6.0)	18.4	21	0.89, d (6.5)	16.8
22	1.54, m & 1.25, m	35.2	22	1.30, m & 1.01, m	33.3
23	2.06, m & 1.87, m	31.3	23	1.15, m	25.5
24		157.0	24	0.91, m	45.1
25	2.19, septet	34.0	25	1.63, m	28.7
26	0.98, d (3.0)	22.0	26	0.81, d (7.5)	18.9
27	0.97, d (3.0)	22.1	27	0.81, d (7.5)	19.6
28	0.86, s	19.4	28	1.25, m & 1.19, m	22.6
29	0.84, s	25.6	29	0.82, d (7.5)	11.7
30	0.92, s	15.5			
31	4.67, s & 4.61, s	106.1	Glc-1′	4.21, d (7.5)	100.7
1′		167.1	2′	2.88, overlapped	73.4
2′	6.24, d (16.0)	116.5	3′	3.11, overlapped	76.6
3′	7.54, d (16.0)	144.4	4′	3.01, overlapped	70.0
4′		127.4	5′	3.05, overlapped	76.7
5′	6.98, d (2.0)	109.5	6′	3.63, overlapped	61.2
6′		146.9		3.39, overlapped	
7′		148.0			
8′	6.86, d (8.0)	114.8			
9′	7.02, dd (8.0, 2.0)	123.1			
OMe	3.87, s	56.1			

[a,] Assignments are based on HMQC and HMBC experiments.

Compounds **5** and **6** were obtained as colorless needles. The ^{1}H NMR spectra of both compounds revealed the presence of an olefinic proton [δ_H 5.72 (1H, s, H-4)] and six methyl groups among which four appeared as doublet [**5**: δ 0.71 (3H, s, Me-18), 0.85 (3H, d, *J* = 7.0 Hz, Me-26), 0.84 (3H, d, *J* = 7.5 Hz, Me-29), 0.83 (3H, d, *J* = 7.0 Hz, Me-27), 0.92 (3H, d, *J* = 7.5 Hz, Me-21), 1.18 (3H, s, Me-19); **6**: δ 0.73 (3H, s, Me-18), 1.01 (3H, d, *J* = 7.5 Hz, Me-26), 0.85 (3H, d, *J* = 7.5 Hz, Me-29), 0.80 (3H, d, *J* = 7.5 Hz, Me-27), 0.83 (3H, d, *J* = 7.5 Hz, Me-21), 1.19 (3H, s, Me-19)]. Additionally, compound **6** showed two olefinic protons signals [δ 5.16 (1H, dd, *J* = 15.5, 8.0 Hz, H-22) and 5.02 (1H, dd, *J* = 15.5, 8.0 Hz, H-

23)] in its ^{1}H NMR data (Table 2). The ^{13}C NMR spectra of both compounds displayed 29 carbon resonances including a carbonyl group at δ_C 199.7 (C-3), indicating to have a stigmastane type skeleton in their structures. Compound **5** showed a pair of olefinic carbon signals at δ_C 123.9 (C-4) and 171.8 (C-5) ppm while **6** displayed two pair of olefinic signals [δ_C 124.0 (C-4), 171.7 (C-5) and 138.3 (C-22), 129.8 (C-23) in the ^{13}C NMR spectra. The above NMR data completely matched with published values of stigmast-4-en-3-one (**5**) and stigmast-4, 22-dien-3-one (**6**) in literatures.[25-27]

Compound **7** was obtained as a white amorphous powder and it showed similar NMR spectra appropriate for stigmastane type skeleton. The ^{1}H NMR spectrum showed an anomeric proton signal [δ 4.21 (1H, d. J = 7.5 Hz, Glc-1′), suggesting a glycosidic linkage at position C-3. The HMBC spectrum of **7** showed the correlation between anomeric proton of glucopyranosyl unit and δ_C 76.9 (C-3) of β-sitosterol structure. Considering the ^{1}H-^{1}H COSY, HSQC and HMBC spectra, **7** was found to be β-sitosterol-3-*O*-glucopyranoside.[28]

Estrogenic/antiestrogenic activity. Literature survey revealed that leaf extract of *P. scandens* is used to induce conception. In order to prove this traditional use, a cell based screening was conducted to determine whether the isolates possess any of the estrogenic/antiestrogenic properties using estradiol (E_2) responsive ATCC breast cancer cells (MCF-7 and T47D). While estradiol (E_2) displayed significant cell proliferation activity in a dose dependent manner (1 to 100 pM), none of the isolates showed estrogen like activity at a concentration of 0.01 to 10 µM (data not shown). Therefore, there is no scientific basis that this plant could be used to induce conception.

Antiestrogenic activities were also investigated for all the isolates. The breast cancer cells (MCF-7 and T47D) proliferation was induced initially by using 100 pM of estradiol (E_2). These cells were co-treated with the each isolates at four different concentrations at 0.01, 0.1, 1.0 and 10.0 µM (Table 4). In order to compare with a positive control, estrogen receptor antagonist (tamoxifen) was used in this assay which suppressed the E_2-enhanced cell proliferation nearly completely ($iEqE_1$) at the concentration of lower than 9.0 µM in both cells. All compounds showed at least 50% inhibition in both cell lines at the lowest tested concentration (Table 4). Among the cycloartane type triterpenoids, 90% inhibition ($iEqE_{10}$) of cell proliferation (induced by 100 pM of estradiol) in T47D and MCF-7 cells were occurred with 24-methylenecycloartanol (**1**) at a concentration of lower than 0.01 µM. On the other hand, 24-methylenecycloartanyl ferulate (**4**) displayed 90% inhibition of cell proliferations (induced by 100 pM of estradiol) in T47D cells only at a concentration of 0.01 µM. Both of the compounds possess either free hydroxyl group or feruloyl moiety attached to oxygen of a hydroxyl group in C-3 position in their structures. Part of the chemical structure is similar as quinona-methide triterpenes tingenone and pristimerin which showed antiestrogenic or cytotoxic activities presenting IC_{50} value 2 and 5 µM, respectively.[29] Due to possible hydrogen bonding with estrogen receptors, these two compounds showed antiestrogenic activity.[30] 24-En-cycloartenone (**3**) suppressed 90% cell proliferation (induced by 100 pM of estradiol) in T47D cells at a concentration less than 0.1 µM. Ninety percent cell proliferation (induced by 100 pM of estradiol) in T47D cells was observed with stigmast-4,22-dien-3-one (**5**) at a concentration less than 0.01 µM whereas in case of MCF-7 cells, the required concentration could not be tested.

Previous study suggested that high levels of estrogen may increase the risk of breast cancer in postmenopausal women.[31] Therefore, compounds **1** and **4** may have the potential as lead compounds for further drug developments. However, detailed studies are required to establish their efficacy in human being.

CONCLUSION

Extensive phytochemical investigation of EtOAc soluble materials of the methanolic extracts of *P. scandens* has revealed seven known cycloartane and stigmastane type triterpenoids (**1**-**7**). All the isolates

were tested for their estrogenic and/or antiestrogenic properties using two different cell lines. All of them showed at least 50% inhibition of estradiol induced cell proliferation. Among them, compound **1** inhibited the cell growth by up to 90% in both cell lines whereas **4** and **6** inhibited 90% the cell growth in T47D cells only.

Table 4. Inhibitory activities of 1-7 against E_2-enhanced cell proliferation.

Cpds	MCF-7				T47D			
	$iEqE_{50}$[a]	$iEqE_{10}$[a]	$iEqE_1$[a]	IL[b]	$iEqE_{50}$[a]	$iEqE_{10}$[a]	$iEqE_1$[a]	IL[b]
24-methylenecycloartanol (**1**)	< 0.01	< 0.01	-	**S**	< 0.01	< 0.01	-	**S**
24-methylenecycloartenone (**2**)	< 0.01	-	-	**M**	< 0.01	-	-	**M**
24-en-cycloartenone (**3**)	< 0.01	-	-	**M**	< 0.01	< 0.1	-	
24-methylenecycloartanyl ferulate (**4**)	< 0.01	10.0	-	**M**	< 0.01	< 0.01	-	**S**
stigmast-4-en-3-one (**5**)	< 0.01	10.0	-	**M**	< 0.01	-	-	**M**
stigmast-4,22-diene-3-one (**6**)	< 0.01	-	-	**M**	< 0.01	< 0.01	-	**S**
β-sitosterol glucoside (**7**)	< 0.01	-	-	**M**	< 0.01	-	-	**M**
Tamoxifen[c]	0.1	0.5	5.0		0.1	0.8	9.0	

[a] $iEqE_{50}$, $iEqE_{10}$, and $iEqE_1$ represent the concentrations of the compounds (μM) that decreased cell proliferation (enhanced by 100 pM of E_2) to equivalent levels induced by 50 pM, 10 pM, and 1 pM of E_2 treatment, respectively. The values were calculated by linear regression analysis using four different concentrations. [b] IL: inhibitory level of the compound. Mild inhibition (M): more than 50% inhibition with the concentration tested, Strong inhibition (S): more than 90% inhibition with the concentration tested. [c] Tamoxifen-positive control.

ACKNOWLEDGEMENTS

This work was supported by a Ministry of Education, Culture, Sports, Science and Technology (MEXT) scholarship from the Japanese government. The authors would like to thank Mr. Narhari Das, Department of Clinical Pharmacy and Pharmacology, University of Dhaka, Bangladesh for his generous support in collecting the samples and Mr. Philip Hawke of the University of Shizuoka, Scientific English Program for his comments on the English in the manuscript.

REFERENCES

1. Russo, J. and Russo, I.H. 2006. The role of estrogen in the initiation of breast cancer. *J. Steroid Biochem. Mol. Biol.* **102**, 89-96.
2. Russo, J., Hu, Y.F. and Russo, I.H. 2000. *Estrogens and breast cancer in humans*, in: M. Metzler (Ed.), Endocrine Disrupters of the Environment, Springer-Verlag, Heidelberg, pp. 1-26.
3. Engel, N., Oppermann, C., Falodun, A. and Kragl, U. 2011. Proliferative effects of five traditional Nigerian medicinal plant extracts on human breast and bone cancer cell lines. *J. Ethnopharmacol.* **137**, 1003-1010.
4. Hussain, S.M.A. 2013. Comprehensive update on cancer scenario of Bangladesh. *South Asian J. Cancer* **2**, 279-284.
5. Buhimschi, C.S. 2004. Endocrinology of lactation. *Obstet. Gynecol. Clin. N. Am.* **31**, 963-979.
6. Luecha, P., Umehara, K., Miyase, M. and Noguchi, H. 2009. Antiestrogenic constituents of the Thai medicinal plants *Capparis flavicans* and *Vitex glabrata*. *J. Nat. Prod.* **72**, 1954-1959.
7. Umehara, K., Nemoto, K., Matsushita A., Terada, E., Monthakantirat, O., De-Eknamkul, W., Miyase, T., Warashina, T., Degawa, M. and Noguchi, H. 2009. Flavonoids from the heartwood of the Thai medicinal plant *Dalbergia parviflora* and their effects on estrogenic-responsive human breast cancer cells. *J. Nat. Prod.* **72**, 2163-2168.
8. Muhit, M.A., Umehara, K., Mori-Yasumoto, K. and Noguchi, H., 2016. Furofuran lignan glucosides with estrogen-inhibitory properties from the Bangladeshi medicinal plant *Terminalia citrina*. *J. Nat. Prod.* **79**, 1298-1307.
9. Muhit, M.A., Umehara, K. and Noguchi, H., 2016. Five furofuranone lignan glucosides from *Terminalia citrina* inhibit in vitro E_2-enhanced breast cancer cell proliferation. *Fitoterapia* **113**, 74-79.
10. Muhit, M.A., Izumikawa, M., Umehara, K. and Noguchi, H., 2016. Phenolic constituents of the Bangladeshi medicinal plant *Pothos scandens* and their anti-estrogenic, hyaluronidase inhibition, and histamine release inhibitory activities. *Phytochemistry*, **121**, 30-37.

11. Muhit, M.A., Umehara, K. and Noguchi, H., 2018. α-Keto tetrahydrofuran lignan glucosides from the Bangladeshi medicinal plant *Terminalia citrina* inhibit estradiol (E_2) induced proliferation in cancer cells. *Phytochemistry* **145**, 161-167.
12. *Banglapedia: National Encyclopedia of Bangladesh*. 2003. Asiatic Society of Bangladesh. Dhaka, Bangladesh.
13. Mia, M.M.K. 1990. *Traditional Medicines of Bangladesh*. Asiatic society of Bangladesh, Dhaka, Bangladesh.
14. Yusuf, M., Begum, J., Hoque, M.N. and Chowdhury, J.U. 2009. *Medicinal Plants of Bangladesh*. Bangladesh Council of Scientific and Industrial Research Laboratories, Chittagong, 2nd ed.
15. Haneefa, M.K.P., Hanan, S.K., Saraswathi, R., Mohanta, G.P. and Nayar, C. 2010. Formulation and evaluation of herbal gel of *Pothos scandens* Linn. *Asian Pac. J. Trop. Med.* **3**, 988-992.
16. Sangwoo, L., Chunjie, X. and Shengji, P. 2008. Ethnobotanical survey of medicinal plants at periodic markets of Honghe prefecture in Yunnan province, SW China. *J. Ethnopharmacol.* **117**, 362-377.
17. Bhandary, M.J., Chandrashekar, K.R. and Kaveriappa, K.M. 1995. Medical ethnobotany of the Siddis of Uttara Kannada district, Karnataka, India. *J. Ethnopharmacol.* **47**, 149-158.
18. Ayyanar, M. and Ignacimuthu, S. 2005. Traditional knowledge of Kani tribals in Kouthalai of Tirunelveli hills, Tamil Nadu, India. *J. Ethnopharmacol.* **102**, 246-255.
19. Lalitharani, S., Mohan, V.R. and Regini, G.S. 2010. Ethnomedicinal plants used by Kanikkar in Karayar of Agasthiamalai biosphere, Western Ghats, Tamil Nadu. *J. Econ. Taxon. Bot.* **34**, 472-477.
20. Lee, S.Y., Choi, S.U., Lee, J.H., Lee, D.U. and Lee, K.R. 2010. A new phenylpropane glycoside from the rhizome of *Sparganium stoloniferum*. *Arch. Pharm. Res.* **33**, 515-521.
21. Yonemoto, R., Shimada, M., Gunawan-puteri, M.D.P.T., Kato, E. and Kawabata, J. 2014. α-Amylase inhibitory triterpene from *Abrus precatorius* leaves. *J. Agric. Food Chem.* **62**, 8411-8414.
22. Lian-Niang, L. and Hong, X. 1986. Triterpenoids from roots and stems of *Kadsura coccinea*. *Planta Med.* **52**, 492-493.
23. Mao-juan, Z., Bing, L., Yan-feng, A., De-qiang, F. and You-kai, J. 2014. Chemical constituents in stems with hooks of *Uncaria sessilifructus*. *Chin Tradit. Herbal Drugs* **24**, 175-180.
24. Muthal, A.P., Rojatkar, S.R. and Bodhankar, S.L. 2016. Isolation and structure setermination of 24-methylenecycloartanyl ferulate from Indian rice bran and its quantitative analysis. *Pharmacogn Mag.* **12**(Suppl 3), 307-314.
25. Ibrahim, S.R.M., Elkhayat, E.S., Mohamed, G.A., Khedr, A.I.M., Fouad, M.A., Kotb, M.H.R. and Ross, S.A. 2015. Aspernolides F and G, new butyrolactones from the endophytic fungus *Aspergillus terreus*. *Phytochem. Lett.* **14**, 84-90.
26. Lone, S.H. and Bhat, K.A. 2015. Phytosterols as precursors for the synthesis of aromatase inhibitors: Hemisynthesis of testololactone and testolactone. *Steroids* **96**, 164-168.
27. Sandjo, L.P., Rincheval, V., Ngadjui, B.T. and Kirsch, G. 2011. Cytotoxic effect of some pentacyclic triterpenes and hemisynthetic derivatives of stigmasterol. *Chem. Nat. Compd.* **47**, 731-734.
28. Jayaprakasha, G.K., Jadegoud, Y., Gowda, G.A.N. and Patil, B.S. 2010. Bioactive compounds from sour orange inhibit colon cancer cell proliferation and induce cell cycle arrest. *J. Agric. Food Chem.* **58**, 180-186.
29. Gomes, J.P.M., Cardoso, C.R.P., Varanda, E.A., Molina, J.M., Fernandez, M.F., Olea, N., Arlos, I.Z. and Vilegas, W. 2011. Antitumoral, mutagenic and (anti)estrogenic activities of tingenone and pristimerin. *Braz. J. Pharmacogn.* **21**, 963-971.
30. Hidalgo, M., Santamaria, S.M., Recio, I., Moreno, C.S., Teresa, B.D.P., Rimbach, G. and Teresa, S.D.P. 2012. Potential anti-inflammatory, anti-adhesive, anti/estrogenic, and angiotensin-converting enzyme inhibitory activities of anthocyanins and their gut metabolites. *Genes Nutr.* **7**, 295-306.
31. Folkerd, E.J., Lonning, P.E. and Dowsett, M. 2014. Interpreting plasma estrogen levels in breast cancer: caution needed. *J. Clin. Oncol.* **32**, 1396-1400.