STRUCTURE MODIFICATION OF ANDROGRAPHOLIDE TO IMPROVE ITS POTENCY AS ANTICANCER

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ABSTRACT

Andrographolide, a diterpenoid lactone isolated from the herb of Andrographis paniculata and known to possess antitumor activity in breast cancer models was subjected to semisynthesis leading to the preparation of a number of derivatives. After protection of the two hydroxyl groups present at C-3 and C-19 to give 3,19-isopropylidene and 3,19-benzylidene andrographolides, the remaining hydroxyl group at C-14 of andrographolide was treated with acid anhydride or acid chloride under base condition. Unfortunately, the reactions gave only 14-dehydroandrographolide as well as unidentified diacyl compounds in replace of the target molecule 14-O-acyl andrographolide. An alternative procedure using neat acetic anhydride under reflux gave the acetyl derivatives. The resulted compounds exhibited cytotoxic activity against MCF-7 breast cancer cells with better growth inhibition than the parent compound andrographolide.

***Keywords**: andrographolide, acylation, anticancer, cytotoxic, breast cancer cells.*

INTRODUCTION

Andrographis paniculata (Burn. f.) Ness from the family Acanthaceae, also known as “sambiloto” or “king of bitters”, is widely found and cultivated in tropical and subtropical Asia, south-east Asia and India in a variety of human illness [1]. These range from antibacterial, antiviral, anti-malarial, anti-inflammatory, immuno-stimulatory, hypoglycemic, hypotensive, hepato-protective and anticancer activities [2–11]. The main components of *A. paniculata* are diterpenes, flavonoids and stigmasterols [11], from which labdane diterpenoids being the major constituents [12,13]. Andrographolide (**Andro-1**) having complete structure as shown in Fig 1 is the major diterpenoid of *A. paniculata* herba and chemically designated as 3-{2-[decahydro-6-hydroxy-5-(hydroymethyl)-5,8a-dimethyl-2-methylene-1-naptha-lenyl]ethylidene}dihydro-4-hydroxy-2(3*H*)-furanone.

Andrographolide is found in the whole plant of *A. paniculata* with the leaves contain the highest amount, while the seeds contain the lowest [14]. Rajani *et al*. developed a rapid method for isolation of andrographolide from *A. paniculata* leaves involving extraction of the leaf powder by cold maceration in a 1:1 mixture of dichloromethane and methanol, and isolation of andrographolide directly from the resulting extract by recrystallization [15]. With respect to the potent anticancer activity of andrographolide, some publications have reported the preparation of numerous novel compounds derived from andrograholide to study the effect of various chemical modifications on andrographolide for the purpose of to improve the activity of andrographolide and to understand the structural requirements for the anticancer activity [16–21]. On the basis of structure-activity relationship (SAR) evaluation, it was showed that an intact γ-butyrolactone ring, double bonds at C-12 and 13 as well as at C-8 and C-17, and hydroxyl group at C-14 of andrographolide are important for its cytotoxic activity [19,20].

In order to determine the importance of the hydroxyl groups present at C-3 and C-19 combined with the presence of acyl functionality at C-14 hydroxyl group of andrographolide for cytotoxic activity, we synthesized andrographolide analogues by protection of the two hydroxyl groups present at C-3 and C-19 followed by treating the remaining hydroxyl group at C-14 with acid anhydride or acid chloride under base condition. An alternative procedure was also attempted by reaction of andrographolide with neat acetic anhydride under reflux to give the tri-*O*-acetyl derivatives. Here, we report the semisynthesis of andrographolide analogues and their *in vitro* anticancer activities in MCF-7 breast cancer cell lines.

EXPERIMENTAL SECTION

Materials

Chemicals

Andrographolide (**Andro-1**) was isolated from the herb of *Andrographis paniculata* under a known method [13], and purified by using column chromatography and recrystallization procedure providing colorless crystals (mp 230–231 ºC) whose NMR data was in appropriate with that of reported data [13,22].

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Solvents and reactants were of the best commercial grade available and used without further purification unless noted. Column chromatography was performed on silica gel (45~75 μm, Wakogel C-300).

Cell culture

Human breast carcinoma MCF-7 cells were purchased from Dainippon Pharmaceutical Co., LTD. (Osaka, Japan). The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and kanamycin (100 μg/mL) at 37 °C in a 5% CO_2 humidified atmosphere.

Instrumentation

Melting point was measured using an Electrothermal melting point apparatus without correction. The ^{1}H NMR spectrum was run in $CDCl_3$ unless otherwise noted. All chemicals shifts are reported as δ values in parts per million (ppm) relative to TMS and residual $CDCl_3$ as internal standards on 270 and 500 MHz spectrometers, and coupling constants (*J*) are expressed in hertz (Hz). Mass spectrum was recorded on a JEOL JMS 600 spectrometer in the chemical ionization (CI) with isobutane and electron impact (EI) methods. The signal splitting in NMR spectral data is characterized as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad.

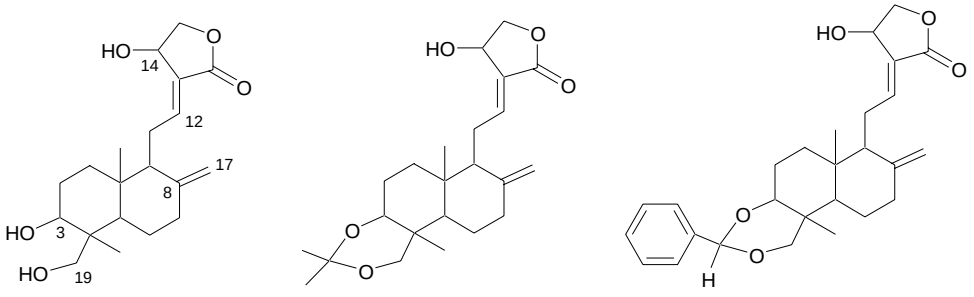
Procedure

Synthesis of 3,19-isopropylidene andrographolide.

A mixture of andrographolide (0.5 g, 1.43 mmol), 2,2-dimethoxypropane (1.27 g, 12.24 mmol) and a catalytic amount of pyridinium *p*-toluenesulfonate (50 mg) in a mixture of benzene (18 mL) and dimethyl sulfoxide (2 mL) was stirred at room temperature under an atmosphere of nitrogen. After completion of the reaction checked by thin layer chromatography method (usually overnight), the contents were treated with excess of triethylamine to quench the remaining catalyst. The reaction mixture was diluted with benzene and washed with water (3 times, 3 x 10 mL). The organic layer was separated, dried over anhydrous sodium sulfate and concentrated by vacuum distillation. The product was purified by column chromatography using chloroform–methanol (20:1) as eluent.

Synthesis of 3,19-benzylidene andrographolide.

A mixture of andrographolide (0.1 g, 0.28 mmol), benzaldehyde (0.3 g, 2.83 mmol) and a catalytic amount of pyridinium *p*-toluenesulfonate (20 mg) in a mixture of benzene (4.5 mL) and dimethyl sulfoxide (0.5 mL) was stirred at room temperature under an atmosphere of nitrogen. After completion of the reaction checked by thin layer chromatography method (usually overnight), the contents were worked up in the same manner as described for the preparation of 3,19-isopropylidene andrographolide.



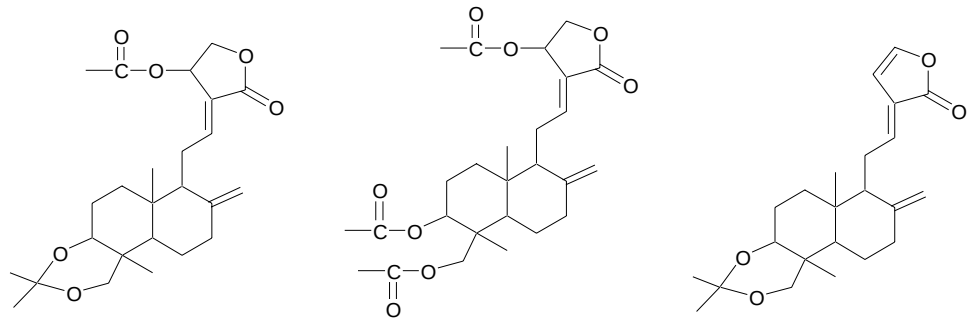


Fig 1. Chemical structures of andrographolide and derivatives

Reaction of 3,19-isopropylidene andrographolide with acetic anhydride and pyridine.

To a solution of 3,19-isopropylidene andrographolide (0.1 g, 0.26 mmol) in dry pyridine (2 mL) under an atmosphere of nitrogen at room temperature, acetic anhydride (0.27 g, 2.64 mmol) was added and stirred until completion of the reaction checked by thin layer chromatography method (usually overnight). The reaction mixture was then poured into ice–water and extracted three times with chloroform. The combined organic layers were extracted successively with 3% HCl, saturated aqueous sodium bicarbonate, and water. The organic layer was then dried with anhydrous sodium sulfate and concentrated by vacuum distillation. The resulting residue was purified by column chromatography using chloroform–methanol (50:1) as eluent.

Reaction of 3,19-isopropylidene andrographolide with benzoyl chloride and pyridine.

To a soluton of 3,19-isopropylidene andrographolide (0.1 g, 0.26 mmol) in dry pyridine (2 mL) under an atmosphere of nitrogen at room temperature, benzoyl chloride (0.37 g, 2.63 mmol) was added and stirred until completion of the reaction checked by thin layer chromatography method (usually overnight). Methanol was added and the solution was concentrated by vacuum distillation. The residue was dissolved in chloroform and washed successively with saturated aqueous sodium bicarbonate and water. The organic layer was dried with anhydrous sodium sulfate and concentrated by vacuum distillation. The resulting residue was purified by column chromatography using chloroform–methanol (50:1) as eluent.

Reaction of 3,19-isopropylidene andrographolide with acetic anhydride and DMAP.

To a solution of 3,19-isopropylidene andrographolide (0.1 g, 0.26 mmol) in dichloromethane (2 mL) under an atmosphere of nitrogen at room temperature, acetic anhydride (0.20 g, 1.96 mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (DMAP) (20 mg) were added and stirred until completion of the reaction checked by thin layer chromatography method (usually overnight). The reaction mixture was diluted with water and extracted three times with chloroform. The combined organic layers were worked up in the same manner as described for the reaction of 3,19-isopropylidene andrographolide with acetic anhydride and pyridine but without purification.

Reaction of 3,19-isopropylidene andrographolide with benzoic anhydride and DMAP.

To a solution of 3,19-isopropylidene andrographolide (0.1 g, 0.26 mmol) in dichloromethane (2 mL) under an atmosphere of nitrogen at room temperature, benzoic anhydride (65 mg, 0.29 mmol) and a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (20 mg) were added and stirred until completion of the reaction checked by thin layer chromatography method (usually overnight). The reaction mixture was worked up in the same manner as described for the reaction of 3,19-isopropylidene andrographolide with benzoyl chloride and pyridine but without purification.

Reaction of 3,19-isopropylidene andrographolide with acetic anhydride under reflux.

A mixture of 3,19-isopropylidene andrographolide (0.2 g, 0.51 mmol) and acetic anhydride (1.5 mL) was refluxed for 1 h. The reaction was monitored by thin layer chromatography method. After completion of the reaction, the contents were cooled to room temperature, diluted with water and extracted with chloroform (3 times). The organic layer was separated, dried over anhydrous sodium sulfate and concentrated to obtain an oily material. The crude material was purified by column chromatography using chloroform–methanol (50:1) as eluent.

Reaction of andrographolide with acetic anhydride under reflux.

A mixture of andrographolide (0.1 g, 0.28 mmol) and acetic anhydride (1.5 mL) was refluxed for 1 h. The reaction was monitored by thin layer chromatography method. After completion of the reaction, the contents were worked up in the same manner as described for the reaction of 3,19-isopropylidene andrographolide with acetic anhydride under reflux.

Sensitivity of andrographolide derivatives to MCF-7 cells.

MCF-7 cells were seeded separately at a density of 1 x 10^4 cells per well in 96-well plates and maintained for 24 h in RPMI medium supplemented with 10% FBS. The cells at 30% confluence in the well were treated with medium containing andrographolide derivatives (3.13 to 100 μM) in three replicates and then incubated for 48 or 72 h. The cytotoxicity was determined by a WST-8 assay (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan).

RESULT AND DISCUSSION

Preparation of 3,19-isopropylidene and 3,19-benzylidene andrographolides.

After purification using column chromatography followed by recrystallization from ethyl acetate–hexane, 3,19-isopropylidene andrographolide (**Andro-2**) was found as colorless crystals (93% yield), mp 193–194 ^oC. For EI-MS analysis, *m/z* (relative intensity): 390 $[M]^+$ (14), 375 $[M - CH_3]^+$ (100), 332 $[M - (CH_3)_2CO]^+$ (59), 314 $[M - (CH_3)_2CO - H_2O]^+$ (33), 302 [M –

$(CH_3)_2CO_2CH_2]^+$ (24). For ^{1}H NMR analysis, (i) CH_3 groups: δ 0.96 (s, 3 H), 1.20 (s, 3 H), 1.37 (s, 3 H), 1.42 (s, 3 H); (ii) ring CH/CH_2 groups and allylic H atoms: δ 1.24 – 1.33 (m, 3 H), 1.72 – 1.87 (m, 4 H), 1.95 – 2.03 (m, 2 H), 2.40 – 2.44 (m, 1 H), 2.56 – 2.59 (m, 2 H); (iii) hydroxyl H atom: δ 2.85 (d, *J* = 7.0 Hz, 1 H); (iv) carbinol H atoms: δ 3.18 (d, *J* = 11.4 Hz, 1 H), 3.50 (dd, *J* = 3.6, 8.6 Hz, 1 H), 3.97 (d, *J* = 11.4 Hz, 1 H), 5.03 (br s, 1 H); (v) lactonyl CH_2 group: δ 4.26 (dd, *J* = 2.0, 10.6 Hz, 1 H), 4.45 (dd, *J* = 6.0, 10.6 Hz, 1 H); (vi) olefinic H atoms: δ 4.63 (s, 1 H), 4.91 (s, 1 H), 6.95 (dt, *J* = 1.6, 7.0 Hz, 1 H).

After purification using column chromatography, 3,19-benzylidene andrographolide (**Andro-2A**) was found as colorless solid (85% yield), mp 142–143 ºC. For EI-MS analysis, *m/z* (relative intensity): 438 $[M]^+$ (45), 420 $[M - H_2O]^+$ (100), 332 $[M - C_6H_5CHO]^+$ (34), 314 $[M - C_6H_5CHO - H_2O]^+$ (97), 296 $[M - C_6H_5CHO - 2\ H_2O]^+$ (27). For ^{1}H NMR analysis, (i) CH_3 groups: δ 0.85 (s, 3 H), 1.48 (s, 3 H); (ii) ring CH/CH_2 groups and allylic H atoms: δ 1.18 – 1.33 (m, 3 H), 1.76 – 2.02 (m, 5 H), 2.36 – 2.61 (m, 4 H); (iii) benzylic H atom: δ 5.76 (s, 1 H); (iv) carbinol H atoms: δ 3.58 (d, *J* = 11.3 Hz, 1 H), 3.66 (dd, *J* = 4.4, 12.6 Hz, 1 H), 4.26 (d, *J* = 11.3 Hz, 1 H), 4.90 (br s, 1 H); (v) lactonyl CH_2 group: δ 4.15 (dd, *J* = 2.0, 10.4 Hz, 1 H), 4.36 (dd, *J* = 6.1, 10.4 Hz, 1 H); (vi) olefinic H atoms: δ 4.63 (s, 1 H), 4.90 (s, 1 H), 6.90 (t, *J* = 6.1 Hz, 1 H); aromatic H atoms: δ 7.33 – 7.39 (m, 3 H), 7.47 – 7.51 (m, 2 H).

Products of reactions between 3,19-isopropylidene andrographolide in pyridine with respective acetic anhydride and benzoyl chloride.

Both reactions of a mixture of 3,19-isopropylidene andrographolide and pyridine with respective acetic anhydride and benzoyl chloride gave unidentified analogous compounds as predominant products (**Andro-3** for acetyl derivative and **Andro-4** for benzoyl derivative). Although the starting material have only one hydroxyl group at C-14 as acylation target site, each of the products of reactions showed the presence of two acetyl groups or two benzoyl groups, respectively, which was concluded from ^{1}H NMR spectra. For CH_3 group of the acetyl: δ 2.02 (s, 3 H), 2.04 (s, 3 H). For aromatic H atoms of the benzoyl: δ 7.36 (dt, *J* = 1.6, 7.8 Hz, 4 H), 7.50 (t, *J* = 7.8 Hz, 2 H), 7.93 (dt, *J* = 0.9, 7.8 Hz, 4 H). The 3,19-isopropylidene functionality was found unchanged that after hydrolysis using a mixture of acetic acid – water (7:3) gave a unprotected 3,19-diol functionality. Due to the presence of more carbon atoms in the products of reaction than that of the target molecule 14-*O*-acetyl or 14-*O*-benzoyl-3,19-isopropylidene andrographolide as can be shown in their ^{13}C NMR spectra, it can be postulated that **Andro-3** and **Andro-4** may be bis-andrographolide like products.

Products of reactions between mixture of 3,19-isopropylidene andrographolide and DMAP with respective acetic anhydride and benzoic anhydride.

Both reactions of a mixture of 3,19-isopropylidene andrographolide and DMAP in CH_2Cl_2 with respective acetic anhydride and benzoic anhydride gave the same 3,19-isopropylidene-14-dehydroandrographolide as predominant product. The structure of resulted compound was confirmed from ^{1}H NMR spectra that showed olefinic H atoms of lactone ring: δ 6.20 (d, *J* = 3.4 Hz, 1 H) and 7.01 (t, *J* = 1.7 Hz, 1 H). The presence of newly formed olefinic H atoms at C-14 and C-15 of the lactone ring as mentioned above is in conformity with the disappearance of both lactonyl CH_2 group at C-15 and 14-*O*-acetyl or 14-*O*-benzoyl functionalities. We suggested that the14-*O*-acetyl and 14-*O*-benzoyl groups were formed previously, and then they became good leaving groups following the removal of one of the two protons of C-15 to give the C-14 and C-15 olefinic system.

Products of reactions between respective 3,19-isopropylidene andrographolide and andrographolide with acetic anhydride under reflux.

After purification using column chromatography, 14-*O*-acetyl-3,19-isopropylidene andrographolide (**Andro-3A**) was found as a pale yellow viscous oil (86% yield). For EI-MS analysis, *m/z* (relative intensity): 432 $[M]^+$ (8), 417 $[M - CH_3]^+$ (100), 372 $[M - (CH_3)_2C - H_2O]^+$ (12), 357 $[M - (CH_3)_2C - CH_3 - H_2O]^+$ (12), 314 $[M - (CH_3)_2CO - CH_3CO_2H]^+$ (45), 297 $[M - (CH_3)_2CO_2 - CH_3CO - H_2O]^+$ (37); 284 $[M - (CH_3)_2CO_2CH_2 - CH_3CO_2H]^+$ (17). For ^{1}H NMR analysis, (i) CH_3 groups: δ 0.94 (s, 3 H), 1.20 (s, 3 H), 1.37 (s, 3 H), 1.41 (s, 3 H), 2.13 (s, 3 H); (ii) ring CH/CH_2 groups and allylic H atoms: δ 1.18 – 1.46 (m, 3 H), 1.67 – 1.88 (m, 4 H), 1.97 – 2.06 (m, 2 H), 2.38 – 2.48 (m, 3 H); (iii) carbinol H atoms: δ 3.18 (d, *J* = 11.4 Hz, 1 H), 3.50 (dd, *J* = 3.6, 8.2 Hz, 1 H), 3.96 (d, *J* = 11.4 Hz, 1 H), 5.93 (d, *J* = 5.9 Hz, 1 H); (iv) lactonyl CH_2 group: δ 4.24 (dd, *J* = 1.8, 11.2 Hz, 1 H), 4.56 (dd, *J* = 5.9, 11.2 Hz, 1 H); (v) olefinic H atoms: δ 4.55 (s, 1 H), 4.90 (s, 1 H), 7.03 (t, *J* = 6.1 Hz, 1 H).

After purification using column chromatography, 3,14,19-tri-*O*-acetyl- andrographolide (**Andro-3B**) was found as a colorless viscous oil (90% yield). For EI-MS analysis, *m/z* (relative intensity): 416 $[M - CH_3CO_2H]^+$ (33), 356 $[M - 2\ CH_3CO_2H]^+$ (33), 341 $[M - 2\ CH_3CO_2H - CH_3]^+$ (4), 314 $[M - CH_3CO_2H - CH_3CO_2 - CH_3CO]^+$ (7), 296 $[M - 3\ CH_3CO_2H]^+$ (100). For CI-MS analysis, *m/z*: 477 $[M + 1]^+$. For ^{1}H NMR analysis, (i) CH_3 groups: δ 0.76 (s, 3 H), 1.04 (s, 3 H), 2.05 (s, 6 H), 2.12 (s, 3 H); (ii) ring CH/CH_2 groups and allylic H atoms: δ 1.22 –

Table 1. Inhibition of MCF-7 cancer cell lines by andrographolide derivatives

Compounds	Cell survival (%)						
	100 μM	50 μM	25 μM	12.5 μM	6.25 μM	3.13 μM	0 μM
Andro-1	3.3 ± 0.3	3.7 ± 0.6	7.8 ± 2.1	41.0 ± 1.6	48.5 ± 3.2	105.7 ± 4.4	100 ± 11.3
Andro-2	2.7 ± 0.9	2.4 ± 0.6	11.1 ± 2.3	39.9 ± 3.4	51.0 ± 1.4	68.0 ± 9.0	100 ± 11.35
Andro-2A	3.0 ± 0.5	2.8 ± 0.3	4.5 ± 1.0	34.5 ± 2.0	48.7 ± 1.7	75.7 ± 4.0	100 ± 11.35
Andro-3A	1.2 ± 0.7	1.5 ± 0.4	2.5 ± 0.0	32.4 ± 1.8	35.1 ± 10.8	46.0 ± 2.6	100 ± 11.35
Andro-3B	2.6 ± 0.6	1.9 ± 0.3	4.9 ± 2.8	16.0 ± 7.4	53.1 ± 10.8	63.4 ± 20.3	100 ± 11.35

1.37 (m, 3 H), 1.75 – 1.92 (m, 4 H), 2.00 – 2.08 (m, 2 H), 2.36 – 2.45 (m, 3 H); (iii) carbinol H atoms: δ 4.12 (d, *J* = 11.7 Hz, 1 H), 4.26 (dd, *J* = 1.5, 10.5 Hz, 1 H), 4.36 (d, *J* = 11.7 Hz, 1 H), 5.92 (d, *J* = 5.7 Hz, 1 H); (iv) lactonyl CH_2 group: δ 4.54 – 4.64 (m, 2 H); (v) olefinic H atoms: δ 4.53 (s, 1 H), 4.91 (s, 1 H), 7.01 (t, *J* = 6.1 Hz, 1 H).

Inhibition of andrographolide derivatives to MCF-7 cells.

The anticancer activity of andrographolide derivatives (**Andro-2**, **Andro-2A**, **Andro-3A** and **Andro-3B**) as well as the parent compound **Andro-1** were evaluated in MCF-7 human breast cancer cell lines. Concentrations of examined compounds were 3.13, 6.25, 12.5, 25, 50, 100 μM, respectively, and accompanied with a blank solution as a reference. The growth inhibitory and cytotoxic properties of these compounds were determined by using a 72 h WST-8 cell viability assay.

As shown in Table I, all of the derivatives exhibited improvement in antitumor activities compared to the parent compound against MCF-7 cell lines. Compound **Andro-3A** was the most potent than the others based on the fact that **Andro-3A** produced 50% growth inhibition at concentration lower than 3.13 μM; while **Andro-1**, **Andro-2**, **Andro-2A** and **Andro-3B** gave similar response at concentration around 6.25 μM.

In this study, we observed that the presence of substituents protecting the hydroxyl groups at both C-3 and C-19 of andrographolide was responsible for enhancement of cytotoxic activity. The presence of alkyl or aryl moieties at C-3 and C-19 of andrographolide as isopropylidene and benzylidene as shown in the molecular structures of **Andro-2** and **Andro-2A** did not show variations in the antitumor activity. Analogous andrographolide synthesized by coupling reaction of the two hydroxyl groups present at C-3 and C-19 of andrographolide with aliphatic or aromatic aldehydes (e.g. **Andro-3A**) showed higher cytotoxic activity than that of uncoupled 3,19-disubstitued andrographolide (**Andro-3B**). Substitution of acyl functionality to hydroxyl group present at C-14 of andrographolide as showed by **Andro-3A** gave improvement in anticancer activitity compared to that of C-14 unsubstituted derivatives (**Andro-2** and **Andro-2A**).

CONCLUSION

In conclusion, in our search for development of anticancer agents from medicinal plants, we have tried to synthesize a number of andrographolide derivatives possessing more potent *in vitro* antitumor activity than that of the parent compound against MCF-7 human breast cancer cell lines. Substitution of acyl functionality to hydroxyl group present at C-14 as well as coupling reaction of the two hydroxyl groups present at C-3 and C-19 with aliphatic or aromatic aldehydes were responsible for increasing the cytotoxicity. Structure elucidation of the unidentified products of reactions as well as further variations of the parent compound to improve both potency and selectivity and to obtain more information on the structure–activity relationship (SAR) are currently being investigated.

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