Journal of Medicinal Plants Research Vol. 6(21), pp. 3781-3785, 9 June, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR12.346
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Cholinesterase inhibitory activities of xanthones from *Anaxagorea luzonensis* A. Gray

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Accepted 12 April, 2012

Five xanthones (1-5) were isolated from the heartwood of *Anaxagorea luzonensis* A. Gray (Annonaceae) and this is the first report of the isolation of xanthone 1 (6-deoxyisojacareubin) from this species. The *in vitro* inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) of these isolated xanthones was highest in 1,3,5-trihydroxy-4-prenylxanthone (3) for both enzymes with IC_{50} values of 56.3±0.4 and 46.0±0.3 µM, respectively. The presence of the C-4 prenyl residue attached to the xanthone core co-orrelated significantly with the AChE and BChE inhibitory activities and was significantly reduced by hydroxylation (removal of the carbon double bond and addition of a hydroxyl group).

Key words: *Anaxagorea luzonensis*, Annonaceae, xanthone, acetylcholinesterase, butyrylcholinesterase.

INTRODUCTION

Anaxagorea luzonensis A. Gray (Magnoliales: Annonaceae) is a deciduous shrub that is indigenous to Sri Lanka, India, Indonesia, Laos, Myanmar, Philippines and Thailand. In Thai folk medicine, the heartwood extract is used as a tonic for blood, a stomachic and antipyretic and for treating torn tendons and giving relief from muscular pain (Na Songkha, 1982). Previous studies have addressed the isolation of simple oxygenated and prenylated xanthones together with various flavonoids (Kitaoka et al., 1998; Gonda et al., 2000, 2001, 2002). Among the isolated compounds, 8-prenylnaringenin was found to possess a strong estrogenic action (Kitaoka et al., 1998), whereas 3,5,7,4′-tetrahydroxy-2′-methoxyflavone revealed the same antioxidative ability as α-tocopherol in terms of the DPPH free radical scavenging assay (Gonda et al., 2000).

Xanthones are natural secondary metabolites found in many plant species. They contain a rigid tricyclic aromatic ring, to which can be attached a variety of different substituent groups. The pharmacological activities of isolated xanthones have been widely reported, and these include antioxidant (Yu et al., 2007), hepatoprotective (Fernandes et al., 1995), cytotoxic (Nkengfack et al., 2002; Sousa et al., 2002), anti-inflammatory (Chung et al., 2002), antimicrobial (Ngoupayo et al., 2009) and α-glucosidase inhibitory (Hari et al., 2011; Ryu et al., 2011) activities. Moreover, some xanthones have been reported to have a potent cholinesterase (ChE) inhibitory activity, which may have important roles in the treatment of Alzheimer's disease (AD) (Brühlmann et al., 2004; Urbain et al., 2004, 2008; Louh et al., 2008). A common approach for treating AD is to enhance the neurotransmitter acetylcholine (ACh) level in the brain using ChE inhibitors (ChEIs). There are two ChE enzyme types responsible for the hydrolysis of acetylcholine, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The application of AChEIs successfully restores the level of ACh in the brain in early-stage AD patients. However, as the AChE activity is diminished, so the BChE activity will increase and then acts as the major degrading enzyme (Greig et al., 2002). Therefore, dual AChEI/BChEI might be of benefit for the treatment of AD patients.

A literature survey indicated that the xanthones isolated from *A. luzonensis* have not yet been examined for their

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ChEI activities. Thus, this plant was fractionated to isolate the components. Five xanthones were obtained in this study and their AChEI and BChEI activities were evaluated.

MATERIALS AND METHODS

Plant materials

The heartwood of *A. luzonensis* was collected from Chachoengsao province, Thailand, in February 2008. The material was identified by Prof. Piya Chalermglin, Thailand Institute of Scientific and Technological Research, and a specimen [herbarium number 013408 (BCU)] was deposited at the Department of Botany, Faculty of Science, Chulalongkorn University.

Extraction and isolation

The air dried heartwood (5 kg) was extracted twice with methanol (MeOH) in a Soxhlet apparatus for 8 h. The methanolic extract was filtered and concentrated *in vacuo*, and the resulting aqueous suspension was partitioned successively with *n*-hexane, dicholoromethane (CH_2Cl_2) and *n*-butanol (*n*-BuOH) to yield *n*-hexane (7.8 g, 0.17% of the initial heartwood mass), CH_2Cl_2 (20.5 g, 0.41% of the initial heartwood mass), and *n*-BuOH (85.3 g, 1.7% of the initial heartwood mass) extracts.

A portion of the CH_2Cl_2 extract (10 g) was fractionated using silica gel column chromatography, eluting first with hexane, and then increasing the eluting mobile phase polarity with ethyl acetate (EtOAc) and methanol (MeOH) to give four fractions (A1 to A4). Fraction A1 (8.7 g) was further fractionated over a silica gel column as above except eluting with a stepwise gradient of 9:1 (v/v) hexane: EtOAc to 1:4 (v/v) MeOH: EtOAc to give fractions A1-1 to A1-4. Fraction A1-2 was refractionated in the same manner, and the major component obtained was then purified by Sephadex LH-20 gel filtration eluting with a 1:1 (v/v) chloroform ($CHCl_3$): MeOH mobile phase to yield xanthone 1 (20.5 mg). Xanthone 2 (75.2 mg) was isolated from fraction A3 by Sephadex LH-20 gel filtration as above.

A part of the *n*-BuOH (50 g) extract was dissolved in water and then subjected to a Diaion HP-20 resin column chromatography, with the column being eluted successively with water (H_2O), 1:1 (v/v) MeOH: H_2O, MeOH, 1:1 (v/v) acetone: MeOH and finally with acetone. The acetone eluate was applied to a silica gel column as above except eluting first with hexane and then increasing the polarity with EtOAc/MeOH to give eight fractions (B1 to B8). Fraction B2 was purified to obtain xanthone 3 (23.2 mg) by Sephadex LH-20 gel filtration using MeOH as the eluting mobile phase. Fraction B4 was first chromatographed on a silica gel column as above except with a linear 1:4 to 0:1 (v/v) hexane: EtOAc gradient to give five sub-fractions (B4-1 to B4-5), which were individually subjected to Sephadex LH-20 gel filtration with MeOH as the eluting mobile phase as above to give xanthones 4 (12.4 mg) and 5 (83.2 mg).

The yield of xanthones 1-4 in terms of the original starting heartwood mass were 4.1×10^{-4}, 1.5×10^{-3}, 4.6×10^{-4}, 2.5×10^{-4} and 1.7×10^{-3}%, respectively.

Compound characterization

The structures of the compounds were established by 1D (proton and carbon) and 2D (gHMBC, gHMQC and gCOSY) NMR spectra, obtained on a Varian model Mercury+400 spectrometer, and ESI-MS data, performed on an electrospray ionization mass spectrometer model VG TRIO 2000, and supported by comparison of their NMR and MS data with that previously reported in the literature. The chemical structures were principally established by the 1D NMR and ESI-MS data, with 2D NMR being used to further support this along with comparison of the reported data for the designated compounds in the literature. However, in the case of xanthones 1 and 3, the position assignments in the literature were not clear, and so the 2D NMR data was more important in the chemical structure assignment.

Xanthone 1

Yellow solid; mp: 237-238 °C; ESI-MS m/z: 311 $[M+H]^+$, $C_{18}H_{14}O_5$; ^{1}H-NMR (400 MHz, $(CD_3)_2CO$, δppm): 13.10 (1H, s, 5-OH), 9.23 (1H, s, 1-OH), 7.69 (1H, dd, J=8, 1.5 Hz, H-7), 7.39 (1H, dd, J=8, 1.5 Hz, H-8), 7.31 (1H, dd, J=8, 1.5 Hz, H-6), 7.05 (1H, d, J=10 Hz, H-2), 6.21 (1H, s, H-11), 5.77 (1H, d, J=10 Hz, H-12), 1.49 (6H, s, H-14, 15). ^{13}C-NMR (100 MHz, $CDCl_3$, δppm): 182.9 (C-9), 165.1 (C-6), 162.7 (C-1), 157.2 (C-10a), 147.9 (C-5), 147.1 (C-4a), 129.2 (C-12), 126.1 (C-7), 123.3 (C-8a), 122.9 (C-6), 117.5 (C-8), 11.7 (C-11), 105.2 (C-9a), 103.2 (C-4), 100.6 (C-2), 80.1 (C-13), 29.4 (CH3×2). These data were in good agreement with those values reported by Helesbeux et al. (2004), and the position assignment was also confirmed by gHMBC and gHMQC experimental data. Thus, xanthone 1 was determined as 6-deoxyisojacareubin.

Xanthone 2

Pale yellow solid; mp: 278-279°C; ESI-MS m/z: 261 $[M+H]^+$, $C_{13}H_8O_6$; ^{1}H-NMR (400 MHz, CD_3COCD_3, δppm): 13.17 (1H, s, 5-OH), 7.62 (1H, d, J=8.8 Hz, H-8), 6.98 (1H, d, J=8.8 Hz, H-7), 6.43 (1H, d, J=2 Hz, H-4), 6.23 (1H, d, J=2 Hz, H-2). ^{13}C-NMR (100 MHz, CD_3COCD_3, δppm): 182.1 (C-9), 166.8 (C-3), 165.7 (C-1), 159.7 (C-6), 153.1 (C-4a), 147.8 (C-10a), 134.2 (C-5), 118.4 (C-8), 115.7 (C-7), 114.6 (C-8a), 104.0 (C-9a), 99.8 (C-2), 95.7 (C-4). These data are in good agreement with those values reported by Sia et al. (1995), and so xanthone 2 was determined as 1,3,5,6-tetrahydroxyxanthone.

Xanthone 3

Yellow solid; mp: 189-191 °C; ESI-MS m/z: 313 $[M+H]^+$, $C_{18}H_{16}O_5$; ^{1}H-NMR (400 MHz, $CDCl_3$, δppm): 12.85 (1H, s, 5-OH), 7.77 (1H, dd, J=8, 1.6 Hz, H-8), 7.32 (1H, dd, J=7.6, 1.6 Hz, H-7), 7.26 (1H, t, J=7.6 Hz, H-6), 6.32 (1H, s, H-2), 5.30 (1H, t, J=6 Hz, H-12), 3.57 (2H, d, J=6.4 Hz, H-11), 1.88 (3H, s, H-15), 1.78 (3H, s, H-14). ^{13}C-NMR (100 MHz, $CDCl_3$, δppm): 183.9 (C-9), 164.9 (C-3), 163.4 (C-1), 157.3 (C-4a), 148.0 (C-5), 146.7 (C-10a), 132.8 (C-13), 125.6 (C-7), 124.3 (C-8a), 122.3 (C-6), 121.5 (C-12), 117.3 (C-8), 108.7 (C-4), 105.6 (C-9a), 99.5 (C-2), 26.9 (C-15), 23.1 (C-14), 19.0 (C-11). These data are in good agreement with those values reported by Helesbeux et al. (2004), and the position assignment was also confirmed by gHMBC and gHMQC experimental data. Thus, xanthone 1 was determined as 1,3,5-trihydroxy-4-prenylxanthone.

Xanthone 4

Yellow solid; mp: 286-287°C; ESI-MS m/z: 331 $[M+H]^+$, $C_{18}H_{18}O_6$; ^{1}H-NMR (400 MHz, CD_3COCD_3, δppm): 12.78 (1H, s, 5-OH), 7.64 (1H, dd, J=7.3,1.8 Hz, H-8), 7.29 (2H, m, H-6,7), 6.34 (1H, s, H-2), 2.98 (2H, m, H-11), 1.79 (2H, m, H-12), 1.33 (6H, s, CH_3×2). ^{13}C-NMR (100 MHz, CD_3COCD_3, δppm): 181.0 (C-9), 162.7 (C-3), 161.1 (C-1), 154.7 (C-4a), 146.2 (C-5), 145.0 (C-10a), 124.0 (C-7),

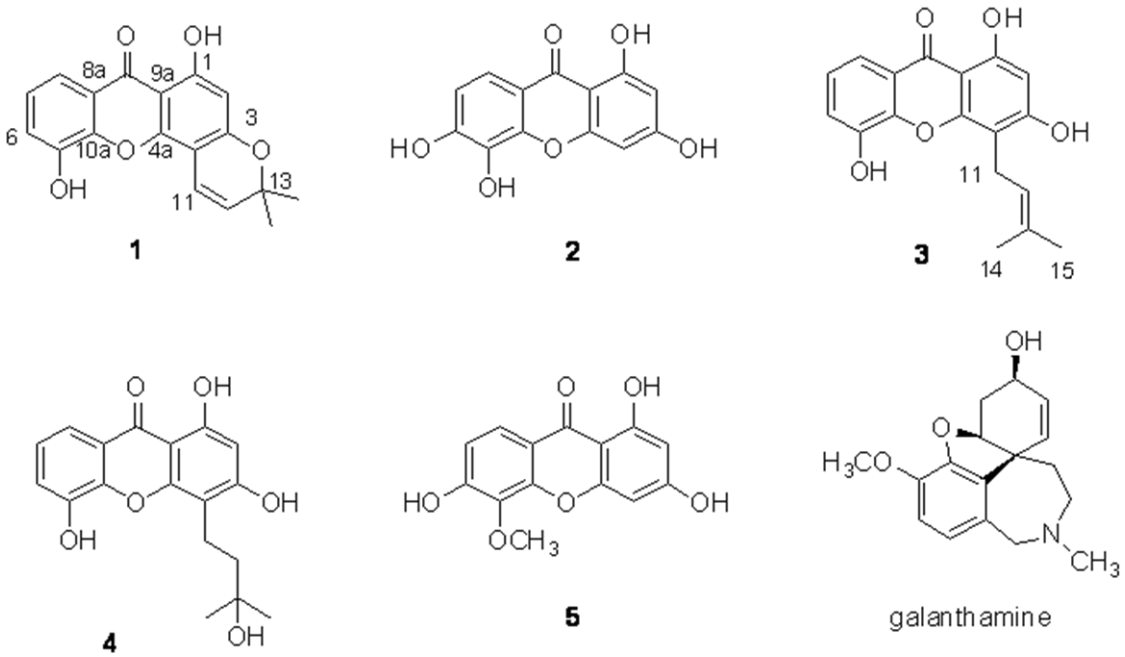

Figure 1. Chemical structures of xanthones 1-5 isolated from *A. luzonensis*, and the reference compound galanthamine.

121.0 (C-8a), 120.2 (C-6), 115.2 (C-8), 107.9 (C-4), 102.9 (C-9a), 97.6 (C-2), 70.7 (C-13), 41.8 (C-12), 28.6 (CH3×2), 16.4 (C-11). These data are in good agreement with those values reported by Gonda et al. (2000), and so xanthone 4 was determined as 1,3,5-trihydroxy-4-(3-hydroxy-3-methylbutyl)xanthone.

Xanthone 5

White needle; mp: 277-278°C; ESI-MS m/z: 275 $[M+H]^+$, $C_{14}H_{10}O_6$; ^{1}H-NMR (400 MHz, CD_3COCD_3, δppm): 13.05 (1H, s, 5-OH), 7.79 (1H, d, J=8.8 Hz, H-8), 6.97 (1H, d, J=8.8 Hz, H-7), 6.45 (1H, d, J=1.3 Hz, H-4), 6.22 (1H, d, J=1.3 Hz, H-2). ^{13}C-NMR (100 MHz, CD_3COCD_3, δppm): 179.8 (C-9), 165.1 (C-3), 163.8 (C-1), 157.7 (C-6), 156.1 (C-4a), 150.6 (C-10a), 134.6 (C-5), 121.1 (C-8), 114.2 (C-7), 113.4 (C-8a), 102.2 (C-9a), 98.1 (C-2), 94.1 (C-4), 60.9 (-OCH_3). These data are in good agreement with those values reported by Zhang et al. (2002), and xanthone 5 was determined as 1,3,6-trihydroxy-5-methoxyxanthone.

Cholinesterase inhibitory (ChEI activity) assay

The AChEI and BChEI activities were determined by a modification to the reported method (Ellman et al., 1961; Ingkaninan et al., 2006). Briefly, 25 μL of 1.5 mM acetylthiocholine iodide in MilliQ water, 125 μL of 3 mM 5,5 dithiobis[2-nitrobenzoic acid] in buffer A (50 mM Tris-HCl, pH 8, 0.1 M NaCl, 0.02 M $MgCl_2 \cdot 6H_2O$), and 50 μL of buffer B (0.1% (w/v) bovine serum albumin in 50 mM Tris-HCl, pH 8) were mixed with 25 μL of sample in a 96-well microplate. Subsequently, 25 μL of 0.22 U/mL of either AChE from electric eel (Type-VI-S, EC 3.1.1.7) or BChE from horse serum (EC 3.1.1.8) was added and the absorbance was measured at 415 nm over 2 min at 5 s time intervals using a Sunrise microplate reader (P-Intertrade Equipments, Australia). The respective enzyme activity was calculated by comparing the rate of reaction for the samples relative to that for the blank (25 μL of solvent, 10% (v/v) methanol in buffer, instead of sample). The percentage AChEI or BChEI activity was calculated by subtracting the percentage of AChE or BChE activity, respectively, from 100%. The experiment was run in triplicate. The concentration of the sample required to inhibit 50% of the maximum observed enzymatic activity (IC_{50}) was determined graphically from log concentration-percent inhibition curves using the GraphPad Prism 5.01 software of GraphPad Software Inc.

RESULTS AND DISCUSSION

The methanolic extract of the heartwood of *A. luzonensis* was successively partitioned with *n*-hexane, CH_2Cl_2 and *n*-BuOH. Fractionation of the CH_2Cl_2 and *n*-BuOH extracts led to the isolation of five xanthones 1-5 (Figure 1). Their AChEI and BChEI activities were evaluated *in vitro* according to Ellman's colorimetric method with some modifications (Ellman et al., 1961; Ingkaninan et al., 2006). The inhibition potency, expressed as IC_{50} values, for the xanthones in comparison with the clinical drug galanthamine, as a reference standard, is shown in Table 1. Xanthone 3 showed the highest AChEI and BChEI activities with IC_{50} values of 56.3 and 46.0 μM, respectively, but these are still 70.3- and 25.6-fold higher, respectively, than that for galanthamine (Figure 2). The cyclization of the C-4 prenyl group to the C-3 and C-4 substituted chromene ring, as seen in xanthone 1, its removal (xanthones 2 and 5) or its hydroxylation (xanthone 4) all resulted in significantly reduced AChEI and BChEI activities. The fact that xanthone 5 was more active than xanthone 2 suggests that the methoxy group

Table 1. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of xanthones 1-5 from *A. luzonensis*.

Xanthones	IC_{50} (μM)[a]	
	AChE	BChE
1	> 1000	138.3 ± 8.4
2	> 1000	> 1000
3	56.3 ± 0.4	46.1 ± 0.3
4	379.3 ± 0.9	514.3 ± 1.7
5	520.4 ± 6.4	430.3 ± 5.1
galanthamine[b]	0.8 ± 0.1	1.8 ± 0.6

[a]Data are reported as the mean ± 1 S.D. and are derived from three repeats. [b], Standard compound for the ChE inhibition assays.

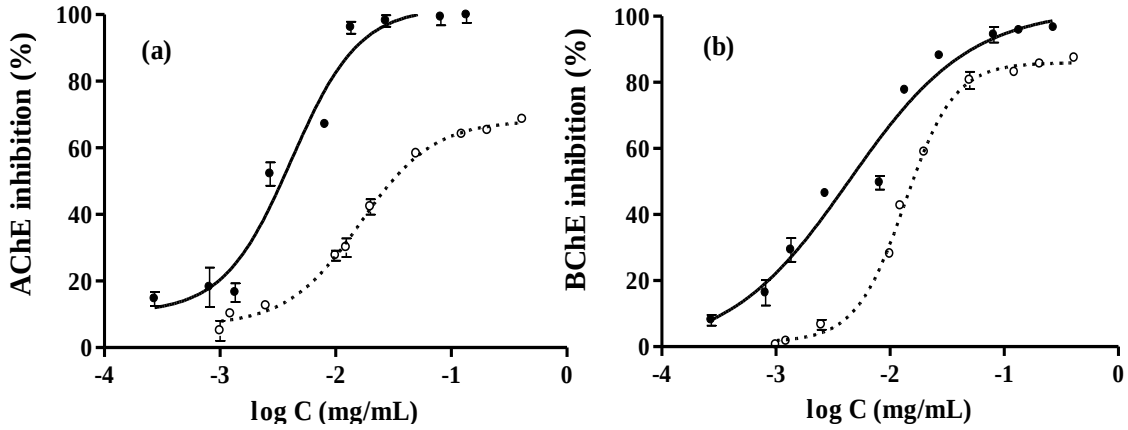

Figure 2. The sigmoidal dose-response curves of the inhibition activities towards (a) AChE and (b) BChE of xanthone 3 (···○···) and galanthamine (–●–). Data are from one typical experiment performed in triplicate, and are shown as the mean ± 1 SD.

at the C-5 position may also be important for AChEI and BChEI activities. Nevertheless, the importance of the presence of the C-4 prenyl and C-5 methoxy groups in xanthone for good AChEI and BChEI activity, although supported here, still remains to be confirmed.

The ChEI activity of xanthones and some essential structural requirements have previously been reported from many plants, such as *Gentiana campestris*, *Gentianella amarella* and *Garcinia polyantha* (Urbain et al., 2004, 2008; Louh et al., 2008). For example, bellidifolin, which was isolated from *G. campestris* together with bellidin and their glycosides, showed similar AChEI and BChEI effects to galanthamine in the TLC-bioautographic method (Urbain et al., 2004). Furthermore, they reported that the methoxy group in C-3 position increased the AChEI and BChEI activities whilst the glycosidic moiety decreased these activities due to its hydrophobicity or steric factors. However, all of the ten xanthones, including bellidifolin, that were obtained from *G. amarella* exhibited only a weak inhibition towards AChE compared to that elicited by galanthamine in the microplate assay (Urbain et al., 2004), suggesting a potential assay-dependent affect upon the relative and absolute ChEI activity, and so we can not reliably compare the activity (and so structure-function relationships) of xanthone 3 here with bellidifolin or indeed the other reported xanthones. Xanthones with different substituent groups (for example, hydroxyl, methoxy and prenyloxy groups) from *G. polyantha* showed moderate to high AChEI and BChEI activities (Louh et al., 2008). Among these, 1,5-dihydroxyxanthone gave a three-fold lower IC_{50} value towards BChE than galanthamine, whilst the insertion of an additional hydroxyl group at the C-3 position resulted in a reduced BChEI activity. Moreover, Ji and Zhang (2006) concluded that the substitution of the C-1 hydroxyl and C-3 methoxy groups resulted in higher AChEI and BChEI activities. The molecular docking study of macluraxanthone, isolated from *Maclura pomifera* concluded that the hydroxyl group substituents provide a strong binding with

the amino acid residues of AChE and BChE *via* hydrogen bonding (Khan et al., 2009). The effect of prenylated cyclization on increasing the AChEI activity was also found in an isoflavone core structure reported by Orhan et al. (2009). They isolated two isoflavones (osajin and pomiferin) from *Maclura pomifera* and their prenylated cyclization derivatives (*iso*-osajin and *iso*-pomiferin) were synthesized. The cyclization of a prenyl group of osajin conferred an increasing AChEI activity, in contrast to that of pomiferin, where *iso*-pomiferin showed a lower AChEI activity than pomiferin. This suggested that the prenyl group influences the AChEI activity but other factors, such as the inhibition mode and binding site are still required to be evaluated to confirm the structure-activity relationship (SAR). Nevertheless, our study potentially does not support the above-mentioned assumptions of the SAR in xanthones. Regardless, this study suggests the potential (correlative) importance of the C-4 prenyl group and the C-5 methoxy group in xanthones for a good AChEI and BChEI activities. Thus, these findings are significant in the search for, and development of, lead-compounds in the field of AD therapeutics.

ACKNOWLEDGEMENTS

This work was supported by Research Funds from the Faculty of Science, Chulalongkorn University (RES-A1B1-21), the Higher Education Research Promotion and National Research University Project of Thailand, the Office of the Higher Education Commission (FW645A), Chulalongkorn University and a Chulalongkorn University Graduate School Thesis Grant. The authors are grateful for the English corrections by Robert Butcher of the Publication Counseling Unit, Faculty of Science, Chulalongkorn University.

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