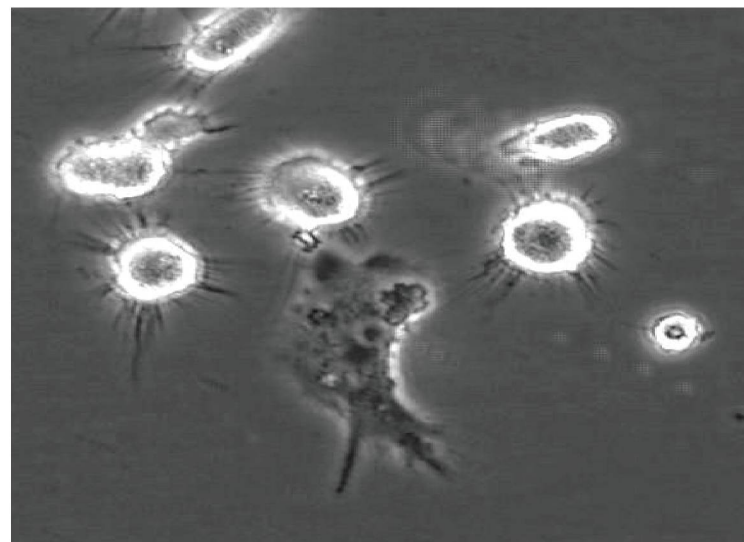
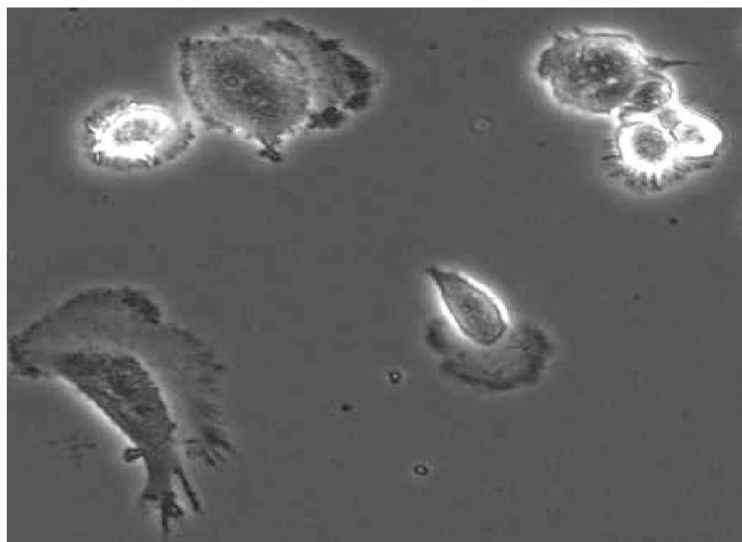
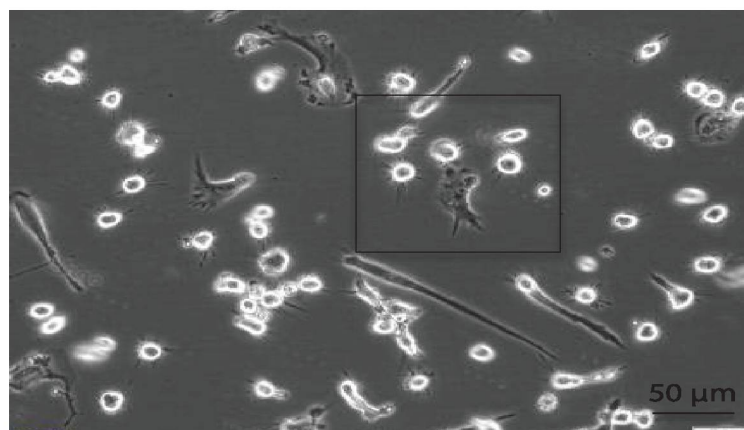
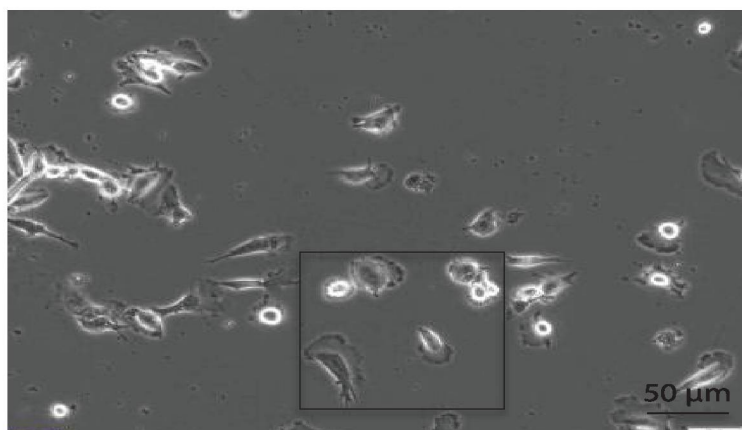


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Initial characterisation of exosomes released by umbilical cord-derived mesenchymal stem cells and mature dendritic cells, under 'Good Manufacturing Practice' conditions

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- 4.2. Geology
- 4.3. Geophysics
- 4.4. Oceanography

5. Environmental Sciences

- 5.1. Ecology
- 5.2. Climatology

PHYSICAL SCIENCES

3 Synthesis and characterisation of dysprosium-doped borate glasses for use in radiation dosimeters.

R.S. Omar, S. Hashim, S.K. Ghoshal

9 Chemical constituents from methanolic extract of *Solanum procumbens* Lour (Solanaceae).

Thi Thanh Thuy Tran, Hoang Phu Dang, Trung Nhan Nguyen

12 Phenolic compounds from *Usnea baileyi* (Stirt.) Zahlbr growing in Lam Dong province.

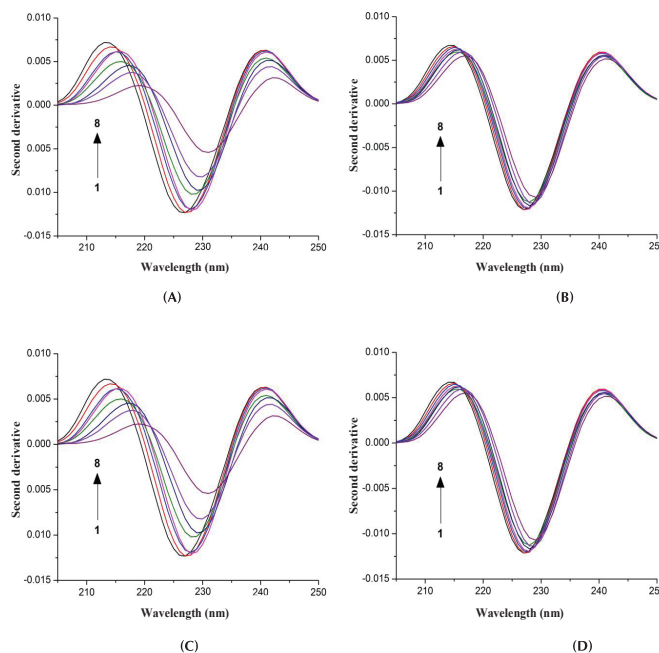
Van Kieu Nguyen, Thuc Huy Duong

16 Partitioning of fluoxetine into mixed lipid bilayer containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC).

Anh T. Sy, Vy T. Pham, Trang T. Nguyen

25 Characterization of optical parameters of breast cancer cell line - BT474 by polarimetry technique.

Thanh-Truc Nguyen, Minh-Vy Huynh, Thanh-Hai Le, Thi-Thu-Hien Pham



LIFE SCIENCES

33 The efficacy of micronano particles across NPK doses and densities on maize growth and yield in Vietnam.

Le Quy Kha, Ngo Quang Vinh, Nguyen Hoai Chau, Pham Vu Bao

40 Melanosis and quality changes of Pacific white shrimps (*Litopenaeus vannamei*) treated with *Houttuynia cordata* extract during cold storage.

Dao Thi Anh Phan, Nga Kieu Thy Vo, Huy Minh Tran, Phan Thi Cam Bui

45 Initial characterisation of exosomes released by umbilical

cord-derived mesenchymal stem cells and mature dendritic cells, under 'Good Manufacturing Practice' conditions.

Hoang Huong Diem, Bui Thi Van Khanh, Hoang Thi My Nhung, Nguyen Thanh Liem, Than Thi Trang Uyen

52 Multiplex polymerase chain reaction (M-PCR) for bacterial vaginosis detection.

Tien Sang Trieu, Van Khoa Tran, Ha My Nguyen, Thi Thu Ha Nguyen, Quang Tung Le, Minh Ngoc Phung, Minh Quang Diep, Huong Ly Vu

CONTENTS

SEPTEMBER 2019 • VOL.61 NUMBER 3

58



57 Two newly recorded species of *Urticaceae* among the flora of Vietnam used as green foods for H'mông beef cattle.

Hung Duy Nguyen, Long-Fei Fu, Yi-Gang Wei, Truong Van Do

61 Cultivation of *Haematococcus pluvialis* for astaxanthin production on angled bench-scale and large-scale biofilm-based photobioreactors.

Hoang-Dung Tran, Thanh-Tri Do, Tuan-Loc Le, Minh-Ly Tran Nguyen, Cong-Hoat Pham, Michael Melkonian

71 Application of biological methods in the treatment of gaseous ammonia.

Pham Thanh Hien Lam, Ngoc Bao Tram Bui, Thanh Tinh Nguyen, Thi Le Lien Nguyen, Thi Thanh Thuy Vo, Nhat Huy Nguyen

ENVIRONMENTAL SCIENCES

77 Assessment and prioritisation of low-carbon technology for the waste sector in Vietnam.

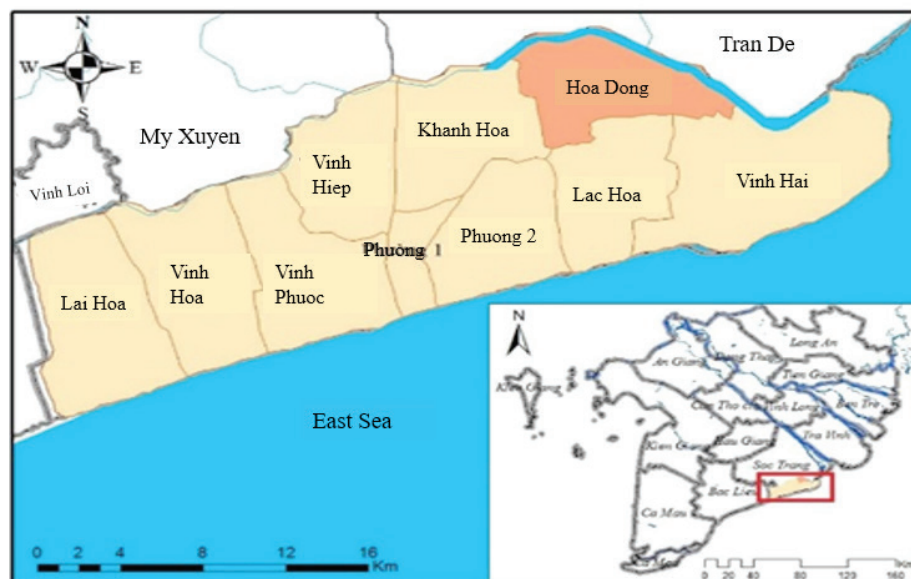
Nguyen Van Thang, Le Ngoc Cau, Makoto Kato, Satoshi Sugimoto

81 Quantification of greenhouse gas emissions from different municipal solid waste treatment methods - case study in Ha Noi, Vietnam.

Thi Mai Thao Pham

90 The current state of domestic water consumption and the feasibility of implementing a rainwater harvesting system in the coastal zone of the Vietnamese Mekong delta: the case study of the Vinh Chau town, Soc Trang province.

Ngoc Diep Nguyen, Thi Le Hang Tran, Van Der Heiden Dion, Diep Anh Tuan Dinh, Pham Dang Tri Van



91

Synthesis and characterisation of dysprosium-doped borate glasses for use in radiation dosimeters

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Abstract:

This paper reports on the synthesis and characterisation of Dy_2O_3 -doped magnesium borate (MB) glasses containing different modifiers, lithium, calcium, and sodium oxides. Glasses composed of $(70-z)\text{B}_2\text{O}_3\text{-}20\text{Li}_2\text{O; CaO; Na}_2\text{O-}10\text{MgO-}z\text{Dy}_2\text{O}_3$ (where $0.05 \leq z \leq 0.7$ mol%) were prepared using the melt-quenching method. X-ray diffraction (XRD) pattern of the as-quenched samples verified their amorphous character. Differential thermal analysis (DTA) confirmed excellent glass-forming ability and thermal stability in the range of 0.60-0.67 and 0.18-0.82, respectively. The energy dispersive X-ray (EDX) spectra verified the precise elemental traces in the studied glasses. Furthermore, MB glasses doped with 0.1 mol% of Dy_2O_3 and modified with lithium oxide were found to have the best soft tissue equivalence ($Z_{\text{eff}} \approx 8.13$). In short, the proposed MB glass system doped with dysprosium ions (Dy^{3+}) was established as effective for accurate radiation detection in emergency situations.

Keywords: dysprosium, MB glass, melt-quenching, radiation detection.

Classification number: 2.1

Introduction

The scientific interest in glassy systems began a few decades ago with the pioneering works of Anderson and Mott on disordered solids as examples of non-crystalline solids [1]. Due to the notable physical and optical properties of borate compounds, new uses of these compounds have gradually emerged [2-4]. Oxide glasses have gained attention due to their structural features [5-7]. The borates containing the isolated planar $[\text{BO}_3]^{3-}$ group in their structure have been shown to be good birefringent materials [8]. The distinguishing feature of the melt-quenching technique used to produce amorphous material is that the amorphous solid can be formed by continuous hardening (increase in viscosity) of the melt [9]. The existence of alkaline metal ions, which act as modifiers in glass systems, build up vacancies and create ionic bonds instead of covalent bonds with the oxygen atoms. This gives the glassy chemical a well-defined shape. The fact that alkaline metal ions have the properties of being small and mobile means these materials are commonly used in thermoluminescent (TL) glass systems. This is because the occurrence of these materials in glass systems introduces a degree of electrical conductivity, particularly in a molten state or at a high temperature. Moreover, the addition of alkaline metal ions creates non-bridging oxygen, the concentration of which increases linearly as the alkaline content increases. Lithium, sodium and calcium are all alkali earth metals and are commonly used in glass systems due to their resistance to corrosion and easier processing. Rare earth elements such as samarium (Sm), europium (Eu), terbium (Tb), dysprosium (Dy) and thulium (Tm) are generally introduced as the doping elements or doping salts, especially in TL dosimetry applications. These lanthanide elements can modify the structure of the glass, as well as its electrical, optical and TL properties. Environmental and personnel monitoring for radiation exposure requires a sensitive TL detector; it should

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be cost effective, have good reproducibility, high sensitivity and tissue equivalence. All of these criteria can be met with the addition of Dy ions to the glass system.

Questions remain regarding the structure of substances, and solving them will facilitate accurate predetermination of the properties of synthetic materials under development. The properties of the glass samples properties are affected by the composition and the various modifying agents of the materials. The very few TL materials (TL dosimeters) appear to be the most attractive due to the fact that they are amorphous materials [10-13]. The most common approach for producing amorphous solid materials (notably, oxide glasses and organic polymers) is to cool the molten form of the material using a melt-quenching technique [14, 15]. Borate glass is relatively chemically stable and does not present any serious problems for doping with impurities such as rare earth, copper, and manganese ions. This study may be useful for future researchers to understand the effects of lithium, calcium, and sodium as modifiers in borate glasses with the presence of dysprosium. The present work attempts to provide new fundamental knowledge about various properties of the proposed glass composition for new TL glass dosimeter applications. In this work, Dy³⁺-doped magnesium borate (MB) glasses with three different modifiers (Na₂O, Li₂O, and CaO) were prepared using a melt-quenching method. The physical properties of the as-quenched samples, including their amorphous state and their glass-forming abilities, were determined. Generally, pure borate glass has certain shortcomings for radiation dosimeter applications due to its highly hygroscopic nature and weak TL glow peak at low temperatures. However, the addition of alkali oxides into borate can overcome these drawbacks as the inclusion of a modifier such as Ca can ensure low hygroscopicity and high chemical stability. The amorphous nature of all the as-quenched samples was verified by X-ray diffraction (XRD) analysis. Differential thermal analysis showed that all the studied glasses obey Kauzmann criterion with excellent *T_g* values and good glass-forming ability. Elemental analyses of glasses were performed using energy dispersive x-ray (EDX) spectroscopy, whereby all the data were used to calculate the effective atomic number (*Z_{eff}*). The obtained results on the proposed glasses may contribute to the study of the TL properties for radiation dosimetry in general and personnel monitoring in particular.

Materials and methods

A brief description of the glass preparation method is presented. A series of Dy₂O₃-doped LMB glasses of nominal

composition (70-*z*)B₂O₃-20Li₂O; CaO; Na₂O-10MgO-*z*Dy₂O₃ (where 0.05 ≤ *z* ≤ 0.7 mol%) were prepared using the melt-quenching method. Analytical grade chemical reagents (in powder form and 99.9% pure) of boron oxide (B₂O₃), magnesium oxide (MgO), lithium oxide (Li₂O), calcium oxide (CaO), sodium oxide (Na₂O) and dysprosium (III) oxide (Dy₂O₃) were used as glass constituents. These chemicals were supplied by Acros Organic and QReC (reagent grade) and were 99.9% pure. Powdered constituents for each batch of 10 g were mixed thoroughly using a milling machine to obtain a homogenous mixture. For each sample, the mixture was placed in a porcelain crucible before being melted inside an electronic furnace (Nabertherm GmbH:SN 299205) at 1100°C for 1 hour and was stirred frequently to ensure complete homogeneity. The resultant melt was annealed at 350°C for 4 hours and allowed to cool gradually (at a rate of 10°C min⁻¹) to room temperature. Finally, the frozen solid was cut into the preferred size and polished for additional spectroscopic analyses. Six samples were prepared and are listed in Table 1. In the case of CMB doped with 0.50 Dy, this concentration was chosen for its optimum concentration at 0.5 mol%, revealed a TL glow curve at a single broad peak, and its *T_m* was around 211°C and it meets the requirements of the ideal TL dosimeter when exposed to such radiation (Cobalt-60 gamma ray). The sample exhibited a stable state when analysed with 0.5 mol% of Dy concentration.

Table 1. Nominal composition of the studied glasses.

Glass Code	Composition (mol%)					
	B ₂ O ₃	MgO	Li ₂ O	CaO	Na ₂ O	Dy ₂ O ₃
LMBDy0	70.00	10.00	20.00	-	-	0.00
LMBDy0.10	69.90	10.00	20.00	-	-	0.10
CMBDy0	70.00	10.00	-	20.00	-	0.00
CMBDy0.50	69.50	10.00	-	20.00	-	0.50
NMBDy0	70.00	10.00	-	-	20.00	0.00
NMBDy0.10	69.90	10.00	-	-	20.00	0.10

The XRD analysis was performed using micro-sized powdered glasses in order to check the amorphous phase of the studied samples. The samples were scanned by mean of the XRD method using an X-ray diffractometer (Siemens Diffractometer D5000 model) with CuK_α radiation operating at 40 kV and 30 mA in Bragg-Brentano geometry at room temperature. The diffraction patterns were measured in steps of 0.05 degree (°) for 1 s counting time per step, with 2θ ranging from 10° to 90°. The inbuilt software in

the diffractogram provided information on atomic pair correlations and bond lengths of the MgO , Li_2O , CaO , Na_2O , B_2O_3 or Dy_2O_3 compounds used as glass constituents.

Supplementary differential thermal analysis (DTA) was used to analyse the heat flows in the glass system as a function of temperature. Thermal behaviour, including the glass transition temperature (T_g), crystallisation temperature (T_c) and melting temperature (T_m), was measured using TG-DTA (Perkin Elmer Pyris Diamond Thermogravimetry - Differential Thermal Analyzer model). This was also used to evaluate glass-forming ability (T_g) and thermal stability in terms of the Hruby parameter (H_R). The TG-DTA was conducting on fine and micro-sized powdered glasses at a temperature range of 50-1000°C (accuracy $\pm 0.1^\circ\text{C}$) with a heating rate of $10^\circ\text{C min}^{-1}$. The glass-forming ability or thermal stability range was determined from the difference between T_c and T_g . The powder (5 mg) was ground from the bulk glass sample and added to the pan. The sample weight was determined to ensure that the total weight of both sample and pan was within 0.1 mg. The low heating rate was chosen to increase the resolution of the system.

The composition of elements present in the prepared glass samples was determined using EDX analysis, which enabled the effective atomic number (Z_{eff}) of the studied samples to be determined. This was achieved using a ZEISS Supra 35 VP scanning electron microscope (SEM) coupled with EDX spectroscopy. Samples were coated with gold using a BIO-RAD Polaron E5400 SEM sputter coating system to ensure good electrical connectivity with the sample holder. The data recorded consisted of spectra presenting peaks corresponding to the elements making up the composition of the sample being examined.

Results and discussion

Figure 1A illustrates the typical XRD patterns of the six studied samples, which consisted of two amorphous halos (broad hump) without any sharp crystalline peaks. These experimental patterns, useful for identification, were obtained using diffractometer methods. The 1976 Interim Report of the National Bureau of Standards was referred to in order to verify the overall results [16]. The magnitude of scattering in a given direction (θ or 2θ) is described in units relative to the scattering from a single electron. The intensity is the quantity measured by the diffraction device and is given as the magnitude of the amplitude squared. The scattering magnitudes are expressed in electron scattering units, and diffraction angles refer to $\text{CuK}\alpha$ X-rays [17, 18]. These broad humps (at 2θ values around 20 - 30° and 40 - 50°) representing the atomic pair correlations of the bond distances of the constituents MgO , Li_2O , CaO , Na_2O , B_2O_3 or Dy_2O_3 confirmed the amorphous nature of the as-quenched sample. However, the intensity of the studied samples gradually decreases with increasing values of 2θ . The scattering factors decrease with increasing 2θ because of destructive interference within the atoms and due to thermal effect. As shown in Fig. 1A, these samples reveal no discrete peaks and a lack of periodicity that is typical for short-range ordered materials, such as glass or liquid that reaches the glassy phase. It is also observed that no sharp peaks were obtained from the XRD analysis. In this case, the broad peaks cannot belong to the glassy phase. The local structure of glass has no long-range order and, therefore, generates only broad features in the diffraction pattern. Therefore, all the glass systems reveal that the samples are glass in nature.

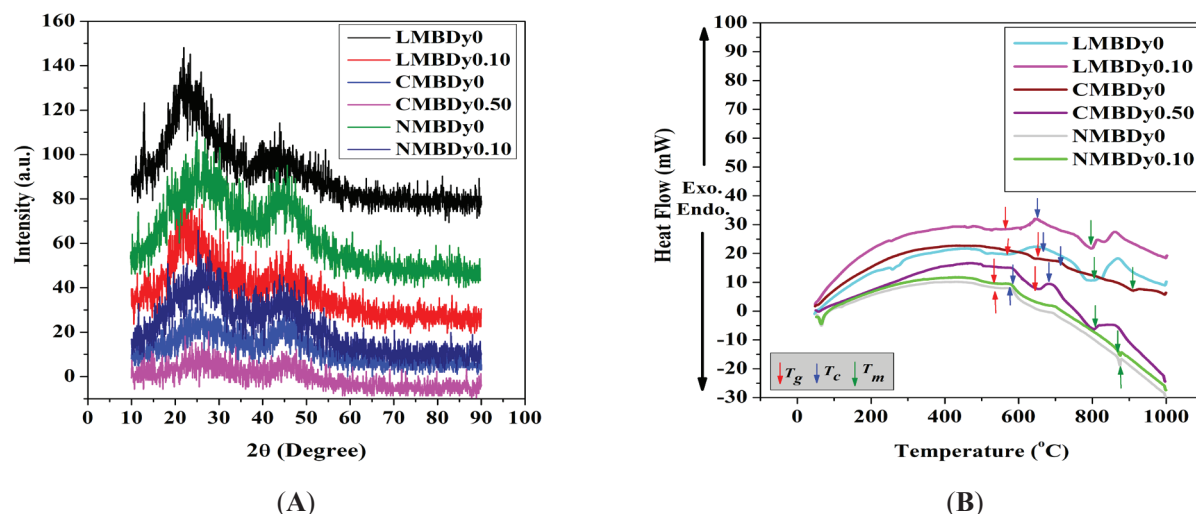


Fig. 1. XRD patterns of LMBDy0, LMBDy0.10, CMBDy0, CMBDy0.50, NMBDy0, and NMBDy0.10 (A); DTA traces of LMBDy0, LMBDy0.10, CMBDy0, CMBDy0.50, NMBDy0, and NMBDy0.10 (B).

Figure 1B shows the DTA curves of LMBDy0, LMBDy0.10, CMBDy0, CMBDy0.50, NMBDy0, and NMBDy0.10 samples with their respective endothermic peaks of T_g at 519.25, 548.87, 626.01, 509.91, 517.01, and 529.68°C, respectively. The exothermic peak of T_c for LMBDy0 and LMBDy0.10 samples appeared at 644.18°C and 670.30°C. Whereas, the exothermic peak of T_c for CMBDy0, CMBDy0.50, NMBDy0, and NMBDy0.10 samples appeared at 744.99, 638.59, 578.99, and 582.75°C, respectively. Meanwhile, the endothermic peak of T_m for the samples occurred at 797.002°C (LMBDy0), 823.12°C (LMBDy0.10), 934.99°C (CMBDy0), 803.77°C (CMBDy0.50), 860.72°C (NMBDy0), and 874.71°C (NMBDy0.10).

The values of T_g , T_c and T_m were found to be sensitive to concentrations of Dy^{3+} ions, as shown in Table 2. Each DTA trace was recorded three times to obtain the average peak value. The estimated values of T_g were found to obey the Kauzmann assumption ($0.5 \leq T_{rg} \leq 0.66$), indicating good glass-forming ability or a lower devitrification tendency [19]. According to Hruby's assumption, a glass system is said to be thermally stable if $H_R \sim 0.5$ and unstable if $H_R \leq 0.1$ [20]. The large values of H_R and T_{rg} obtained clearly indicate excellent thermal stability and glass-forming ability, respectively (Table 2). However, Table 2 shows that the NMBDy0 and NMBDy0.10 samples were found not to meet the glass thermal stability requirement and, therefore, cannot be considered good glass formers. Hence, the glass samples require higher cooling rates.

Table 2. DTA thermal analysis of the studied glasses.

Glass code	T_{rg} value	H_R value
LMBDy0	0.65	0.82
LMBDy0.10	0.67	0.79
CMBDy0	0.67	0.62
CMBDy0.50	0.63	0.78
NMBDy0	0.60	0.22
NMBDy0.10	0.61	0.18

EDX emissions of the LMBDy0, LMBDy0.10, CMBDy0, CMBDy0.50, NMBDy0, and NMBDy0.10 samples are shown in Fig. 2 (A, B, C, D, E, F). The peak height of the spectra represents the abundance of each element in the glass samples, with the x-axis representing the X-ray energy (keV). In this case, lithium (Li) was not easy to detect (Fig. 2A and 2B), due to the very low energy of characteristic radiation. The data from the EDX analysis were used to calculate the effective atomic number (Z_{eff}). The value of the experimental fractional weights, W_{iE} (from the EDX analysis), is compared with the nominal fractional weights, W_{iT} for all the glass samples (Table 3). All these values were compared to calculate the effective atomic number (Z_{eff}), as shown in Table 4.

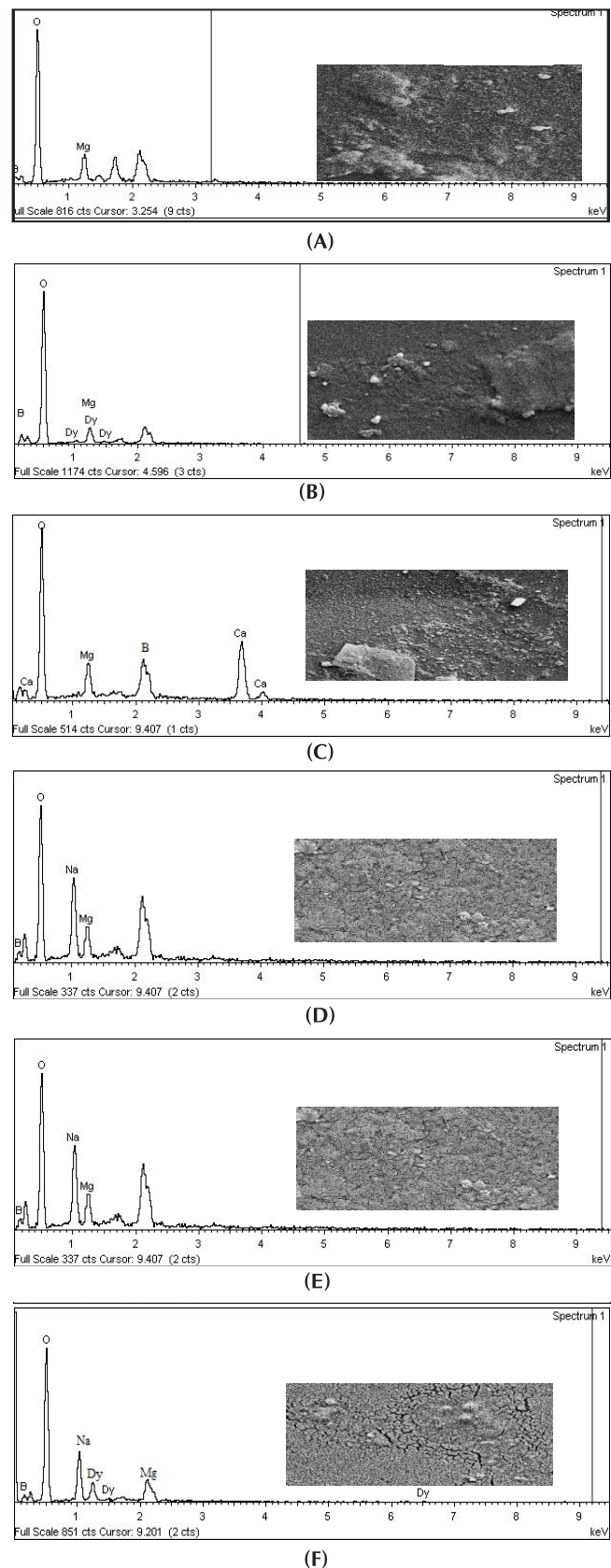


Fig. 2. EDX spectrum of LMBDy0 (A), LMBDy0.10 (B), CMBDy0 (C), CMBDy0.50 (D), NMBDy0 (E), and NMBDy0.10 (F) glass.

Table 3. Nominal and experimental value of fractional weights of each element of the studied samples.

Element	Nominal, W _{IT}	Experimental, W _{IE}	Nominal, W _{IT}	Experimental, W _{IE}
<i>LMBDy0</i>		<i>LMBDy0.10</i>		
Mg	0.0603	0.0260	0.0603	0.0110
Li	0.0929	0.1667	0.0929	0.1667
B	0.2174	0.1955	0.2171	0.2512
O	0.6293	0.6118	0.6288	0.5703
Dy	-	-	0.0008	0.0008
<i>CMBDy0</i>		<i>CMBDy0.50</i>		
Mg	0.0603	0.0217	0.0603	0.0226
Ca	0.1667	0.1853	0.1667	0.0670
B	0.2174	0.2870	0.2158	0.2580
O	0.5556	0.5060	0.5528	0.6358
Dy	-	-	0.0043	0.0166
<i>NMBDy0</i>		<i>NMBDy0.10</i>		
Mg	0.0603	0.0203	0.0603	0.0168
Na	0.1484	0.0606	0.1484	0.0651
B	0.2174	0.2261	0.2171	0.2288
O	0.5739	0.6930	0.5734	0.6790
Dy	-	-	0.00087	0.0097

A dosimeter material should have a Z_{eff} as close as possible to the Z_{eff} of human tissue and is called a tissue-equivalent material. According to the International Commission on Radiological Protection, for human tissue, $Z_{eff}=7.4$. For a mixture or composite such as glass, an equation defined by Mayneord (1937) [21] can be used to determine the single index of Z_{eff} number for a given composite of materials. This is adopted in Eq. (1).

$$Z_{eff} = (a_1 Z_1^m + a_2 Z_2^m + a_3 Z_3^m + \dots + a_n Z_n^m)^{1/m} \quad (1)$$

where $a_1, a_2, \dots, a_n$ are the weight fraction of each component of the glass material, which depend on the total number of electrons in the mixture, and Z_n is the atomic number of the element n . The value of m adopted for photon purposes is 2.94.

The experimental and theoretical results for the Z_{eff} of LMBDy0, LMBDy0.10, CMBDy0, CMBDy0.50, NMBDy0, and NMBDy0.10 samples are given in Table 4.

Table 4. Z_{eff} (theoretical) and Z_{eff} (experimental) of the samples.

Glass Code	Z_{eff} (experimental)	Z_{eff} (theoretical)	Percentage deviation (%)
LMBDy0	7.34	7.74	5
LMBDy0.10	8.13	8.67	6
CMBDy0	12.19	12.03	1
CMBDy0.50	16.64	13.92	16
NMBDy0	7.94	8.53	7
NMBDy0.10	13.78	9.32	32

It can clearly be seen that the Z_{eff} of all the glass samples depends on the concentration of dysprosium, which increases with the addition of dysprosium concentrate. Of the three modifiers, lithium, calcium and sodium, the closest tissue-equivalent properties were recorded for LMBDy0, LMBDy0.10 glass samples as these materials had Z_{eff} values near to that of soft tissue. By contrast, calcium magnesium borate and sodium magnesium borate glass systems are considered suitable TL materials with bone-equivalent performance when dopant is added. These results support the study of TL properties for radiation dosimetry in general and personnel monitoring in particular [22].

Conclusions

A series of Dy_2O_3 -doped MB glasses modified with lithium, calcium, and sodium oxides were prepared using the melt-quenching method and characterised to determine their feasibility for use in radiation dosimeters. Differential thermal analysis confirmed their excellent glass-forming ability and thermal stability. Energy dispersive X-ray spectra verified the elemental traces in the sample. Furthermore, MB glasses doped with 0.1 mol% of Dy_2O_3 and modified with lithium were found to have the closest soft tissue equivalency ($Z_{eff} \approx 8.13$). The proposed MB glasses doped with dysprosium ions (Dy^{3+}) were established as effective for accurate radiation detection in personnel monitoring.

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Chemical constituents from methanolic extract of *Solanum procumbens* Lour (Solanaceae)

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Abstract:

Solanum procumbens L. (Solanaceae) were extracted respectively with *n*-hexane, ethyl acetate, and methanol to obtain the corresponding extracts. The methanol-soluble extract was acidified by adding HCl 0.5M; this was followed by liquid-liquid partition with CHCl₃ to afford CHCl₃-soluble fraction. From this fraction, four compounds were isolated and identified as ergosterol peroxide (1), 9,11-dehydroergosterol peroxide (2), *N*-(*N*-benzoyl-L-phenylalanyl)-L-phenylalanol (3), and moupinamide (4). Their structures were elucidated based on the NMR spectroscopic analysis and comparison with the literature. All isolated compounds were first reported from *S. procumbens*.

Keywords: ergosterol peroxide, moupinamide, Solanaceae, *Solanum procumbens*.

Classification number: 2.2

Introduction

Solanum procumbens L., which belongs to the genus *Solanum* (Solanaceae), was collected at Tinh Bien district, An Giang province, Vietnam. In traditional medicine, the entire plant of *S. procumbens* is used for curing arthritis and hepatitis treatments as well as for supportive treatment of the liver [1]. Based on the literature, *S. procumbens* demonstrated potential antimicrobial activities [2]. The previous investigations regarding chemical constituents of *S. procumbens* precipitated the isolation of flavonoids, steroids, phenols, coumarins, and glycoalkaloids [3]. This paper reports the extraction and structural elucidation of isolated compounds from methanol extract of *S. procumbens*. As a result, four compounds were identified as ergosterol peroxide (1), 9,11-dehydroergosterol peroxide (2), *N*-(*N*-benzoyl-L-phenylalanyl)-L-phenylalanol (3), and moupinamide (4).

Experimental

Plant materials

The entire plant of *S. procumbens* was collected at Tinh Bien district, An Giang province, Vietnam. The plant was identified by the Institute of Drug Quality Control, Ho Chi Minh city, Vietnam. The plant sample was identified by Dr. rer. nat. Anh Tuan Dang-Le, Lab. of Botany, Department of Ecology and Evolutionary Biology, Faculty of Biology-Biotechnology, University of Science, Vietnam National University, Ho Chi Minh city.

General procedures

NMR spectra were taken using a Bruker Avance III 500 spectrometer, with tetramethylsilane (TMS) as an internal standard, and chemical shifts are expressed in ppm. All of these spectroscopic data were recorded in VNUHCM-University of Science. Column chromatography was performed on silica gel (Kieselgel 60, 40-63 μm, Merck)

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and Lichroprep RP₁₈ (40-63 µm, Merck). Analytical and preparative TLC (Thin-Layer Chromatography) were performed on precoated Kieselgel 60F₂₅₄ or RP₁₈ plates (0.25 mm, Merck).

Extraction and isolation of compounds

The entire plants of *S. procumbens* were dried and cut into small pieces (4.0 kg) and then were Soxhlet extracted respectively with *n*-hexane, ethyl acetate, and methanol to yield the corresponding extracts. The methanol-soluble extract (89.0 g) was acidified by acid HCl 0.5M (pH 2-3). Subsequently, the water was added, and the liquid was then partitioned with CHCl₃. The CHCl₃-soluble extract was evaporated under reduced pressure to afford CHCl₃ fraction (7.8 g). This fraction was subjected to a normal phase silica gel column chromatography eluted with *n*-hexane/EtOAc (0-100% EtOAc) and EtOAc/MeOH (0-30% MeOH) mixtures to afford nine fractions (C1-C9). Fraction C2 (378 mg) was repeatedly chromatographed over a silica gel column and eluted with *n*-hexane/EtOAc/Et₃N (80:10:10) mixtures to produce five fractions (C2.1-C2.5). Fraction C2.4 was purified by preparative TLC with *n*-hexane/EtOAc (80:20) as an eluent, to yield compounds **1** (7.0 mg) and **2** (3.5 mg). Fraction C3 (675 mg) was chromatographed over a silica gel column eluted with petroleum ether/EtOAc (0-50% EtOAc) mixtures to obtain compound **3** (12.0 mg). Fraction C4 (798 mg) was separated by column chromatography eluted with CHCl₃/MeOH (95:5) mixtures to create compound **4** (8.6 mg) (Fig. 1).

Ergosterol peroxide (**1**): ¹H NMR (acetone-*d*₆, 500 MHz): δ_H (ppm) 6.49 (1H, *d*, *J* = 8.5 Hz, H-7), 6.23 (1H, *d*, *J* = 8.5 Hz, H-6), 5.28 (1H, *dd*, *J* = 15.3, 7.5 Hz, H-23), 5.21 (1H, *dd*, *J* = 15.3, 8.2 Hz, H-22), 3.76 (1H, *m*, H-3), 3.70 (1H, *d*, *J* = 5.0 Hz, 3-OH), 1.97 (1H, *dd*, *J* = 9.1, 2.9 Hz, H-16a), 1.93 (2H, *m*, H-4a, -11a), 1.90 (1H, *m*, H-4b), 1.87 (1H, *m*, H-24), 1.74 (1H, *m*, H-12a), 1.67 (2H, *dt*, *J* = 13.2, 3.4 Hz, H-1), 1.54 (2H, *m*, H-15), 1.50 (2H, *m*, H-12b, -14), 1.48 (1H, *m*, H-25), 1.41 (1H, *m*, H-9), 1.27 (1H, *m*, H-17), 1.25 (2H, *m*, H-11b, -16b), 1.03 (3H, *d*, *J* = 6.4 Hz, H-21), 0.93 (3H, *d*, *J* = 6.8 Hz, H-28), 0.89 (3H, *s*, H-19), 0.86 (3H, *s*, H-18), 0.85 (3H, *d*, *J* = 6.7 Hz, H-27), 0.84 (3H, *d*, *J* = 6.7 Hz, H-26). ¹³C NMR (acetone-*d*₆, 125 MHz): δ_C (ppm) 136.6 (C-6), 136.5 (C-22), 133.0 (C-23), 131.3 (C-7), 82.5 (C-5), 79.5 (C-8), 66.5 (C-3), 57.1 (C-17), 52.8 (C-14), 52.6 (C-9), 45.3 (C-13), 43.8 (C-24), 40.6 (C-20), 40.4 (C-12), 38.1 (C-4), 37.9 (C-10), 35.7 (C-1), 33.9 (C-25), 31.1 (C-2), 29.1 (C-16), 24.1 (C-15), 21.4 (C-11, -21), 20.3 (C-26), 20.0 (C-27), 18.5 (C-19), 18.1 (C-28), 13.3 (C-18). HRESIMS *m/z* 451.3183 [M+Na]⁺ (calcd for C₂₈H₄₄O₃Na, 451.3188).

9,11-Dehydroergosterol peroxide (**2**): ¹H NMR (acetone-*d*₆, 500 MHz): δ_H (ppm) 6.62 (1H, *d*, *J* = 8.5 Hz, H-7), 6.29 (1H, *d*, *J* = 8.5 Hz, H-6), 5.46 (1H, *dd*, *J* = 6.0, 2.0 Hz, H-11), 5.29 (1H, *dd*, *J* = 15.3, 7.4 Hz, H-22), 5.22 (1H, *dd*, *J* = 15.3, 8.2 Hz, H-23), 3.81 (1H, *m*, H-3), 1.09 (3H, *s*, H-19), 1.03 (3H, *d*, *J* = 6.6 Hz, H-21), 0.94 (3H, *d*, *J* = 6.8 Hz, H-28), 0.86 (3H, *d*, *J* = 6.6 Hz, H-27), 0.84 (3H, *d*, *J* = 6.6 Hz, H-26), 0.78 (3H, *s*, H-18). ¹³C NMR (acetone-*d*₆, 125 MHz): δ_C (ppm) 136.8 (C-7), 136.4 (C-22), 133.0 (C-23), 131.3 (C-6), 119.5 (C-11), 116.0 (C-9), 83.1 (C-8), 78.5 (C-5), 66.1 (C-3), 56.7 (C-17), 49.3 (C-14), 44.3 (C-13), 43.7 (C-24), 42.0 (C-12), 40.8 (C-20), 38.1 (C-10), 37.2 (C-4), 33.9 (C-25), 33.5 (C-1), 31.7 (C-2), 29.5 (C-16), 25.9 (C-19), 21.5 (C-15), 21.1 (C-21), 20.3 (C-26), 19.9 (C-27), 18.0 (C-28), 13.3 (C-18). HRESIMS *m/z* 449.3027 [M+Na]⁺ (calcd for C₂₈H₄₂O₃Na, 449.3032).

N-(*N*-Benzoyl-L-phenylalanyl)-L-phenylalanol (**3**): ¹H NMR (chloroform-*d*, 500 MHz): δ_H (ppm) 7.71 (2H, *d*, *J* = 7.2 Hz, H-3''), 7.52 (1H, *t*, *J* = 7.4 Hz, H-5''), 7.43 (2H, *t*, *J* = 7.6 Hz, H-4'', -6''), 7.34-7.08 (10H, *m*, H-5,5' → 9,9'), 5.97 (1H, *d*, *J* = 8.0 Hz, 1-NHCO), 6.80 (1H, *d*, *J* = 7.6 Hz, 1''-NHCO), 4.78 (1H, *ddd*, *J* = 8.7, 7.6, 5.9 Hz, H-2), 4.09 (1H, *qt*, *J* = 7.7, 4.1 Hz, H-2'), 3.44 (2H, *m*, H-1'), 3.26 (1H, *dd*, *J* = 13.5, 5.9 Hz, H-3a), 3.05 (1H, *dd*, *J* = 13.5, 8.7 Hz, H-3b), 2.78 (1H, *dd*, *J* = 13.7, 7.5 Hz, H-3'a), 2.69 (1H, *dd*, *J* = 13.7, 7.3 Hz, H-3'b), 1.98 (1H, *t*, *J* = 5.9 Hz, 1'-OH). ¹³C NMR (chloroform-*d*, 125 MHz): δ_C (ppm) 170.9 (C-1), 167.3 (C-1''), 137.4 (C-4), 136.9 (C-4'), 133.8 (C-2''), 132.0 (C-5''), 129.5 (C-6', -8'), 129.3 (C-6, -8), 129.0 (C-4'', -6''), 128.8 (C-5, -9), 128.7 (C-5', -9'), 127.4 (C-7'), 127.2 (C-3'', -7''), 126.8 (C-7), 63.7 (C-1'), 55.4 (C-2), 53.0 (C-2'), 38.8 (C-3), 37.0 (C-3'). HRESIMS *m/z* 425.1836 [M+Na]⁺ (calcd for C₂₅H₂₆N₂O₃Na, 425.1841).

Moupinamide (**4**): ¹H NMR (acetone-*d*₆, 500 MHz): δ_H (ppm) 8.17 (1H, *s*, 7-OH), 7.97 (1H, *s*, 6'-OH), 7.44 (1H, *d*, *J* = 15.6 Hz, H-3), 7.16 (1H, *d*, *J* = 1.9 Hz, H-5), 7.06 (2H, *d*, *J* = 8.5 Hz, H-5', -7'), 7.04 (1H, *dd*, *J* = 8.2, 1.9 Hz, H-9), 6.83 (1H, *d*, *J* = 8.1 Hz, H-8), 6.76 (2H, *d*, *J* = 8.5 Hz, H-4', -8'), 6.49 (1H, *d*, *J* = 15.6 Hz, H-2), 3.88 (3H, *s*, -OCH₃), 3.49 (2H, *m*, H-1'), 2.75 (2H, *t*, *J* = 7.4 Hz, H-2'). ¹³C NMR (acetone-*d*₆, 125 MHz): δ_C (ppm) 166.4 (C-1), 156.7 (C-6'), 149.1 (C-6), 148.6 (C-7), 140.3 (C-3), 131.2 (C-3'), 130.5 (C-5', -7'), 128.4 (C-4), 122.6 (C-9), 120.1 (C-2), 116.1 (C-8, -4', -8'), 111.3 (C-5), 56.3 (-OCH₃), 41.9 (C-1'), 35.8 (C-2'). HRESIMS *m/z* 336.1207 [M+Na]⁺ (calcd for C₁₈H₁₉NO₄Na, 336.1212).

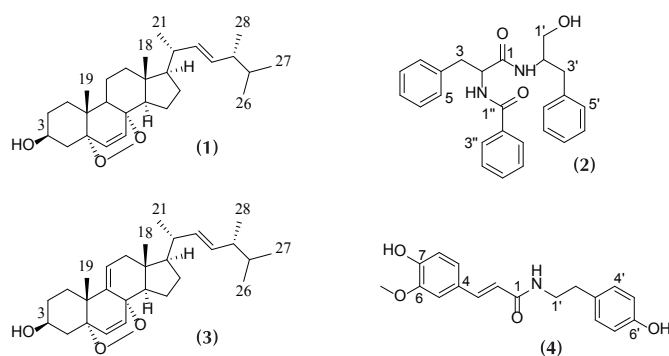


Fig. 1. The chemical structures of compounds 1-4.

Results and discussion

Compound **1** was isolated as a white solid. The ^1H NMR spectrum exhibited the signals for two *cis*-olefinic protons [δ_{H} 6.49 (1H, *d*, $J = 8.5$ Hz, H-6) and 6.23 (1H, *d*, $J = 8.5$ Hz, H-7)], two *trans*-olefinic protons [δ_{H} 5.28 (1H, *dd*, $J = 7.5, 15.3$ Hz, H-23)] and 5.21 (1H, *dd*, $J = 8.2, 15.3$ Hz, H-24)], one oxymethine proton (δ_{H} 3.76, H-3), and six methyl groups (δ_{H} 0.84-1.03). The ^{13}C NMR spectrum illustrated the presence of 28 carbons, including the signals for four olefinic carbons (δ_{C} 130.0-137.0), three oxygenated carbons (δ_{C} 66.5-82.5), six methyl carbons, and other aliphatic carbons. All above NMR data suggested that the structure of **1** was the ergostan-type steroid. These data closely resembled those of ergosterol peroxide [2]. Therefore, the structure of **1** was established as indicated.

Compound **2** was isolated as a white solid. The ^1H NMR spectrum displayed the closely similar signals relative to that of compound **1**, aside from the presence of an additional olefinic signal at δ_{H} 5.46 (1H, *dd*, $J = 6.0, 2.0$ Hz, H-11). The ^{13}C NMR spectrum of **2** exhibited 28 resonance signals, including the presence of two olefinic carbons [δ_{C} 116.0 (C-9), 119.5 (C-11)] instead of the C-9 methine and C-11 methylene signals in **1**. These data were also characteristic of the ergostan-type steroid and closely resembled those of 9,11-dehydroergosterol peroxide [4]. Therefore, the structure of **2** was concluded.

Compound **3** was isolated as a white solid. The ^1H NMR spectrum of **3** exhibited the signals of 15 aromatic protons (δ_{H} 7.00-7.80 ppm), one methine proton [δ_{H} 4.78 (1H, *ddd*, $J = 8.7, 7.6, 5.9$ Hz, H-2)] coupled with one methylene group [δ_{H} 3.26 (1H, *dd*, $J = 13.5, 5.9$ Hz, H-3a), 3.05 (1H, *dd*, $J = 13.5, 8.7$ Hz, H-3b)], one nitrogenated methine [δ_{H} 4.09 (1H, *qt*, $J = 7.7, 4.1$ Hz, H-2')], one methylene [δ_{H} 2.78 (1H, *dd*, $J = 13.7, 7.5$ Hz, H-3'a) and 2.69 (1H, *dd*, $J = 13.7, 7.3$ Hz, H-3'b)], and one hydroxymethyl [δ_{H} 3.44 (2H, *m*, H-1')]. Its ^{13}C NMR spectrum indicated the occurrence of 18 aromatic carbons (δ_{C} 125.0-136.9), two carbonyl carbons (δ_{C} 170.9 and 167.3), one hydroxymethyl (δ_{C} 63.7), two methylene (δ_{C} 38.8 and 37.0), and two nitrogenated carbons (δ_{C} 55.4 and 53.0). By comparing these data with those reported for *N*-(*N*-benzoyl-

L-phenylalanyl)-*L*-phenylalaninol [5], the structure of **3** was concluded as illustrated.

Compound **4** was isolated as a white solid. The ^1H NMR spectrum of **4** exhibited the signals for two *trans*-olefinic protons [δ_{H} 7.44 (1H, *d*, $J = 15.6$ Hz, H-3) and 6.49 (1H, *d*, $J = 15.6$ Hz, H-2)], one methoxy group [δ_{H} 3.88 (3H, *s*)], and two methylene groups [δ_{H} 3.49 (2H, *m*, H-1') and 2.75 (2H, *t*, $J = 7.4$ Hz, H-2')]. The signals of one pair of *ortho*-coupled protons [δ_{H} 7.06 (2H, *d*, $J = 8.5$ Hz, H-5', -7') and 6.76 (2H, *d*, $J = 8.5$ Hz, H-4', -8')] indicated the presence of 1,4-disubstituted benzene ring. Moreover, three aromatic protons of the 1,3,4-trisubstituted benzene ring was indicated [δ_{H} 6.83 (1H, *d*, $J = 8.1$ Hz, H-8), 7.04 (1H, *dd*, $J = 8.2, 1.9$ Hz, H-9) and 7.16 (1H, *d*, $J = 1.9$ Hz, H-5)]. The ^{13}C NMR spectrum of **4** displayed resonances for one carbonyl (δ_{C} 166.4), 12 aromatic carbons (δ_{C} 95.4-167.2), two *trans*-olefinic carbons (δ_{C} 120.1, 140.3), one methoxy carbon (δ_{C} 56.3), and two methylene carbons (δ_{C} 35.8, 41.9). Therefore, the structure of **4** was determined as moupinamide, based on a comparison between these NMR data and the literature [6].

Conclusions

From the methanolic extract of *Solanum procumbens* (Solanaceae), four compounds were isolated, including ergosterol peroxide (**1**), 9,11-dehydroergosterol peroxide (**2**), *N*-(*N*-benzoyl-*L*-phenylalanyl)-*L*-phenylalaninol (**3**), and moupinamide (**4**). The structures of isolated compounds were established based on NMR, MS data, and comparison with those in the literature. Moreover, all isolated compounds were first reported from *S. procumbens*.

The authors declare that there is no conflict of interest regarding the publication of this article.

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Phenolic compounds from *Usnea baileyi* (Stirt.) Zahlbr growing in Lam Dong province

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Abstract:

This study entails a continuation of the phytochemical study regarding the lichen *Usnea baileyi* collected in Lam Dong province. Eight compounds, 8'-O-methylprotocetraric acid (1), protocetraric acid (2), virensic acid (3), subvirensic acid (4), barbatic acid (5), diffractaic acid (6), 4-O-demethylbabartiac acid (7), and atranorin (8), were isolated using various chromatographic methods. Their chemical structures were elucidated through spectroscopic analysis as well as through a comparison of their data with that in the literature.

Keywords: depside, depsidone, lichen, phenolic compound, *Usnea baileyi*.

Classification number: 2.2

Introduction

The genus *Usnea* encompasses over 350 species across the world [1]. They produce diverse lichen metabolites which are endowed with various bioactivities. The fruticose lichen *Usnea baileyi* has proliferated in Lam Dong province, Vietnam. Our previous study concerning this lichen precipitated the isolation of several depsidones from the ethyl acetate [2]. The present research reports the isolation and structure elucidation of eight phenolic compounds (1-8) from the remaining fractions of the ethyl acetate and dichloromethane extracts (Fig. 1).

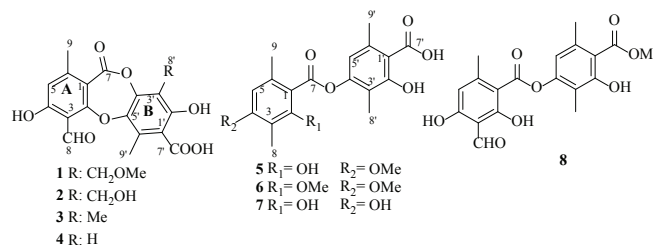


Fig. 1. Chemical structures of 8'-O-methylprotocetraric acid (1), protocetraric acid (2), virensic acid (3), subvirensic acid (4), babartiac acid (5), diffractaic acid (6), 4-O-demethylbabartiac acid (7), and atranorin (8).

Materials and methods

General experimental procedures

The NMR spectra were measured on Bruker Advance (400 MHz for ¹H NMR and 100 MHz for ¹³C NMR) spectrometers. Proton chemical shifts were referenced to the solvent residual signal of CD₃SOCD₃ at δ_H 2.50 and of CDCl₃ at δ_H 7.26. The ¹³C NMR spectra were referenced to the central peak of CD₃SOCD₃ at δ_C 39.52 and of CDCl₃ at δ_C 77.16. The HR-ESI-MS were recorded on a HR-ESI-MS Bruker microTOF Q-II. All NMR and HR-ESI-MS spectra were recorded in the Chemistry Department, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Thin layer chromatography (TLC) was conducted

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on precoated silica gel 60 F₂₅₄ or silica gel 60 RP-18 F₂₅₄S (Merck Millipore, Billerica, Massachusetts, USA), and spots were visualised as a result of spraying with 10% H₂SO₄ solution followed by heating.

Plant material

Thalli of lichen *U. baileyi* were collected from the bark of trees at Tam Bo mountain, Di Linh district, Lam Dong province, Vietnam in May 2015. The scientific name of this lichen was authenticated by Ms. Natwida Dangphui and Assistant Professor Dr. Ek Sangvichien of Lichen Research Unit, Department of Biology, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand.

Extraction and isolation

The air-dried lichen powder (800.0 g) was macerated with acetone (3x10 l) at room temperature. The filtered solution was then evaporated to dryness to yield 80.0 g of crude acetone extract. This extract was washed three times by acetone to obtain a precipitate P (23.8 g). The remainder of the solution was further concentrated to afford the crude acetone extract (56.2 g).

The precipitate P (23.8 g) was subjected to silica gel CC and eluted with a solvent system of CH₂Cl₂: MeOH: AcOH (9.0: 0.2: 0.06) to afford three fractions, P1 (10.7 g), P2 (7.2 g), and P3 (5.8 g). Fraction P3 (5.8 g) was fractionated by CC and eluted with CH₂Cl₂: MeOH: AcOH (9.5: 0.5: 0.07) to afford P3.1 (1.8 g) and P3.2 (3.9 g). Purification of P3.1 (1.8 g) by CC led to the isolation of compounds **1** (4.6 mg), **2** (8.0 mg), and **3** (6.5 mg).

The crude acetone extract (56.2 g) was applied to silica gel quick column and eluted with CH₂Cl₂, EtOAc, acetone and MeOH to obtain four extracts, DC (31.2 g), EA (9.6 g), Ac (6.5 g), and Me (4.6 g), respectively. The EA extract was washed by acetone (3x100 ml) to obtain the precipitate EA-P (1.0 g) and a filtrated solution. The solution was then evaporated to dryness to induce fraction EA-L (7.8 g). The solvent system of CH₂Cl₂: MeOH: AcOH (9.0:0.2:0.06) was then applied for the entire purification process of fraction EA-L. Three fractions EA-L1-3 were obtained by subjecting fraction EA-L to column chromatography. Purifying the fraction EA-L2 (1.2 g) by CC resulted in two compounds, namely **5** (14.1 mg) and **6** (18.4 mg). The extract DC was fractionated by CC and eluted with a gradient of *n*-hexane: EtOAc (8:2-0:10) to obtain four fractions DC1-4, respectively. Applying CC on fraction DC1 (7.8 g) with the mobile phase of *n*-hexane: EtOAc: AcOH (9.0:1.0:0.1) produced five fractions, DC1.1-5. Compound **8** (6.2 mg) and **4** (15.3 mg) were isolated from the purification of DC1.2 (0.6 g), while **7** (5.2 mg) was obtained from the purification of DC1.4.2 using silica gel

column chromatography with the same solvent system of *n*-hexane: EtOAc: AcOH (7.5:2.5:0.06).

- 8'-*O*-methylprotocetraric acid (**1**). White amorphous powder; the ¹H and ¹³C NMR (DMSO-*d*₆) spectroscopic data, see Table 1;

- Protocetraric acid (**2**). White amorphous powder; the ¹H and ¹³C NMR (DMSO-*d*₆) spectroscopic data, see Table 1;

- Virensic acid (**3**). White amorphous powder; the ¹H and ¹³C NMR (DMSO-*d*₆) spectroscopic data, see Table 1;

- Subvirensic acid (**4**). White amorphous powder; the ¹H and ¹³C NMR (DMSO-*d*₆) spectroscopic data, see Table 1;

- Babartic acid (**5**). White colorless needle; the ¹H and ¹³C NMR (DMSO-*d*₆) spectroscopic data, see Table 2;

- DiffRACTAIC acid (**6**). White colorless needle; the ¹H and ¹³C NMR (DMSO-*d*₆) spectroscopic data, see Table 2;

- 4-*O*-demethylbabartic acid (**7**). White colorless needle; the ¹H and ¹³C NMR (DMSO-*d*₆) spectroscopic data, see Table 2;

- Atranorin (**8**). White colorless needle; the ¹H and ¹³C NMR (CDCl₃) spectroscopic data, see Table 2.

Results and discussion

Compound **1** was obtained as a white amorphous powder. The ¹H NMR and HSQC spectra of **1** demonstrated the presence of one formyl (δ_H 10.55, 1H, s), one aromatic proton (δ_H 6.78, 1H, s), one oxymethylene group (δ_H 4.43, 2H, s), one methoxy group (δ_H 3.19, 3H, s), and two methyl groups (δ_H 2.45, 3H, s and 2.34, 3H, s). The ¹³C NMR spectrum in accordance with HSQC spectrum confirmed the presence of 19 carbons comprising one aldehyde carbon (δ_C 191.8), two carboxyl carbons (δ_C 170.4 and 161.3), 12 aromatic carbons (δ_C 164.4, 163.8, 158.2, 151.8, 145.1, 141.2, 131.2, 116.9, 115.5, 115.1, 112.3, and 111.8), one oxygenated methylene carbon (δ_C 62.4), one methoxy group (δ_C 57.3), and two methyls (δ_C 21.3 and 14.4). HMBC cross peaks of both H-5 (δ_H 6.78) and 3-CHO (δ_H 10.55) to C-3 (δ_C 112.3), H-5 to C-9 (δ_C 21.3) and H₃-9 (δ_H 2.45) to C-1 (δ_C 111.8), C-5 (δ_C 116.9) and C-6 (δ_C 151.8) defined the connectivity through C-3-C-4-C-5-C-6-C-1 in the A-ring (see Fig. 2). In addition, the cross peaks of H₃-9' (δ_H 2.34) to C-1' (δ_C 115.5), C-5' (δ_C 141.2), and C-6' (δ_C 131.2) confirmed its position in the B-ring. The ¹H NMR chemical shift of H₂-8' along with the HMBC cross peaks of H₂-8' to C-2' (δ_C 158.2), C-3' (δ_C 115.1), and C-4' (δ_C 145.1) determined the linkage of this group at C-3. The comparison of NMR data of **1** and those of 8'-*O*-methylprotocetraric acid [3] indicated that they were identical; therefore, **1** was elucidated as 8'-*O*-methylprotocetraric acid.

Table 1. ^1H and ^{13}C NMR of **1-4**^a.

Position	1		2		3		4	
	δ_{H} J(Hz)	δ_{C}	δ_{H} J(Hz)	δ_{C}	δ_{H} J(Hz)	δ_{C}	δ_{H} J(Hz)	δ_{C}
1		111.8		111.9		111.9		111.8
2		164.4		164.8		164.0		164.1
3		112.3		112.7		112.3		112.3
4		163.8		163.9		163.8		163.8
5	6.78 s	116.9	6.82 s	115.6	6.83 s	115.1	6.83 s	117.0
6		151.8		152.2		152.1		152.0
7		161.3		161.5		161.3		161.5
8	10.54 s	191.8	10.58 s	191.9	10.59 s	191.7	10.57 s	191.7
9	2.45 s	21.3	2.42 s	21.5	2.43 s	21.4	2.42 s	21.4
1'		111.5		111.9		111.9		111.8
2'		158.2		155.9		155.1		156.2
3'		115.1		117.5		115.7	6.67 s	105.8
4'		145.1		144.5		144.7		144.5
5'		141.2		140.7		141.8		141.0
6'		131.2		127.5		127.6		128.8
7'		170.4		170.3		170.8		168.2
8'	4.43 s	62.4	4.64 s	52.9	2.14 s	9.3		
9'	2.34 s	14.4	2.41 s	14.4	2.41 s	14.3	2.27 s	14.0
OCH ₃	3.19	57.3						

^a: these were recorded in DMSO-*d*₆.

Compound **2** was obtained as a white amorphous powder. Both its ^1H and ^{13}C NMR spectroscopic data were similar to those of **1**; the only difference was the absence of the methoxy moiety (δ_{H} 3.19 and δ_{C} 57.3, 8'-OMe in **1**), which demonstrated the replacement of 8'-OH for 8'-OMe in the B-ring of **2**. The comparison of NMR data of **2** with those of protocetraric acid [3] illustrated that they were identical; therefore, **2** was elucidated as protocetraric acid.

Compound **3** was isolated as a white amorphous powder. Examination of the ^1H NMR and ^{13}C NMR spectra of **3** revealed signal patterns resembling those of **2**, with the exception of the replacement of the methyl group (δ_{H} 2.14 and δ_{C} 9.3, 8'-Me) rather than the oxygenated methylene moiety (δ_{H} 4.64 and δ_{C} 52.9, 8'-CH₂OH) in the B-ring. The comparison of NMR data of **3** with those of virensic acid [3] demonstrated that they were identical; accordingly, **3** was elucidated as virensic acid.

Compound **4** was yielded as a white amorphous powder. The 1D NMR data of **4** were reminiscent of that of **3** (Tables 1 and 2); the primary difference was the presence of H-3 (δ_{H} 6.83, 1H, s) in lieu of the methyl 8'-Me (δ_{H} 2.05, 3H, s and

δ_{C} 9.3, 8'-Me). The NMR data of **4** were identical to that of subvirensic acid [4]. Combined, the chemical structure of **4** was elucidated as subvirensic acid.

Compound **5** was isolated as a white amorphous powder. The ^1H NMR and HSQC spectra of **5** demonstrated the presence of one hydroxy proton (δ_{H} 10.74, 1H, s), two aromatic protons (δ_{H} 6.68, 1H, s and 6.60, 1H, s), one methoxy group (δ_{H} 3.86, 3H, s), and four methyl groups (δ_{H} 2.57, 2.48, 2.00, 1.99, 3H for each, s). The ^{13}C NMR spectrum combined with HSQC spectrum revealed the presence of 19 carbons comprising two carbonxyl carbons (δ_{C} 173.1 and 168.6), 12 aromatic carbons (δ_{C} 161.3, 161.1, 159.5, 151.8, 139.0, 139.0, 115.9, 115.7, 111.4, 110.0, 107.0, and 106.3.8), one methoxy group (δ_{C} 55.7), and four methyls (δ_{C} 23.0, 22.7, 9.04, and 7.99). HMBC cross peaks of both H-5 (δ_{H} 6.60) and H₃-OMe (δ_{H} 3.86) to C-4 (δ_{C} 161.3) and both H-5 and H₃-9 (δ_{H} 2.57) to C-1 (δ_{C} 107.0), C-5 (δ_{C} 106.3), and C-6 (δ_{C} 139.0) defined the positions of these groups (Fig. 2). Moreover, the HMBC cross peaks of 2-OH (δ_{H} 10.74) to C-1, both 2-OH and H₃-8 (δ_{H} 2.00) to C-2 (δ_{C} 159.5) and C-3 (δ_{C} 110.0) totally defined the connectivity through C-1–C-2–C-3–C-4–C-5–C-6 in the A-ring. Furthermore, the HMBC correlations of both H-5' (δ_{H} 6.68) and H₃-9' (δ_{H} 2.48) to C-1' (δ_{C} 111.4), C-5' (δ_{C} 115.9), and C-6' (δ_{C} 139.0) and both H-5' and H₃-8' (δ_{H} 1.99) to C-3' (δ_{C} 115.7) and C-4' (δ_{C} 151.8) defined the system through C-3'–C-4'–C-5'–C-6'–C-1' in the B-ring. The ^{13}C NMR chemical shift of C-7 (δ_{C} 168.6) and C-4' characterised for the ester linkage between C-7 and C-4' of a depside scaffold. The comparison of NMR data of **5** with those of barbatic acid [5] indicated that they were identical; **5** was elucidated as barbatic acid.

Compound **6** was obtained as colorless needle. The ^1H and ^{13}C NMR spectrum of **6** were highly similar to those of **5**, with the exception of the absence of 2-OH (δ_{H} 10.74, 1H, s), replaced by the methoxy group (δ_{H} 3.68 and δ_{C} 61.8) in **6**. The NMR data of **6** resembled that of diffractaic acid [6]. Therefore, **6** was elucidated as diffractaic acid.

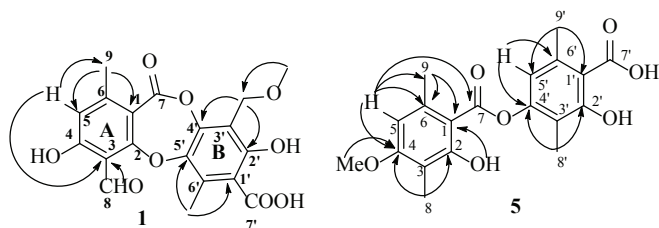
Compound **7** was obtained as colorless needle. The ^1H and ^{13}C NMR spectrum of **7** were identical to that of **5**; the sole difference was the absence of the 4-OMe group (δ_{H} 3.86, 3H, s) rather than one hydroxy group at (δ_{H} 11.13, 1H, s) in **7**. The NMR data of **7** closely resembled that of 4-*O*-demethylbabartic acid [7]. Consequently, **7** was elucidated as 4-*O*-demethylbabartic acid.

Table 2. ^1H and ^{13}C NMR of 5-8.

Position	5 ^a		6 ^a		7 ^a		8 ^b	
	δ_{H} J(Hz)	δ_{C}	δ_{H} J(Hz)	δ_{C}	δ_{H} J(Hz)	δ_{C}	δ_{H} J(Hz)	δ_{C}
1		107.0		119.3		108.6		108.7
2		159.5		156.4		160.7		169.2
3		110.0		116.4		110.9		110.4
4		161.3		161.3		161.9		167.7
5	6.68 s	106.3	6.45 s	108.5	6.63, s	110.9	6.51, s	116.2
6		139.0		134.8		139.0		152.2
7		168.6		165.5		169.2		169.8
8	2.00 s	8.0	1.90 s	8.7	1.94, s	8.0	10.36, s	194.0
9	2.57 s	22.7	2.23 s	19.5	2.44, s	22.7	2.69, s	25.7
2-OMe			3.68 s	61.8				
4-OMe	3.86 s	55.7	3.60 s	55.8				
2-OH	10.74 s				11.13, s		12.54, s	
4-OH							12.50, s	
1'		111.4		111.5		111.6		103.0
2'		161.1		159.5		161.3		163.0
3'		115.7		116.0		115.7		116.9
4'		151.8		152.2		151.7		152.6
5'	6.60 s	115.9	6.62 s	115.7	6.36, s	115.9	6.40, s	113.0
6'		139.0		139.0		139.0		140.0
7'		173.1		173.1		173.1		172.3
8'	1.99 s	9.0	1.98 s	8.9	1.94 s	9.1	2.10 s	9.5
9'	2.48 s	23.0	2.34 s	22.8	2.44 s	23.5	2.54 s	24.1
2'-OH					10.33 s		11.94 s	
COOMe							3.97 s	52.3

^a: these were recorded in DMSO- d_6 .^b: these were recorded in CDCl_3 .

Compound **8** was obtained as colorless needle. The ^1H and ^{13}C NMR spectra of **8** closely resembled those of **7**, with two differences. Firstly, the 3-Me group (δ_{H} 1.94, 3H, s, H_3 -8) in **7** was replaced by the formyl proton (δ_{H} 10.36, 1H, s). Secondly, the presence of one additional methoxy group at δ_{H} 3.98 and δ_{C} 52.4 suggested the methyl ester at C-7'. The NMR data of **7** closely resembled that of atranorin [8]. Therefore, **8** was confirmed as atranorin.

Fig. 2. Key HMBC correlations of **1** and **5**.

8'-O-methylprotocetraric acid (**1**), virensic acid (**3**), barbatic acid (**5**), diffractaic acid (**6**), and 4-O-demethylbarbatic acid (**7**) were discovered for the first time from *Usnea baileyi*. It should be noted that this is the first time that subvirensic acid (**4**) was isolated from the genus *Usnea* [9].

Conclusions

From *Usnea baileyi* collected in Lam Dong province, eight phenolic compounds were isolated and elucidated, including 8'-O-methylprotocetraric acid (**1**), protocetraric acid (**2**), virensic acid (**3**), subvirensic acid (**4**), barbatic acid (**5**), diffractaic acid (**6**), 4-O-demethylbarbatic acid (**7**), and atranorin (**8**).

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Partitioning of fluoxetine into mixed lipid bilayer containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)

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Abstract:

In this study, the partitioning of fluoxetine, an antidepressant of selective serotonin reuptake inhibitor class into a mixture containing anionic and zwitterionic lipid vesicles was evaluated using second derivative spectrophotometry. The partition coefficients (K_p) of fluoxetine into the large unilamellar vesicles (LUVs) composed of zwitterionic 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) containing 0 mol%, 10 mol%, 20 mol%, and 30 mol% of anionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were measured in HEPES buffer at pH 7.4. The result revealed that when more negatively charged lipids incorporated into the LUVs, the condensing effect on the binary phospholipid membrane impeded the partitioning of positively charged fluoxetine, resulting in the decrease in the K_p values. This study adds a deeper understanding of how antidepressant fluoxetine exerts its effect on anionic-containing biological membranes, shedding light onto drug delivery systems in the pharmaceutical field.

Keywords: binary phospholipid membrane, electrostatic interaction, fluoxetine, partition coefficient, second derivative spectrophotometry.

Classification number: 2.2

Introduction

Depression is one of the most widespread mental disorders among humanity and up to 15% of the population might experience a series of symptoms ranging from the persistent state of low mood to suicidal behaviors during their lifetime [1]. The discovery of SSRIs (selective serotonin reuptake inhibitors) in the late 1980s marked a milestone in the therapeutic orientation towards depressive disorder [2]. In recent times, SSRIs have emerged as the most prescribed antidepressants [3] since they have better efficacy, tolerability, lower cost and fewer side effects compared to the old generation depression-resistant drugs [4, 5]. Fluoxetine is a well-known antidepressant, which belongs to the SSRI group that serves as a highly active serotonin reuptake blocker in vitro and in vivo by impeding the action of serotonin transporter [2, 6, 7] (Fig. 1). The therapeutic mechanism of fluoxetine is closely associated with TREK-1 ion channel protein, which is highly distributed in the central nervous system and the cell membrane [8-11]. Fluoxetine blocks the activity of the TREK-1 channel by truncating the C-terminal domain, which causes the loss of channel function, resulting in the depression-resistant phenotype [12, 13]. Being a lipophilic compound, fluoxetine must enter the interior of the lipid membrane to perform the inhibition [14]; hence, the study of fluoxetine partitioning into lipid bilayer could provide a better understanding of how such a common antidepressant exerts its therapeutic effect.

Liposomes are artificially prepared vesicles consisting of natural and synthetic phospholipids and are widely used as cell membrane mimicking platforms to study the drug delivery systems [15-17]. Drug partitioning, a powerful indicator to evaluate the physical activity of drugs towards lipid membranes is obtained by liposome/water partition coefficient (K_p) of drugs. In previous studies, the partition

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coefficient of drugs was determined by different methods such as phase separation, hygroscopic desorption and the octanol/water system [18-21]. As the lipid vesicles cause high apparent background signals derived from the light scattering, these techniques aimed to separate drugs and vesicle suspensions into aqueous and lipid phases [22]. However, they were either time-consuming, disturb the equilibrium state of sample solutions, and more importantly were too simplified to study the drug and membrane interactions or might introduce a huge discrepancy between K_p values of different drugs [23, 24]. Later, the second derivative spectrophotometry was employed as a newly developed method to eliminate the background signals from the absorption spectra without the old methods' drawbacks [25-27].

The lipid bilayer, a core component of the cell membrane, is made of two layers of lipid molecules and each molecule has a hydrophilic headgroup and two hydrophobic tails. The properties of highly dominant lipids in the cell membrane have been in the spotlight for a certain time. Despite the fact that charged lipids are seemingly minor but incident to many crucial biologically relevant processes, the understanding of how they function solitarily and collectively with other cell components is still at the tip of the iceberg [28]. Consequently, examining the role of charged lipids especially the negative ones in form of liposomes mimicking the cell membranes has risen as a great biological interest in recent times [28]. Heterogeneities in lipid membranes comprising of negatively charged lipids have recently attracted considerable attention [29] including lipid-protein interactions e.g. the interplay of peripheral proteins with phosphatidylinositol (PI) [30], the interactions of phosphatidylserine (PS) with the Tim4 protein characterized by all-atom molecular dynamics data combined with interfacial X-ray scattering and membrane binding essays [31], and lipid-cholesterol interactions e.g. the behaviors of cholesterol towards the PC/PS asymmetric model bilayers [32]. However, the interplay between mixed protein-free lipid bilayers comprising of a negatively charged lipid and nanosized molecules, like drugs, are considerably few [28]. For the above reasons, this study aimed to examine the partitioning of fluoxetine, a positively charged drug molecule, into a mixture of anionic-zwitterionic lipid bilayers via derivative spectrophotometry under the viewpoint of electrostatic interactions. By incorporating charged lipids into the membrane components, the lipid-water interface region might unveil some interesting features. The partition coefficients of fluoxetine into LUVs composed of pure zwitterionic 1,2-distearoyl-*sn*-glycero-3-

phosphocholine (DSPC) and DSPC containing 10 mol%, 20 mol%, 30 mol% of anionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) (Fig. 1) were determined using second derivative spectrophotometry. Phosphatidylcholine (PC) is the most abundant constituent of cell membranes which has a zwitterionic headgroup [33, 34]. Though the anionic phosphatidylglycerol (PG) is reported to account for a minority in cells, it is commonly representative of the charged lipids [29]. PG is fairly distributed in the pulmonary surfactant [35] and the thylakoid membrane of the chloroplast [36], it also has a part in ATP production via the cooperative function with the pulmonary surfactant proteins and cardiolipin [37, 38]. This study focused on the partitioning of fluoxetine in the mixtures of DSPC:DPPG bilayers at a molar ratio of 7:3, which is the ideal molar fraction between the zwitterionic and anionic lipid species in the lung surfactant [38]. DSPC and DPPG transition temperatures are 55°C and 41°C, respectively; thus at the experimental temperature of 37°C, they both remain in the solid-gel state.

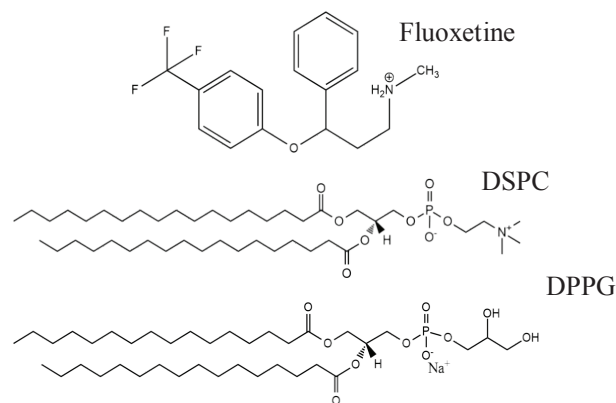


Fig. 1. Molecular structures of fluoxetine, DSPC and DPPG.

With pK_a 10.1, fluoxetine carries a net positive charge at pH 7.4 [27]. Thus, at this physiological pH the presence of the anionic DPPG in the DSPC bilayer would induce the electrostatic interaction between the drugs and the lipid bilayers, affecting the partitioning of fluoxetine into the lipid bilayers. There were few works paid attention to the interaction of fluoxetine with LUVs by differential scanning calorimetry and spin labeling EPR techniques [39-42] but still, the insight of fluoxetine partitioning into LUVs under the electrostatic perspective has not been profusely investigated. Therefore, this study was carried out to add further understanding of how fluoxetine interacts towards heterogeneous anionic membranes.

Experimental

Materials

Fluoxetine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. The buffer was composed of 50 mM NaCl and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES buffer) and adjusted to pH 7.4. 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol C16:0 (DPPG) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine C18:0 (DSPC) of 99% purity were purchased from Avanti Polar-Lipids Inc. (Alabaster, AL, USA) and used without further purification. Stock DSPC (20 mg/ml) was supplied as a 2% (w/v) chloroform solution and stock DPPG (20 mg/ml) was supplied as a 10% (w/v) chloroform solution containing 5% methanol. Both solutions were stored at -20°C before usage. Nanopure water distilled from the Nanopure system with an impedance of 18.2 MΩ (Ultrapure, USA) was used to prepare all solutes.

Methods

Stock solution of drug preparation: a stock solution of the drug was prepared at 1 mg/ml concentration in 50 mM NaCl - 10 mM HEPES buffer.

Liposome preparation: appropriate amounts of DSPC and DPPG stock solutions were mixed and evaporated with a gentle stream of nitrogen. Further removal of the solvent residue was performed by applying a high vacuum at room temperature for more than 4 hours. Thereafter, the unused dried lipid cakes were stored at -20°C for further use.

The resulting dried lipid cake was dispersed with 50 mM NaCl - 10 mM HEPES buffer to produce large homogeneous multilamellar vesicles (LMVs). The suspension was subsequently vortexed. Five consecutive cycles of 5 min freeze at -20°C and 5 min thaw at 60°C were repeated. Suspensions of LMVs were extruded 30 times through 100 nm pore size polycarbonate filters (Avanti Polar-Lipids Inc., AL, USA) at a temperature which is higher than both lipids' transition temperatures to produce LUVs.

Drug-Liposome environment preparation: the extruded suspensions were diluted to different concentrations for further analysis. Sample solutions were prepared by mixing a known volume of the drug solution (67.5 μM) and a suitable aliquot of the vesicle suspensions in HEPES buffer. The correspondent reference solutions were prepared identically but without the drug. Sample solutions and reference solutions were all prepared in 1-ml Eppendorf and

the final volume was 800 μl. All samples were vortexed 5 min and incubated at 37°C for 30 min before being measured to collect the UV-Vis absorption spectra.

UV-Vis absorption spectra collection and second derivative spectrophotometry: each absorption spectrum of the sample solution was measured against the correspondent reference solution by using a microcell cuvette with the chamber volume of 700 μl on the Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA), with a temperature regulated cell holder set at 37°C. The spectral window was from 190 nm to 300 nm. Thereafter, the second derivatives of absorption spectra were obtained from Origin 9.1.0 software (Origin Lab, WA, USA) based on the Savitzky-Golay method [42], in which the second-order polynomial convolution of 20 points was employed. A wavelength interval ($\Delta\lambda$) of 1 nm was incorporated in the calculation.

Partition coefficient determination: the partition coefficient of drugs between lipid bilayer vesicle suspensions and aqueous solutions is defined as [23].

$$K_p = \frac{\text{fraction of drug in lipid} / [\text{lipid}]}{\text{fraction of drug in aqueous phase} / [\text{aqueous phase}]} \quad (1)$$

where:

[lipid]: lipid molar concentration [43]

[aqueous phase]: water molar concentration (55.3 mol/dm³ at 37°C)

The fraction of the bound fluoxetine is defined as $\Delta D / \Delta D_{\max}$, where $\Delta D = D - D_0$ is directly proportional to the fluoxetine concentration in the membrane [26] and ΔD is the second derivative intensity difference between absorption in the presence and absence of liposomes.

From equation (1), K_p value could be presented as

$$K_p = \frac{\left(\frac{\Delta D}{\Delta D_{\max}}\right) [\text{aqueous phase}]}{\left(\frac{1 - \Delta D}{\Delta D_{\max}}\right) [\text{lipid}]} \quad (2)$$

After simple transformations of the equation (2), equation (3) was obtained as follows

$$\Delta D = \frac{K_p \Delta D_{\max} [\text{lipid}]}{[\text{aqueous phase}] + K_p [\text{lipid}]} \quad (3)$$

The value of K_p and $\Delta D_{\max}$ can be calculated from the experimental values of [lipid] and ΔD by employing a non-linear least-squares fitting to equation (3).

The second derivatives of the absorption spectra were obtained from Origin 9.1.0 software (Origin Lab, WA, USA) and the K_p values were calculated by Sigmaplot 12.0 software (Systat Software Inc., CA, USA).

Results

Absorption spectra of fluoxetine in LUVs

Absorption spectra of fluoxetine at a concentration of 67.5 μM recorded in the presence of different liposome concentrations of pure DSPC and mixed DSPC:DPPG = 9:1, 8:2, and 7:3 are depicted in Fig. 2, respectively. It is requisite to point out that the concentration of fluoxetine used in this study conforms to the Beer-Lambert Law for absorbance. The curves (2-8) in Fig. 2 were obtained by subtraction of the absorption spectrum of liposomes without fluoxetine from the absorption spectrum of liposomes with the drug recorded at the same lipid concentration.

In respect to four different compositions i.e. pure DSPC, DSPC:DPPG = 9:1, 8:2, 7:3 with increased lipid concentrations, absorption maxima (λ_{max}) of fluoxetine decreased and shifted to the longer wavelength (bathochromic shift) as compared to the maximum in the buffer solution (spectrum 1). The bathochromic shift was caused by the decrease of polarity in fluoxetine molecules' surrounding, indicating the incorporation of fluoxetine into the hydrophobic cores of the lipid bilayers. This behavior are observed on other drugs namely phenothiazine (Poła et al. 2004), chlorpromazine and methochlorpromazine [43], trifluoperazine [44], and promazine [26] when they partitioned into lipid membranes.

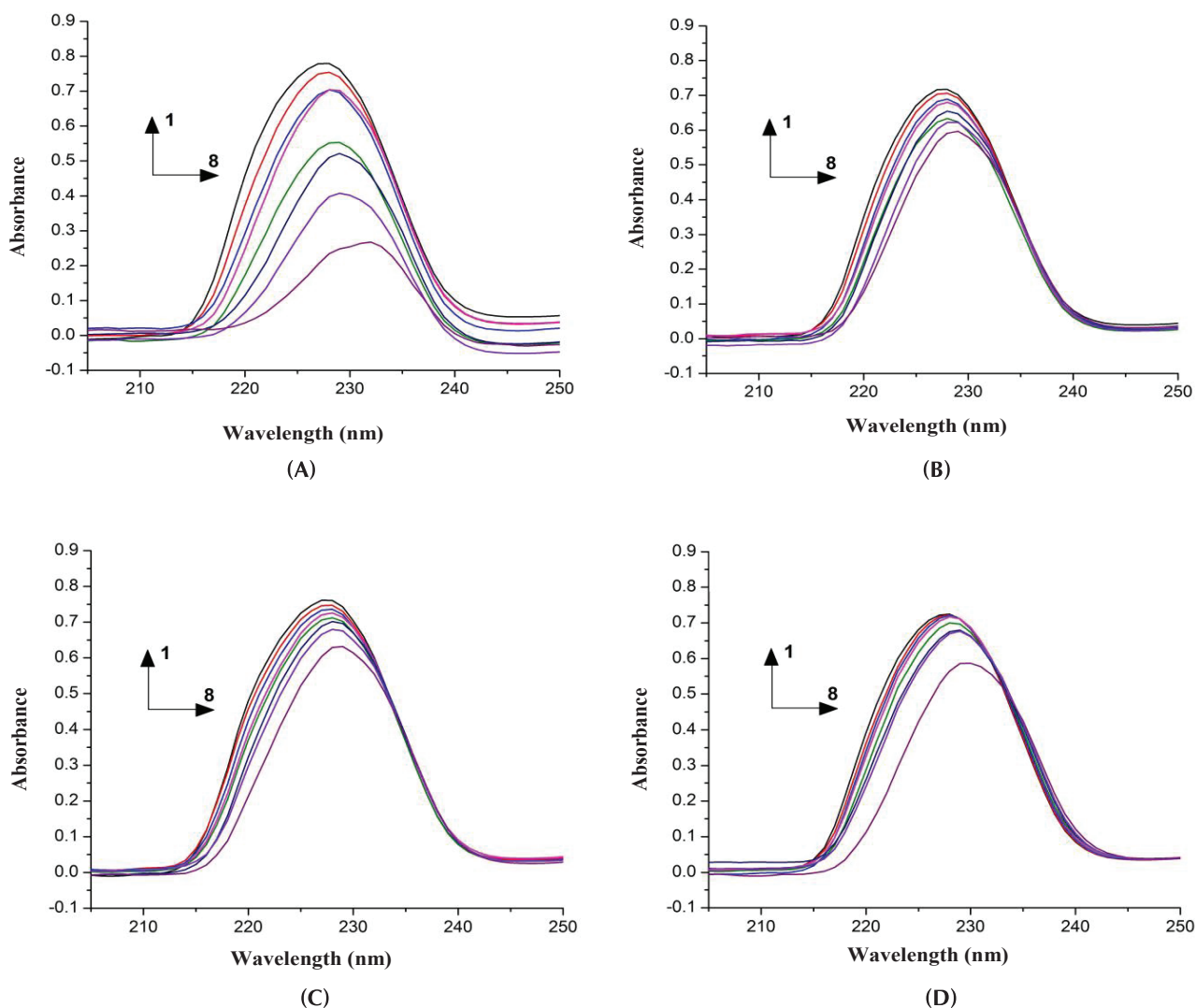


Fig. 2. Absorption spectra of fluoxetine in HEPES buffer (pH 7.4, 37°C) containing various amounts of pure DSPC (A), and mixtures of DSPC: DPPG = 9:1 (B), 8:2 (C), 7:3 (D). LUVs concentrations (mM) (1) 0; (2) 0.025; (3) 0.050; (4) 0.075; (5) 0.10; (6) 0.15; (7) 0.20; (8) 0.30.

Second derivative spectra of absorption

The apparent background signals caused by light scattering in the liposome suspensions could be eliminated by applying the second derivative spectrophotometric method [45, 46]. The second derivatives of the absorption spectra of fluoxetine in the HEPES buffer containing the pure DSPC and the mixtures of DSPC and DPPG LUVs are depicted in Fig. 3.

Despite the fact that the same amount of LUVs was purposely prepared in the sample and reference solutions, no isosbestic points are observed in the absorption spectra figures. It was obvious that strong background signals impeded the complete baseline correction. Therefore, the

second derivative spectrophotometric method was then applied to eliminate those background noises, allowing isosbestic points to be obtained, and enabling the partition coefficients to be determined. In previous studies, the partition coefficient of drugs into the DMPC liposomes [23] and phenothiazine into the phosphatidylcholine bilayer vesicles and water [47] were also determined by using second derivative spectrophotometry. With the increasing of lipid concentrations, the second derivative minima increase in intensity and shift toward the longer wavelength in all four conditions (Fig. 3). Two isosbestic points at 218 nm and 229 nm were obtained, proving that the apparent background signals were entirely eliminated [24-26, 48].

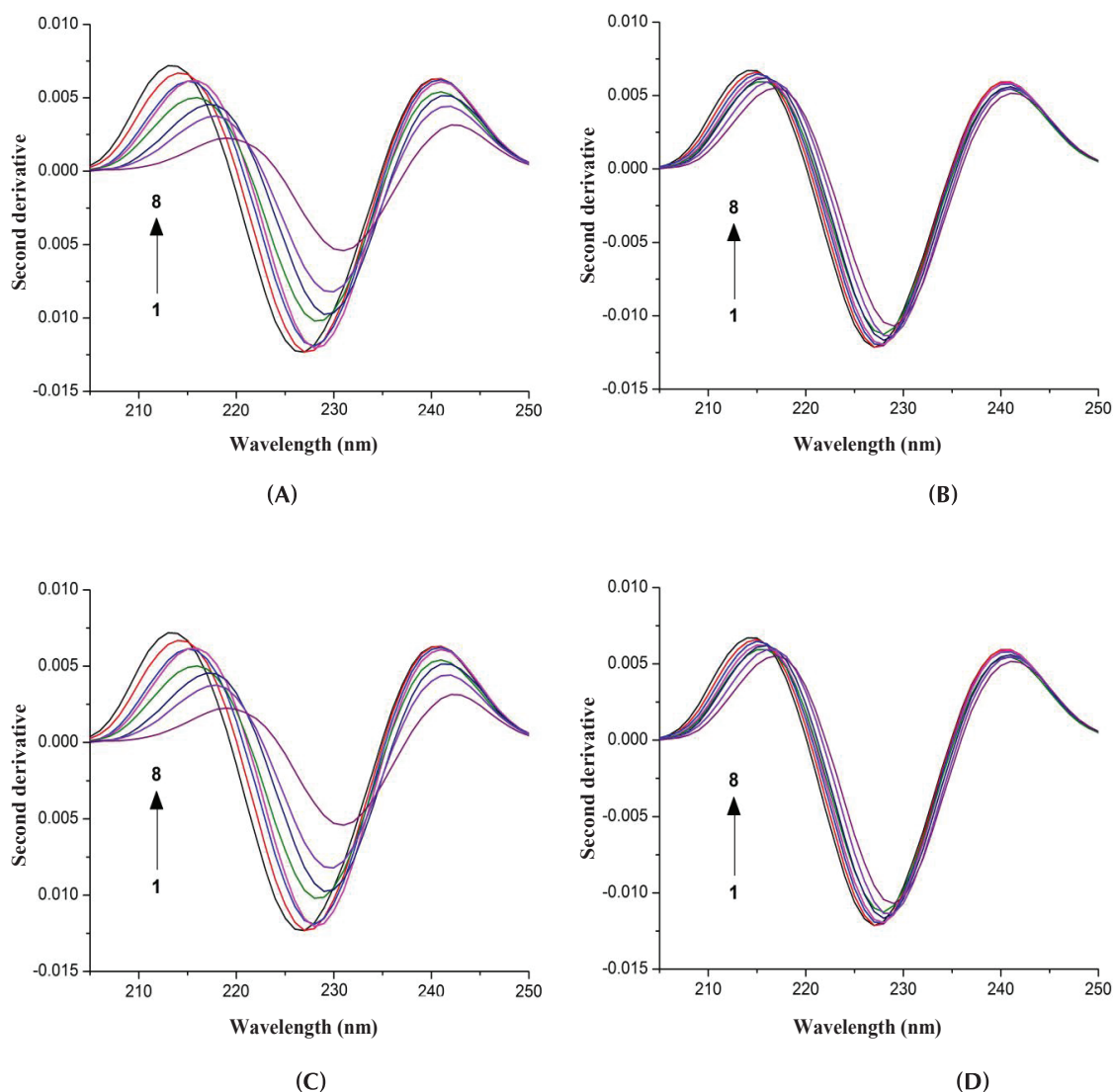


Fig. 3. Second derivative spectra of fluoxetine in HEPES buffer (pH 7.4, 37°C) calculated from the absorption spectra in Fig. 2: pure DSPC (A), and mixtures of DSPC:DPPG = 9:1 (B), 8:2 (C), 7:3 (D).

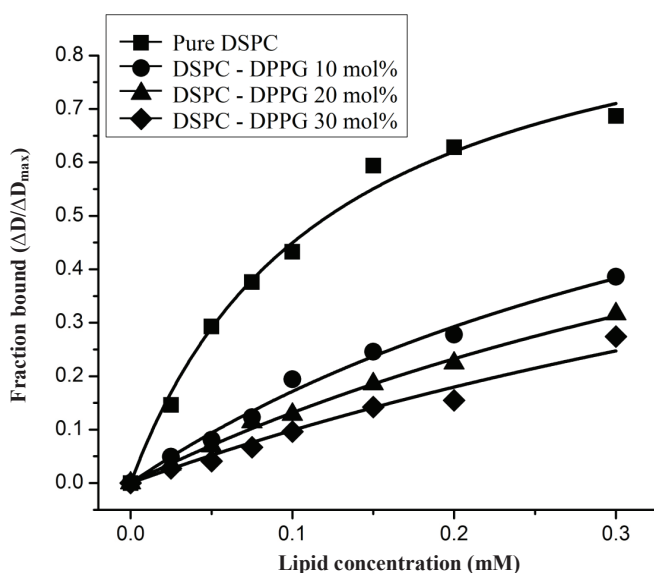


Fig. 4. The fraction bound of fluoxetine in LUVs ($\Delta D/\Delta D_{\max}$) as a function of lipid concentration (mM).

The fraction bound of partitioned fluoxetine in the lipid vesicles ($\Delta D/\Delta D_{\max}$)

The values of $\Delta D/\Delta D_{\max}$, i.e. the fraction of fluoxetine partitioned into the LUVs, were plotted against the concentrations of the LUVs and shown in Fig. 4. The partition coefficients were obtained by non-linear fitting the ΔD values and the LUVs' concentration to equation (3) and listed in Table 1.

Table 1. The partition coefficient of fluoxetine at a concentration of 67.5 μM into mixed lipid vesicles composed of DSPC and DPPG at pH 7.4, 37°C.

Liposome component(s)	Partition coefficient ($K_p \times 10^{-5}$)*
Pure DSPC	2.04 $\pm$ 0.17
DSPC:DPPG = 9:1	1.32 $\pm$ 0.26
DSPC:DPPG = 8:2	0.69 $\pm$ 0.17
DSPC:DPPG = 7:3	0.53 $\pm$ 0.02

*The value shown in the results section was the mean of at least two independent determinations. K_p value was presented as mean $\pm$ s.d.

Discussion

As seen in Table 1, the K_p values tendentially decrease with the increase of the molar fraction of the negatively charged DPPG in the binary membrane of DSPC - DPPG LUVs. This event indicates that fluoxetine had lower affinity to the binary DSPC - DPPG bilayer membrane than

the pure DSPC membrane. The figures of K_p themselves do not provide insights into how each lipid species was arranged on the liposome membrane. For this reason, three potential regions which were believed to have great impact on the final K_p values should be taken into account: DSPC - rich regions, DPPG - rich regions, and DSPC - DPPG rich regions. On each lipid region, different driving forces were responsible for the partitioning of fluoxetine into the lipid hydrophobic core.

In the DSPC - rich regions, the zwitterionic PC headgroup of DSPC lipids composed of a positive choline group and a negative phosphate group is being ionized at physiological pH [33]. The electrolytes were found not to have any interactions with the functional groups of the lipids [38]. The sodium and the chloride ions were shown to remain homogeneously in the buffer and no headgroup modification was recorded in the presence of salt in the binary lipid system [38]. DSPC itself has strong steric headgroup repulsions between the same charges of two adjacent lipids [49]. It also has two attractive intermolecular forces that help to stabilize the membrane. The first intermolecular force is the hydrogen bonds that are formed between the water molecules and two phosphocholine molecules, and the second one is the weak electrostatic interactions between the positive choline and the negative phosphate groups of the neighboring lipids [50]. The electrolytes that are distributed homogeneously in the aqueous media indeed has no influences on physical properties in general and the packing density of DSPC lipids in specific. Besides that, the electrostatic interaction was further demonstrated not to be the driving force for the partitioning of charged molecular particles. Phan, et al. and Pola, et al. reported that the disordering in the lipid acyl chains, in which the hydrophobic part of fluoxetine interacts with the hydrophobic tails of DSPC lipids, gives rise to the partitioning of fluoxetine into the lipid hydrophobic core of DSPC [39, 44]. The DSPC - rich regions were believed to be the main K_p contributor since the deduction of DSPC molar fractions in the binary lipid system led to the significant decrease in the K_p values.

Regarding the DPPG - rich regions, these regions are anionic since DPPG itself possesses a net negative charged at physiological pH [29]. Despite the fact steric repulsions exist between the neighboring lipids that push them apart from each other, there is a source of attraction in the lateral directions which helps to stabilize the membrane [38]. Dicko, et al. proposed that glycerol hydroxyl is hydrogen-bonding to phosphate or carbonyl groups of the phospholipids [51]. Later research confirmed the experimental suggestion and stated that this specific hydroxyl-phosphate molecular interaction accounts for the event [38]. Do, et al. suggested that at 50 mM NaCl, fluoxetine was found to be located at

the interfacial part of the DPPG - rich regions due to the strong electrostatic attraction between the positive NH_3^+ moiety of fluoxetine and the negative phosphate moiety of the DPPG lipid [41]. This attractive force could lead to the drug accumulation on the interface of the lipid membrane, which interfered with fluoxetine penetrating more deeply into the lipid bilayers' hydrophobic core. This event led to the fact that the natural negative charges of DPPG - rich regions were neutralized by the screening effect upon the addition of positive fluoxetine molecules. Indeed, the steric repulsions between two adjacent lipid headgroups were significantly reduced by the addition of the positive-charge drug agents into the media, thus more densely packed regions on the bilayer surface were expected. Thanks to the neutralizing effect, the phase separation caused by the DPPG lipids in an anionic-zwitterionic mixture was prevented, which further helped to stabilize the membrane structure [29]. In short, the electrostatic interactions were responsible for the partitioning of fluoxetine into DPPG lipid bilayers. As similar to the case of DSPC lipids, the electrolytes were found not to have any great impact on the lipid membrane surface [41]. The DPPG - rich regions by some means or other have a part in the final partition coefficients. However, no matter how much DPPG lipids were added to the binary mixtures, the partitioning of fluoxetine into the LUVs decreased, indicating that the contribution of such regions to the final K_p values was negligible.

In regards to the DSPC - DPPG rich regions, the zwitterionic PC headgroup of DSPC lipids consists of a single dipole including an immobile negative phosphate moiety and a mobile positive amine moiety [49]. The PC headgroup orientation was found to remain essentially unaffected in the anionic-zwitterionic membrane [49, 52]. This indicates that there was no attraction between the positive end of DSPC lipid and the negative end of the adjacent DPPG lipid. Thus, hardly any cluster was formed in terms of a strong electrostatic interaction between these two lipid species. Indeed, as in the case of the DMPC/DMPG mixture, only few and short-lived hydrogen bonds between them were recorded [52]. However, there must have a structure that keeps the lipid system in shape and prevents the phase separation. In this case, the electrolytes are believed to play an essential role in stabilizing and increasing the packing density of the DSPC - DPPG rich regions. Before the drug is added to the vesicle suspensions, in the area where DSPC and DPPG lipids are held next to each other, the sodium cations neutralize the negative moieties of each lipid (Fig. 5). This screening effect could prevent the repulsion between two adjacent negative phosphate groups; therefore, it helps to tighten the membrane structure. Later on, upon the addition of fluoxetine, the positively charged fluoxetine could be repelled by the positive choline moieties of DSPC lipids, which inhibits the partitioning of fluoxetine. Besides that, the sodium cations which occupied considerable

spaces on the membrane created the steric hindrances that further impeded the partitioning of fluoxetine into the lipid bilayers. With the decline in K_p values, the data in Table 1 clearly shows that the DSPC - DPPG rich regions were not the main K_p contributors.

On the whole, not only is electrostatic interaction indispensable in a homogenous lipid bilayers but its role is also recognizable in a heterogenous lipid system which has at least a charged lipid agent in the combination. Once the positive fluoxetine makes its appearance, it initially targets the DPPG - rich regions on the liposomes due to the strong attractive forces between the opposite charged species. When all DPPG - rich regions were thoroughly absent, the two remaining areas were DSPC - rich regions and DSPC - DPPG rich regions, which would compete for contacting with the drug molecules. Nevertheless, both the impeditive coordination of the sodium ions association on the vesicles and the repulsions between same charged species evidently demanded more energy for fluoxetine to partition into this area. Therefore, the DSPC - rich regions should interact with the drugs prior to the DSPC - DPPG rich regions.

To sum up, the decline in molar fractions of the main K_p contributor, DSPC, causes the reduction of the final K_p values. The DPPG - rich region shows not to contribute much to the outcome since its increased molar fractions still associates with a lessened partition of fluoxetine. The DSPC - DPPG rich regions are probably formed more than the DPPG - rich regions. This could occur because the hindering effects are much greater than the contribution of DPPG lipids themselves, which results in a significant reduction of the final K_p values. Electrostatic interaction between the sodium cations and the natural negative moieties of each lipid plays an important role in the binary anionic-zwitterionic lipid system. It helps to prevent phase separation caused by the repulsion between two adjacent negative moieties of each lipid. It also increases the packing density of the lipid bilayers, which hinders the drug partitioning.

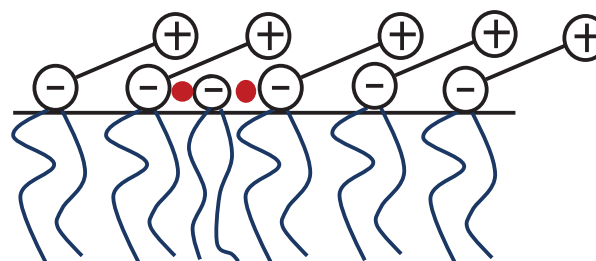


Fig. 5. Schematic illustration of a DSPC - DPPG rich membrane leaflet. The headgroup of DPPG lipid species is presented as a single negative charge, whereas the headgroup of DSPC lipid species consists of a mobile positive choline group and a fixed negative phosphate group. Sodium cations are depicted as red dots.

Conclusions

In this study, the partitioning of fluoxetine into DPPG - DSPC binary lipid bilayers was investigated under the viewpoint of electrostatic interaction by varying the molar fractions of DPPG in the lipid system. It was found that the increase of negative charges on the membrane surface impeded the partitioning of fluoxetine into the anionic DPPG - zwitterionic DSPC LUVs. As the molar fraction of DPPG increased, the partition coefficient decreased. The condensing effect on the membrane under the impact of electrolytes strongly demonstrated that the electrostatic interaction between the oppositely charged ions in the aqueous solution played such an important role in the partitioning of the positive charged drug into binary membranes composed of anionic and zwitterionic lipids. This study also highlighted how seemingly small variations in the lipid system could affect biophysical membrane properties and proved how fundamental membrane measurements were crucial in the interpretation of lipid-drug delivery mechanisms.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Characterization of optical parameters of breast cancer cell line - BT474 by polarimetry technique

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Abstract:

Breast cancer is a well-known health issue that has been a major focus for healthcare professionals for quite some time. Still, the most common noninvasive diagnostic tool - mammography - results in a high false positive rate along with risks of exposure to radiation. These disadvantages are magnified and become more severe when screenings are done repeatedly. To tackle this problem, we introduce a novel framework for uncomplicated diagnosis of breast cancer. Our method utilizes the analytical technique of Mueller matrix decomposition and Stokes vector polarimetry from a polarized light system consisting of a helium-neon laser (wavelength of 632.5 nm), a quarter-wave plate, polarizers, and a Stokes polarimeter. Thus, this technique introduces no radiation. We extracted nine optical parameters of a breast cancer cell line - BT474 - and determined the relationship and separation power of these parameters to cancerous cells and healthy cells. Specifically, the samples were designed as a two-dimensional cellular model of malignant breast tumours that combined a range of four cell densities - 10^4 , 10^5 , 10^6 , and 10^7 cells - per an area of 9 cm². Nine optical parameters - orientation angle of linear birefringence (α), retardance or linear birefringence (β), optical rotation angle or circular birefringence (γ), orientation angle of linear dichroism (θ_d), linear dichroism (D), circular dichroism (R), degrees of linear depolarization (e_1 and e_2), and degree of circular depolarization (e_3) - were extracted from a total of 40 samples using the polarized light system. The results revealed the positive correlations between three cell densities (10^4 , 10^5 , and 10^6) and the orientation angle of linear birefringence ($R^2 = 0.8038$), linear birefringence ($R^2 = 0.8627$), and linear dichroism ($R^2 = 0.9662$). Meanwhile, both the orientation angle of linear dichroism and circular dichroism illustrated the negative correlation with that range of cell densities with $R^2 = 0.9983$ and 0.9447 , respectively. This proves that the optical parameters measured demonstrate significant association with the cells' characteristics and thus, the proposed method could pave the way for an accessible diagnosis of breast cancer.

Keywords: breast cancer cell line, BT474, circular birefringence, linear birefringence, linear dichroism, Mueller matrix decomposition, optical properties, polarized light, Stokes polarimeter.

Classification number: 2.3

Introduction

Breast cancer, which takes place when cells in the breast begin to grow uncontrollably, has developed into one of the major public health problems worldwide, with 50-80% of breast cancer cases being invasive ductal carcinoma. In spite of being the gold standard among diagnostic tools, conventional imaging examinations (i.e., X-ray mammography, ultrasonography, and magnetic resonance imaging - MRI) have their disadvantages. In fact, X-ray mammography has been reported to have low sensitivity and specificity [1-3]. To overcome low accuracy and limited availability of breast cancer diagnostics, various optical techniques have recently been proposed for screening

breast cancer, including near-infrared optical tomography, optoacoustic imaging, Raman spectroscopy, diffuse optical spectroscopy, time-resolved diffuse optical spectroscopy, and polarized light. Their advantages in comparison with the standard techniques are enhanced accuracy, speed, and cost-effectiveness. Additionally, due to their simple facilities, they are more suitable for continuous bedside monitoring.

Polarized light, a different dimension of biomedical photonics, has offered new possibilities for noninvasively diagnostic approaches. Recently, various methodologies have been proposed for determining the optical properties of turbid media. To date, the polarized light method has been applied successfully to investigate optical properties of

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several types of biological and pathological samples, such as collagen components [4, 5], diabetes [6], cancer [7], and partial bladder outlet obstruction [8]. Pham, et al. proposed the decoupled analytical technique to extract nine effective optical parameters of turbid media utilizing Mueller matrix decomposition and Stokes vector polarimetry [9-11]. This method determined Mueller matrices of the parameters in a decoupled manner [9]; hence, it could reduce error as well as solve the problem of multiple solutions in previous works. Additionally, the technique was successful in extracting the full polarization properties of a turbid medium in one measurement [10, 11].

The challenges of applying polarized light in screening for cancer include the method of extracting full-range optical values of the biological samples, data bank building, and early detection when malignant cells are in low density. The process of collecting pathological tissues for building a data bank has limitations, as cancerous tumours could be polyclonal [12], various in types and grades [13], and present in a finite supply. In this work, we apply Mueller matrix decomposition and Stokes vector polarimetry to screening for breast cancer. This method is a novel technique using polarized light to solve the problem of extracting full-range optical parameters. Meanwhile, there was no report on utilizing Mueller matrix decomposition to investigate breast cancer. The research was expected to provide a measurement range of the optical system and effective parameters of a mammary carcinoma cell line - BT474. That investigation would be useful for the optical system upgrade, the understanding of cancer-light interaction, and the building of a data bank for breast cancer detection through sample taxonomy.

The samples used in the research were two-dimensional cellular models of breast cancer. The cell line was BT474. In histological taxonomy, BT474 is derived from invasive ductal carcinoma, which accounts for 50-80% of breast cancer types [14]. In molecular classification, BT474 is in luminal B, one of five subtypes, with the expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [15].

Methodology

Optical system set-up and measurement

The technique applied in the research was Mueller matrix decomposition and Stokes vector polarimetry. Fig. 1 illustrates the schematic of the optical system. Nine effective parameters can be extracted for each sample. Table 1 presents names, symbols, ranges, and the formulas for nine effective parameters. More details about the technique can be found in the previous work of Pham and Lo [9-11].

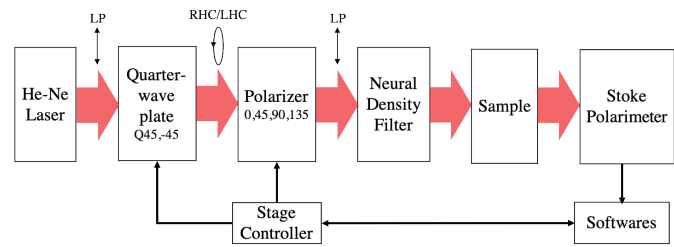


Fig. 1. The set-up of the optical system and measurement. A helium-neon (He-Ne) laser generates linearly polarized output that is characterized when passing through a series of optical elements. This light interacts with an anisotropic biological sample, then relays the optical information to a Stoke polarimeter. The nine effective optical parameters formulating linear birefringence, linear dichroism, circular birefringence, and circular dichroism are extracted by a software developed in Pham and Lo's laboratory. A stage controller rotates the quarter-wave plate and the polarizer to the exact angles needed during measurement. LP: linearly polarized. RHC/LHC: circularly right-hand-side/circularly left-hand-side.

Table 1. Symbols, ranges, and definitions of nine effective parameters.

Name	Symbol	Range	Definition(*)
Orientation angle of linear birefringence	α	$[0^\circ, 180^\circ]$	
Linear birefringence	β	$[0^\circ, 360^\circ]$	$2\pi(n_s - n_f)l/\lambda_0$
Optical rotation or circular birefringence	γ	$[0^\circ, 180^\circ]$	$2\pi(n_- - n_+)l/\lambda_0$
Orientation angle of linear dichroism	θ_d	$[0^\circ, 180^\circ]$	
Linear dichroism	D	$[0, 1]$	$2\pi(\mu_s - \mu_f)l/\lambda_0$
Circular dichroism	R	$[-1, 1]$	$2\pi(\mu_- - \mu_+)l/\lambda_0$
Linear depolarization	e_1 and e_2	$[-1, 1]$	
Circular depolarization	e_3	$[-1, 1]$	

*n is the refractive index; μ is absorption coefficient; l is path length through a medium (thickness of material); and λ_0 is vacuum wavelength. Furthermore, subscript f and s represent the fast and slow linearly polarized waves, respectively, when neglecting the circular effects. Finally, + and - represent the right and left circularly polarized waves, respectively, when neglecting the linear effects.

The polarized light system included a helium-neon laser with a wavelength of 632 nm and power < 5 mW (Thorlabs, Inc., America), a quarter-wave plate (Thorlabs, Inc., America), a polarizer (Thorlabs, Inc., America), a detector (Thorlabs, Inc., America), two stage controllers (OptoSigma), a Stokes polarimeter (Thorlabs, Inc., America), and a computer. Fig. 2 illustrates the installation of the system. The He-Ne laser was placed at the first position; the quarter-wave plate and the polarizer were set at the second and third positions, respectively; the sample was at the fourth position; and the detector stood at the fifth position. A stage controller was used to automatically

control the lens rotation of the quarter-wave plate and the polarizer. A Stokes polarimeter analyzed signals received from the detector to provide six polarization angles. A computer displayed a graphic user interface of the stage controller, the Stokes polarimeter, and calculated a package of polarization angle data to extract nine effective parameters of the sample by an algorithm written on Matlab (MATLAB, The MathWorks, Inc.).

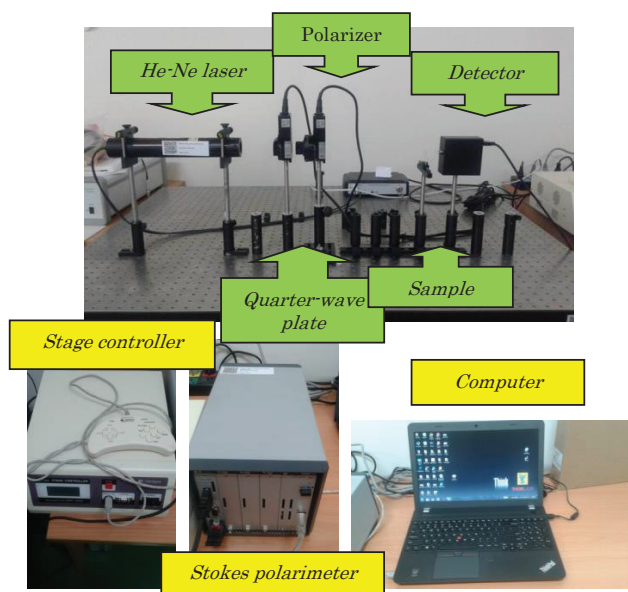


Fig. 2. Installation of the optical polarization system, including He-Ne laser, a quarter-wave plate, polarizer, sample holder, detector, stage controller, Stokes polarimeter, and computer.

Experiment design

To confirm the optimal condition for the research, a preliminary investigation was conducted. The design of the cellular model was based on the property of sample containers (standing quartz slides), available cell culture technique, and facilities. Specifically, we investigated cell densities, cell concentrations, medium volume, spreading area, and additive substances that could enhance the adhesion of breast cancer cells to a 9 cm² area on the quartz slide. Preliminary investigation revealed the appropriate parameters for cell adhesion; meanwhile, preliminary results demonstrated the correspondingly approachable cell densities of the optical system.

The design for cell adhesion is illustrated in Fig. 3. One layer of fibronectin solution was coated on the quartz slide. It has been proved that this substance enhances the adhesion of cells to surfaces. After removing the excess solution, one layer of cell medium was placed above the dried fibronectin layer.

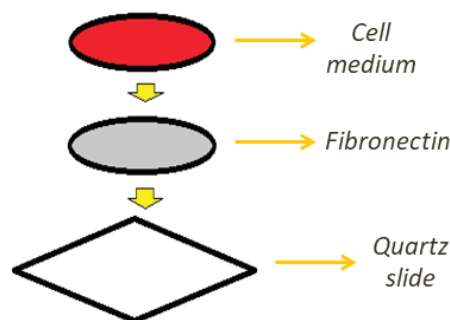


Fig. 3. Cell adhesion design that includes one layer of fibronectin and another layer of cells on quartz slide.

Table 2 presents appropriate parameters of the adhesive area, cell densities, the volume of medium applied to the adhesive area, and concentration of cells on the medium. It is noticeable that with the area of the quartz slide (9 cm²), the suitable cell densities were 10⁴ cells, 10⁵ cells, and 10⁶ cells, and the medium volume needed to be 500 µl. It was found that these numbers of cells, as well as the volume, resulted in a full two-dimensional cover for the area. Corresponding with cell densities and medium volume, cell concentrations should be 10⁴ cells/500 µl, 10⁵ cells/500 µl, and 10⁶ cells/500 µl. For fibronectin, the suitable concentration and the appropriate volume per quartz slide were 10 µg/ml and 500 µl, respectively.

Table 2. Appropriate parameters for cell adhesion.

Parameter	Value
Adhesive area	9 cm ²
Cell density	10 ⁴ cells/9 cm ² 10 ⁵ cells/9 cm ² 10 ⁶ cells/9 cm ²
Medium volume	500 µl/quartz slide
Cell concentration	10 ⁴ cells/500 µl 10 ⁵ cells/500 µl 10 ⁶ cells/500 µl
Additive substance	Fibronectin
Concentration	10 µg/ml
Volume	500 µl/quartz slide
Incubating time	60 minutes

To confirm the study's findings, we also extended the model to 10⁷ cells/500 µl. However, due to the mass amount of cells, we scaled the model 10 times, which was 10⁶ cells/0.9 cm² with the equal volume of 50 µl for both fibronectin and cell medium.

Sample preparation

Cell culture: the BT474 human breast cancer cells were cultured in Dulbecco's modified Eagle medium (DMEM;

Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), and 1% penicillin-streptomycin (PS) (Sigma-Aldrich). Cells were kept at 37°C in humidified air with 5% CO₂.

Adhesion assays: firstly, the quartz plates were cleaned with alcohol 70° and distilled water, sterilized with ultraviolet light for 20 minutes, coated by filling with 500 µl of fibronectin in concentrations of 10 µg/ml, and incubated at 37°C for one hour for the binding of the fibronectin. Then, the excess fibronectin was removed. While incubating fibronectin, we prepared a suspension of the cells with specific concentrations for the examination. Next, a volume of 500 µl was added for each concentration (that is 500 µl for 10⁴ cells, 500 µl for 10⁵ cells, and 500 µl for 10⁶ cells) into each plate. The cells were allowed to adhere to the surface of the plate for one hour in a 37°C-5% CO₂ incubator. Subsequently, the unbound cells were removed from the plate. The plates were put on Petri dishes and carried to the optical system in the Medical photonics laboratory - International University. For density 10⁷, both the volume and the area were scaled down 10 times, which means 10⁶ cells, volume of 50 µl, and an area of 0.9 cm². The process of the cell adhesion is illustrated in Fig. 4.

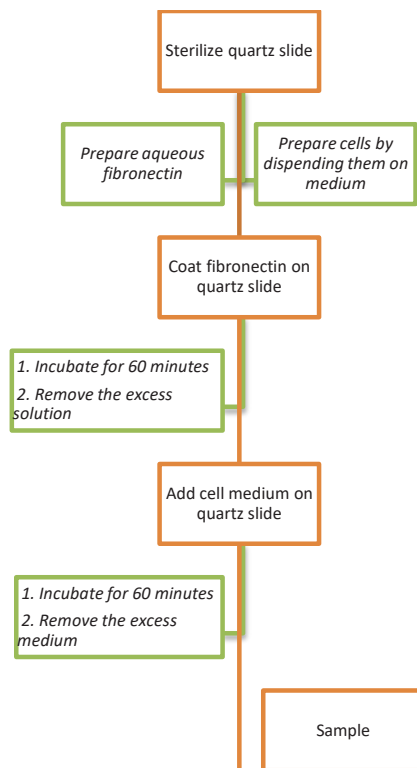


Fig. 4. Flowchart of the sample preparation process.

Figure 5 provides images of control samples and cell samples. To control the number of cells in a unit of area, the cell models were contained on transparent square slides kept in Petri dishes. A cell-counting technique was used in a unit of area under a modern microscope. From the results, we can determine the exact density of the cells in a sample. As can be seen, that density - 10⁷ - was found in circles while control samples and other density samples covered the entire slide surface.

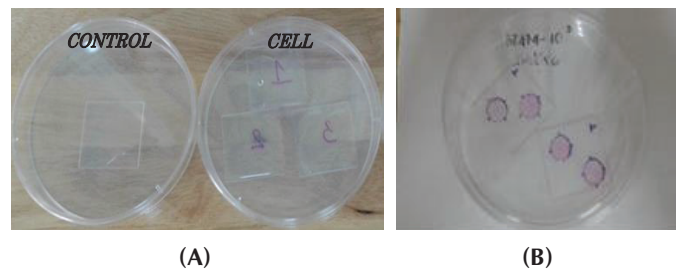


Fig. 5. Control sample and cell samples after cell adhesion: control sample, density 10⁴, density 10⁵, density 10⁶ (A), and density 10⁷ (B).

It is noted that in Fig. 5, the control sample contains only one layer of fibronectin and the cell sample contains one layer of fibronectin and one layer of the cells with different specific concentrations.

Data acquisition and analysis

Six angles (i.e., right-hand-side and left-hand-side for circular polarization, 0°, 45°, 90°, and 135° for linear polarization) were collected from three to five different points of each sample (i.e., control samples, samples of density 10⁴, of 10⁵, of 10⁶, and 10⁷). Six control samples; 12 samples of each density - 10⁴, 10⁵, 10⁶; and six samples of density 10⁷ were measured for the statistical significance.

Each data package of six polarization angles for each point on a sample was calculated by an algorithm written in a Matlab program (MATLAB, The MathWorks, Inc., Natick, Mass., United States) to extract nine effective parameters representing full optical properties of the measured samples.

Experimental results

Two-dimensional cellular models of breast carcinoma

This part presents the results of building cellular models for the investigation. Fig. 6 provides the images of four cell densities under the optical microscope (Nikon Corp., Minato, Tokyo, Japan) with the magnification of 40, 100, and 200. The photos were taken immediately after cell adhesion. From the photos, it was evident that the number of the cells was highest in photos of density 10⁷, followed

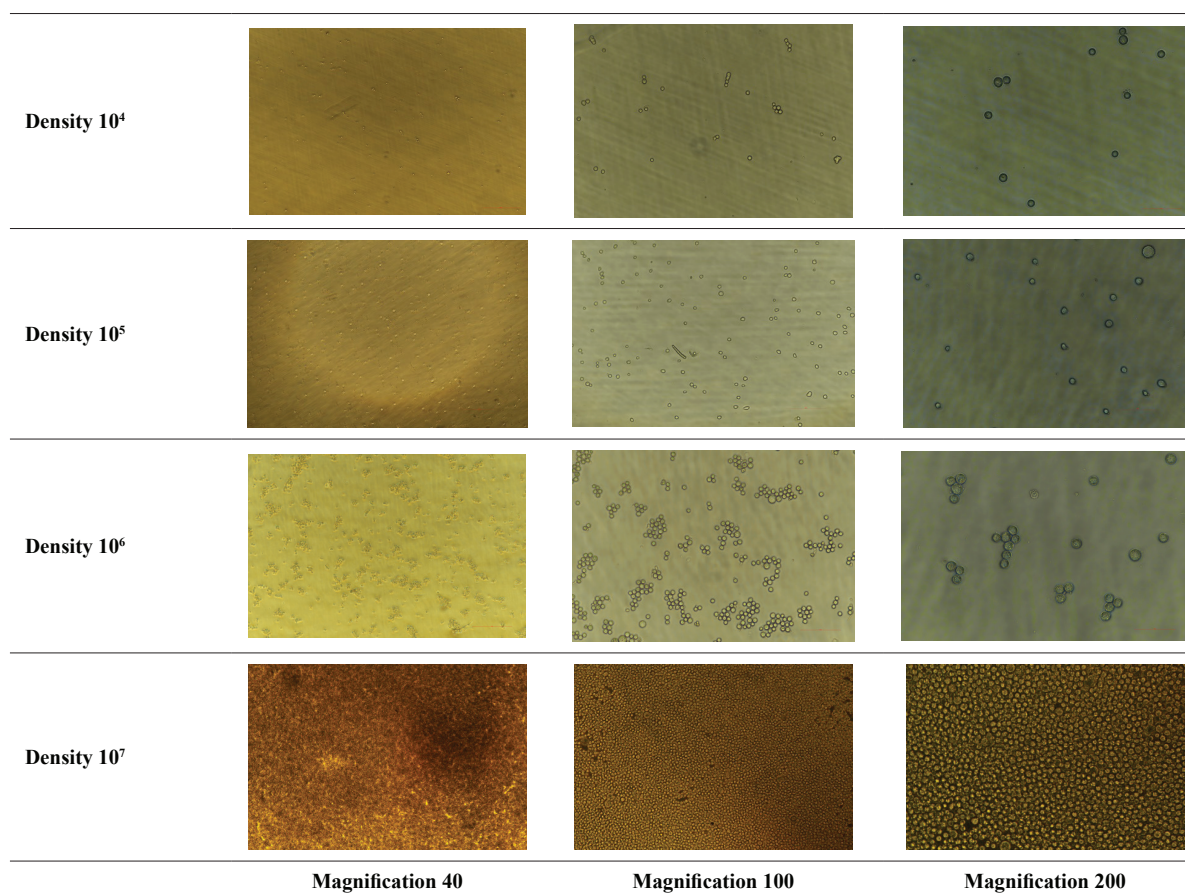


Fig. 6. Microscope images of BT474 after adhesion on quartz plates with different densities, i.e., 10^4 , 10^5 , 10^6 , and 10^7 , respectively.

by those of 10^6 , lower in photos of density 10^5 , and lowest in those of density 10^4 . Alternatively, an increase in cell numbers corresponded with cell densities in the models. In addition, it is noticeable that density 10^7 was the maximum cell number entirely covering the surface of the quartz slide.

Effective optical parameters

This section reveals the values of effective optical parameters extracted from the investigated samples. Although 48 samples were built, only 40 samples were successfully measured. Nine effective parameters - orientation angle of linear birefringence (α), retardance or

linear birefringence (β), optical rotation angle or circular birefringence (γ), orientation angle of linear dichroism (θ_d), linear dichroism (D), circular dichroism (R), degrees of linear depolarization (e_1 and e_2), and degree of circular depolarization (e_3) - were extracted from a total of 40 samples by the polarized light system. Those samples included six each for control, density 10^4 , and density 10^7 , and 11 each for density 10^5 and density 10^6 , respectively. Table 3 lists the sample number collected from each density to calculate the average value and standard deviation for effective optical parameters.

Table 3. A summary of all nine effective optical parameters of BT474 extracted using the decoupled analytical method.

Cell density	Nine effective optical parameters of BT474								
	α (deg)	β (deg)	θ_d (deg)	D (deg)	γ (deg)	R (deg)	e_1 (deg)	e_2 (deg)	e_3 (deg)
10^4 cells/ml	121.55±20.542	0.934±0.4053	106.81±19.269	0.1446±0.0139	22.973±34.125	-0.0256±0.0647	1.0114±0.0124	-1.019±0.0468	3.5999±0.5799
10^5 cells/ml	146.3±23.219	1.6915±1.2625	93.936±30.066	0.1809±0.0201	7.7493±16.869	0.0152±0.0304	1.0133±0.089	-1.0194±0.0602	2.8651±1.0321
10^6 cells/ml	148.22±30.556	1.8298±1.3375	82.77±34.43	0.2518±0.0397	1.5173±4.0789	0.01±0.0355	0.974±0.0136	-1.009±0.3974	3.0097±0.7924

α : orientation angle of linear birefringence; β : linear birefringence; θ_d : orientation angle of LD; D : linear dichroism; γ : circular birefringence CB; R : circular dichroism CD; e_1 : linear depolarization; e_2 : linear depolarization; e_3 : circular depolarization. N = 60 for each cell density.

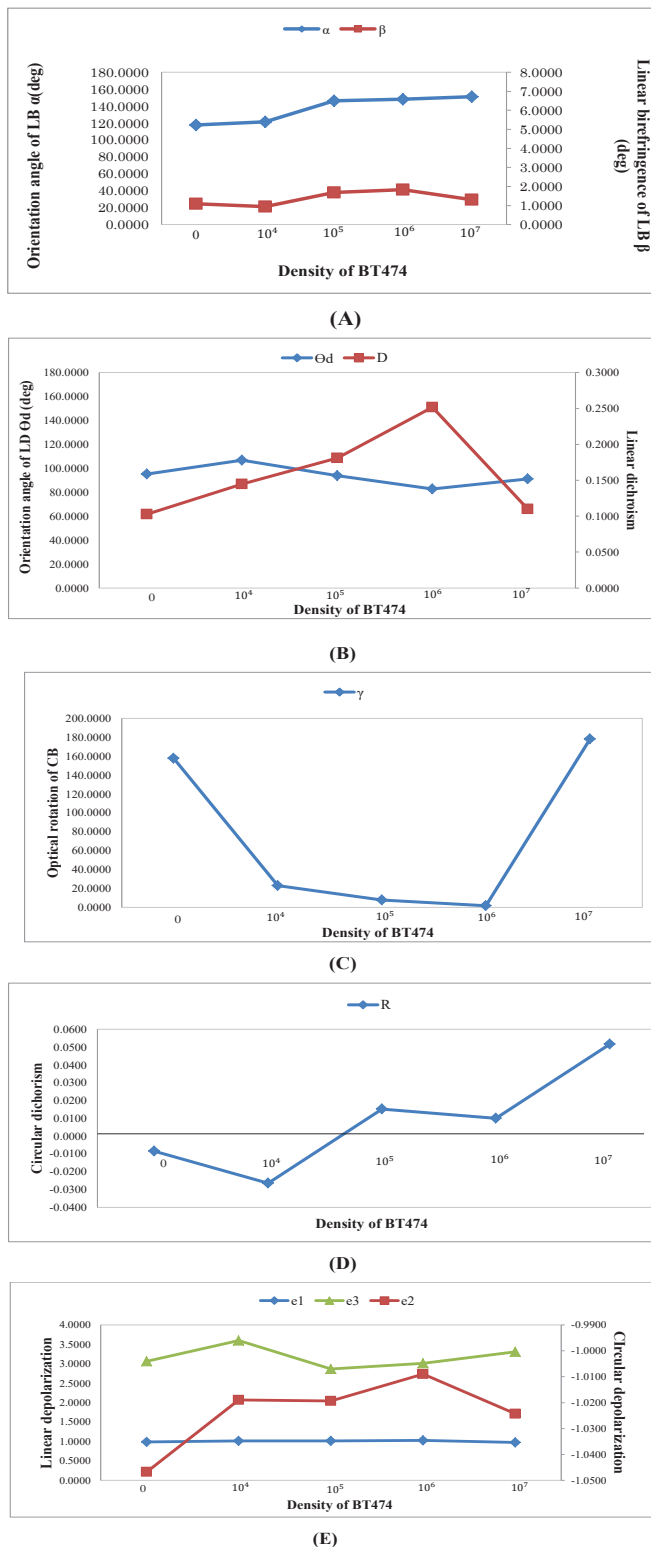


Fig. 7. Effective parameter values of five cell densities - density 0, density 10^4 , density 10^5 , density 10^6 , and density 10^7 . Orientation angle of linear birefringence (A), orientation angle of linear dichroism and linear dichroism linear (B), optical rotation or circular birefringence (C), circular dichroism (D), linear and circular depolarization (E).

Figure 7 illustrates the graphic results of extracted parameters for different cellular densities of BT474.

From the graphic illustration of Fig. 7, linear birefringence, linear dichroism, and circular birefringence values of three cell densities - 10^4 , 10^5 , and 10^6 - were plotted in Fig. 8 to illustrate the linear relationship of optical parameters with the increment of density of BT474. For example, Fig. 8 demonstrates that the orientation angle and linear birefringence of LB and optical rotation angle of CB increase linearly with the change of BT474 density, respectively. In Fig. 8B, the measured value of LD increases as the cell density increases. Meanwhile, the orientation angle of LD decreases when the BT474 density increases.

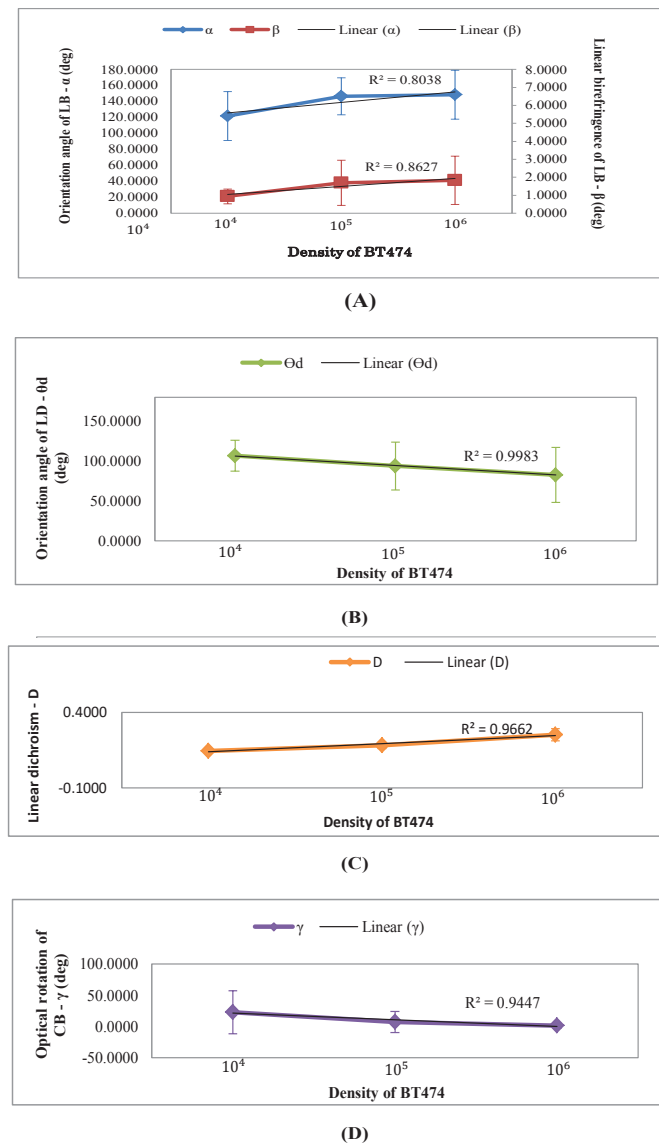


Fig. 8. Linear relationship between the range of cell density [10^4 , 10^6] and orientation angle of linear birefringence and linear birefringence (A), orientation angle of linear dichroism (B), linear dichroism (C), circular birefringence (D).

Discussion and implementation

The possible explanation for the positive correlation between breast cancer cell densities and linear birefringence as well as linear dichroism is based on the work of Angelskaya, et al. [16]. In the study, the authors determined which substances in the tumour played the role of optical indicators of cancer changes in biological tissues. In particular, they concluded that two types of fluorophore in the tumour - NADH and collagen - resulted in an increase in linear birefringence and linear dichroism. However, the study focused on murine models and two types of cancer - prostate and esophageal - hence, there is a need for verification of breast cancer samples.

The negative correlation between the density range and circular birefringence was thought to be due to the decline in glucose concentration in cells when cancer developed. In other words, circular birefringence values decreased along with the rise of cell density. Meanwhile, there was no possible explanation for the decrease of linear birefringence's orientation angle corresponding with the exponential increase of cell density.

The lowest density (i.e., 10^4 cells/9 cm²) reveals the potential of the polarized light system in early detecting breast cancer whereas the highest density (i.e., 10^7 cells/9 cm²) reveals the threshold for both the two-dimensional model and the instrument. Specifically, that low density provides the sensitivity of the optical system in screening the malignant transition of mammary cells. In terms of the model, 10^7 cells are sufficient to build a two-dimensional layer on an area of 9 cm². In the context of the technique, concentration and thickness of the layer created by that cell density reach the threshold of the helium-neon laser (wavelength 632.5 nm, power < 5 mW). Alternatively, the laser is unable to penetrate through or will interact with the sample of that high density to provide good signals.

In terms of the cellular model, the cell adhesion process needs to be modified. Firstly, from microscopic photos (Fig. 1), BT474 did not spread well on the surface, and that made standard deviation of parameter values high. Additionally, the considerable discrepancy between cell numbers on the sample before and after removing floating cells requires cell counting for the sample. Besides, it is promising to extend the model to lower densities (i.e.,

10^2 and 10^3 cells) for the primitive prognosis of breast cancer. When it comes to the technique, it can be seen that the values of the depolarization are out of the range. The reason is thought to come from the calculation. We suggest processing the data by alternative algorithms (i.e., genetic algorithm).

Conclusions

In conclusion, the optical system of a helium-neon laser (wavelength of 632 nm, power < 5 mW), a quarter-wave plate, polarizers, and a Stokes polarimeter utilizing the Mueller matrix decomposition and Stokes vector polarimetry was able to detect in-vitro mammary cancer through effective optical parameters. Specifically, there was a linear relationship between five parameters - linear birefringence (α and β), linear dichroism (θ_d and D), and circular birefringence (γ) - and the range of cell densities [10^4 , 10^6]. The R-squared values were approximately over 0.8 and over 0.9. The system is promising for uncomplicated cancer diagnostics. In addition, the high threshold for two-dimensional cellular models was revealed to be 10^7 cells/9 cm² or 10^6 cells/0.9 cm².

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The authors declare that there is no conflict of interest regarding the publication of this article.

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The efficacy of micronano particles across NPK doses and densities on maize growth and yield in Vietnam

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Abstract:

Increasing the efficiency of maize production in Vietnam is a prerequisite for increasing the quantity of maize for animal feed. With a view to reducing nitrogen-phosphorus-potassium (NPK) doses for maize production, a two-factor experiment using complex micronano particles across three NPK fertiliser doses and three densities on maize hybrid PAC999 was conducted following a randomized complete block design with plot size of 21.84 m², in Binh Dinh province of Vietnam from May to September 2017. Three NPK fertiliser doses (kg/ha) constitute the first factor, as P1: 156 N - 86 P₂O₅ - 84 K₂O (100% normal dose); P2: 140 N - 77 P₂O₅ - 76 K₂O (90%); and P3: 125 N - 69 P₂O₅ - 67 K₂O (80%). Three densities constitute the second factor, as M1: 71,429 plants/ha (100% normal density); M2: 64,935 plants/ha (90%), and M3: 57,143 plants/ha (80%). Nine combinations of M and P were developed into nine treatments; the control involved spraying with water rather than nano foliar fertiliser. Nano particles were applied as a foliar fertiliser solution at 20 days after sowing with 300 litres/ha, 30 days after sowing with 500 litres/ha, and 40 days after sowing with 700 litres/ha. The results show that 80% and 90% of normal NPK doses combined with 90% and 80% normal density produced a grain yield of 6.52 and 6.63 tons per ha, respectively, which is 14-16% higher than that of the control (5.71 tons/ha). The results of the experiment were demonstrated on large plots of 500 m² each in summer/autumn 2018 in Binh Dinh and Hau Giang provinces, spring/summer 2018 in Long An, and winter/spring 2017-2018 in Dong Nai province. Over an area of 500 m², 75% of farmers' NPK doses and the micronano solution were applied; the control involved applying 100% NPK doses. The results of the demonstration showed that the grain yield of maize (tons/ha) with the nano fertiliser solution (9.44 in Binh Dinh, 9.2 in Dong Nai, 9.52 in Long An, and 8.7 in Hau Giang) was 0.95, 0.28, 0.68, and 0.3 tons higher than that of the control (8.49, 8.92, 8.84, and 8.4 tons/ha, respectively).

Keywords: demonstration, factors, fertiliser, foliar, micronano.

Classification number: 3.1

Introduction

Maize production in Vietnam is not only facing biotic and abiotic stresses, but also high costs of production due to minimal application of advanced technological fertilisers, low levels of mechanization, and post-harvest losses. Survey data from FAO Statistical Data [1] shows that the cost of production for one ton of maize grain was US\$138 in Brazil, US\$142 in the USA, US\$225 in Thailand, US\$275 in the Philippines, US\$282 in Indonesia, but US\$329 in Vietnam. Regarding the components involved in maize production in Vietnam [2], the average cost of fertilisers

constitutes 30-35.5% of total costs; labour costs constitute 38.2%; mechanisation (machine hire) is between 5.0 to 8.7%; and pesticides vary from 4.9 to 12.2%.

Due to limited land area for maize production in Vietnam, one of the options that has emerged to increase the yield to meet demand is to study the efficacy of micronano particles (40-80 nm) manufactured by the Institute of Environmental Technology in the form of a foliar fertiliser for spraying maize plants in Binh Dinh province. The experimental results were demonstrated in Binh Dinh, Hau Giang, Long An, and Dong Nai provinces of Vietnam.

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Materials and methods

Materials

Seeds of maize hybrid PAC999 were provided by Advanta Seed Company. The micronano particles were manufactured by the Institute of Environmental Technology of the National Academy of Science and Technology of Vietnam. Nano size is 40-80 nm and the nutrient solution was applied in the form of foliar a fertiliser spray. The components of nano particles are listed in Table 1.

Methods

Experimental design: the plants in the experiment were planted as three replications on plots of 21.84 m² (5.2 m x 4.2 m), laid out in a randomized complete block design. Each plot was planted with six rows, each of which were 5 m long, with 0.7 m between the rows and 0.2 m between each plant in a row. The experiment consisted of 10 treatments of two factors. The first factor comprised three fertiliser doses: P1: 156 N - 86 P₂O₅ - 84 K₂O (100% normal dose); P2: 140 N - 77 P₂O₅ - 76 K₂O (90%); and P3: 125 N - 69 P₂O₅ - 67 K₂O (80%). The second factor comprised three densities: M1: 71,429 plants/ha (100% normal density); M2: 64,935 plants/ha (90%), and M3: 57,143 plants/ha (80%). Nine combinations of M and P were developed into nine treatments; the control involved spraying water instead of nano nutrients on P1M1. The three sprays of foliar nano nutrients are also presented in Table 1.

Duration of experiment: 24 April to December 2018.
Location of experiment: Nhon Hau commune, An Nhon town, Binh Dinh province, Vietnam.

Temperature and rainfall at the sites of the experiment in Binh Dinh and demonstrations in Dong Nai, Long An and Hau Giang provinces: according to weather and climate data from Climate-Data.Org (2019) [3], in Binh Dinh province in the southern central region, the average annual temperature is 26.8°C. About 1,630 mm of precipitation falls annually. The warmest month of the year is August, with an average temperature of 30.0°C. In January, the average temperature is 23.0°C. Such data for the provinces in which the results of the experiment demonstrated is presented in Fig. 1.

In Dong Nai province, in the southeastern region, the average annual temperature is between 23.9 and 29.0°C,

Table 1. Constituents of foliar micronano stock solution sprayed on maize plants.

Nutrient	1 st spray	2 nd spray	3 rd spray
N	200,000	150,000	150,000
P	200,000	50,000	50,000
K	200,000	450,000	450,000
Mg	15,700	11,800	11,800
S	20,000	14,000	14,000
Si(OH) ₄	50,000	50,000	50,000
Fe	3,600	7,100	7,100
Cu	400	4,700	4,700
Zn	102,260	111,800	11,800
Mn	1,800	11,200	11,200
B	1,100	5,700	90,700
Mo	60	110	110
Se	20	20	20
Cytokinin	25.2	25.2	25.2
Gibberellin A3	14	14	14
IAA	14	14	14
IBA	14	14	14
Amino Acid	12,000	12,000	12,000
Chitosan	2,500	2,500	2,500

Note: 1st spray1: 1 litre of stock solution/ha, at six-leaf stage (22-25 days after sowing, DAS), diluted in 300 litres of water; 2nd spray: 2 litres of stock solution/ha, at 12-leaf stage (35-37 DAS), diluted in 500 litres of water; 3rd spray: 2 litres of stock solution/ha, at VT stage - a vegetative growth stage description that all branches of tassel visible (47-50 DAS), diluted in 700 litres of water.

somewhat lower than the standard level of the tropical regions (26-30°C). Rainy days in a year number between 120 and 170, with total rainfall of some 1,500-2,750 mm (Fig. 1). The average humidity is around 80-82%; in the dry season it is 10-12%, lower than in the rainy season; humidity varies considerably between the areas. In Hau Giang province, in the Mekong delta region of Vietnam, the summers are much rainier than the winters. The temperature here averages 27.1°C. The average annual rainfall is 1,589 mm (Fig. 1). In Long An province, the summers are much rainier than the winters. The average annual temperature is 27.5°C. About 1,809 mm of precipitation falls annually (Fig. 1).

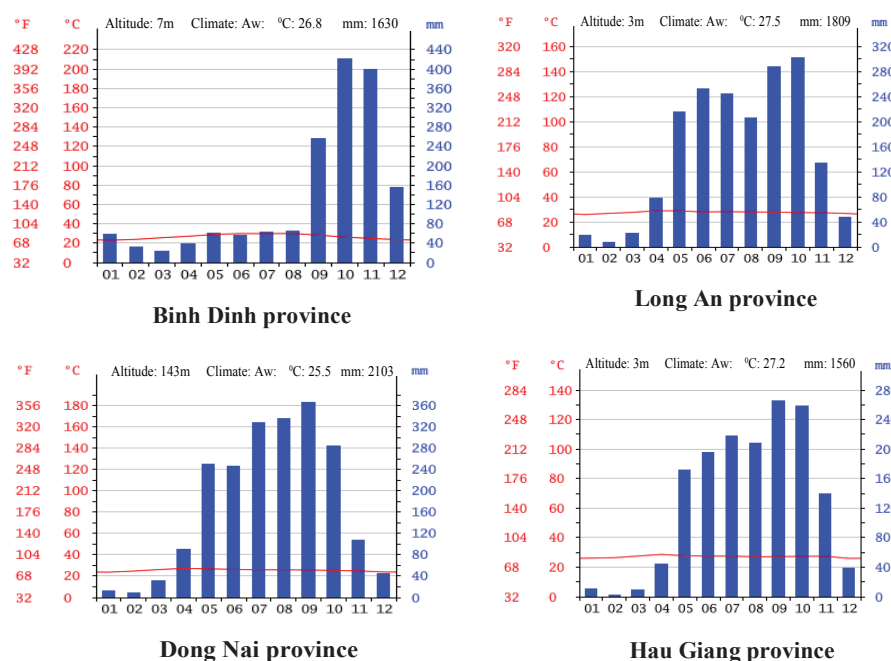


Fig. 1. Temperature and rainfall at the sites of the experiment in Binh Dinh and demonstrations in Dong Nai, Long An and Hau Giang provinces, 2017-2018.

Demonstration fields for applying nano foliar fertiliser: the plot size of the demonstration field was 500 m² for PAC999, with 75% NPK doses (126 kg N - 75 kg P₂O₅ - 75 kg K₂O/ha) and nano solution applied; in the control plot, 100% NPK doses (180 kg N - 100 kg P₂O₅ - 100 kg K₂O/ha) without nano solution were applied. Both testing and control fields were planted with 57,143 plants/ha (80%).

The method for spraying the nano foliar fertiliser was similar to that applied in the experiment. The locations of the demonstrations were Binh Dinh, Hau Giang, Long An, and Dong Nai provinces in Vietnam, in summer/autumn 2018 and winter/spring 2018-2019.

Data collection and analysis: for the experiment, data was collected from the two middle rows. Guidelines from CIMMYT were applied to the collection of agronomy and yield characteristic data [4]. For the demonstration plot, five grain samples were taken from the field and the data collected was based on five samples across the field of the experimental and control plots. The area of each sample was 11.0 m² (three rows x 5.25 m long x 0.7 m between rows).

MS Excel and IRRISTAT 5.0 were used for ANOVA analysis and the Duncan multiple test was used for comparing the means.

Time and place of the study

The experiment was conducted in summer/autumn season in Binh Dinh province. Large-scale demonstrations were implemented in summer/autumn 2018 in Binh Dinh and Hau Giang provinces, as well as in spring/summer 2018 in Long An province and winter/spring 2017-2018 in Dong Nai province.

Results

Results of soil analysis in Binh Dinh for experiment and in Dong Nai, Long An and Hau Giang provinces for demonstrations

According to the soil micronutrient classification for meeting demand for crop production in Vietnam by Pham Dinh Thai (2017) [5], the micronutrient content of soil in Binh Dinh is low in Mn, Zn, B, and high in Cu. The soil in Hau Giang province is acidic, poor in K, very poor in P, average in total N, with available Mn, Cu, and Zn, and rich in B. The soil in Long An province is acidic, very poor in total N, poor in K and B, average in Zn, and rich in P. The soil in Dong Nai province is slightly acidic, poor in total N total, with available Cu, average in Zn and B, and rich in available K (Table 2).

Table 2. Result of soil analysis in four provinces in Vietnam.

Criteria	Unit	Binh Dinh	Hau Giang	Long An	Dong Nai	Testing methods
pH (KCl)		4.91	3.45	4.31	5.19	TCVN 5979-2007
N (total)	%	0.108	0.177	0.091	13.3	TCVN 6498-1999
P (available)	mg/kg	44.8	14.2	157	454	TCVN 8942-2011
K (available)	mg/kg	48	63	53	226	TCVN 8662-2011
B (available)	mg/kg	0.775	0.405	0.38	0.56	Australian Manual for Soil and Water Analysis, 2011 (12C1)
Cu (available)	mg/kg	1.88	1.85	5.3	8	TCVN 7727-2007
Zn (available)	mg/kg	2.81	4.46	9.0	33.7	TCVN 7727-2007
Fe (available)	mg/kg	*	*	1.68	1.8	
Mn (available)	mg/kg	21.8	11.6	0.301	*	TCVN 7727-2007

Source: soil analysis laboratory of the Institute of Agricultural Sciences for Southern Vietnam. Note: *: not available.

Results of the experiment

Efficacy of micronano nutrients across densities and NPK fertiliser applications on the growth and development of maize hybrid PAC999 in Binh Dinh province: there was no significant difference between treatments for the number of days (Table 3) from sowing to emergence (6 days) and from sowing to pollen-shedding (50-51 days), meaning that no effects of nano across plant densities and NPK doses on growth of maize were found. There was no clear effect of the micronano nutrients across fertiliser applications and densities on plant height of the PAC999 maize hybrid. At 15 DAS, the shortest plant height was that of treatment M1P1 sprayed with micronano solution (35 cm), 89% compared to the control (M1P1 sprayed with water only); at 45 DAS, the shortest treatments were M1P1, M2P2, and M3P3 (141-142 cm); and at maturity the shortest treatment was M2P3 (192 cm). These may be the result of soil variation being higher than that of the fertiliser, density, and nano effects (Table 3).

Table 3. Effects of micronano nutrients across densities and fertiliser applications on the growth and development of maize hybrid PAC999 in Binh Dinh province, 2017.

Treatments	Days to emergence (days)	Days to pollen shedding (days)	Plant height					
			15 DAS		45 DAS		At maturity	
			cm	% check	cm	% check	cm	% check
M1P1	6	50	35c	89	141bcde	92	190ab	100
M1P2	6	50	39ab	98	156a	101	191a	101
M1P3	6	51	38abc	95	147abc	96	192a	102
M2P1	6	50	35bc	89	133e	87	189ab	100
M2P2	6	50	39ab	98	142bcde	92	191a	101
M2P3	6	50	37abc	93	147abcd	95	184b	97
M3P1	6	50	38abc	95	147abcd	95	192a	101
M3P2	6	50	36abc	90	138de	90	189ab	100
M3P3	6	50	36abc	90	141cde	92	192a	102
Control	6	50	40a	100	154ab	100	189	100
CV (%)			6.24		4.51		1.97	
LSD _{0.05}			3.99		11.74		6.46	

Note: Duncan's multiple range test: similar character in the column (a, or b, c, d or e) means no significant difference among the average data.

Effects of micronano nutrients across plant densities and fertiliser application doses on abiotic and biotic stress tolerance: for the summer/autumn maize crop in Binh Dinh (2017), there were no storms or typhoons and therefore the maize plants were not broken, resulted in good plant aspects (score of 1). A similar trend can be seen for insects and diseases of maize crops in this season in Binh Dinh province (Table 4).

Table 4. Effects of micronano nutrients across densities and fertiliser applications on plant aspect and infection by insects and diseases of maize hybrid PAC999 in Binh Dinh province, 2017.

Treatments	Plant aspect (1-5)	Stalk lodg (%)	Root lodg (%)	Stem borer (%)	BLSB (%)	<i>H. turci</i> (%)	Rust (%)
M1P1	1	0	1	1	0	0	0
M1P2	1	0	1	1	0	0	0
M1P3	1	0	1	1	0	0	0
M2P1	1	0	1	1	0	0	0
M2P2	1	0	1	1	0	0	0
M2P3	1	0	1	1	0	0	0
M3P1	1	0	1	1	0	0	0
M3P2	1	0	1	1	0	0	0
M3P3	1	0	1	1	0	0	0
Control	1	0	1	1	0	0	0

Note: Lodg: lodging; *H. turci*: *helminthosporium turcicum*; BLSB: banded leaf spot blight.

Effects of micronano nutrients across densities and fertiliser applications on yield components of maize hybrid PAC999 in Binh Dinh province: the ratio of ears per plant varied from 0.97 to 1.02, leading one to infer that there was no effect of the nano solution across treatments of densities and fertiliser applications (Table 5). The shelling percentage of treatment M3P1 (57,143 plants/ha and 156 kg N - 86 kg P₂O₅ - 84 kg K₂O/ha (100% normal dose) was highest, at 84%, 5% superior to the control, to which 156 N - 86 P₂O₅ - 84 K₂O was applied without nano fertiliser at a density of 71,429 plants/ha (100% normal density). This can be explained by the fact that at low density and the normal NPK dose, with the support of nano fertiliser, a maize plant could absorb enough nutrients so that the shelling percentage was the highest. For the treatment of M3P2: 57,143 plants/ha (80%) and 140 kg N - 77 kg P₂O₅ - 76 kg K₂O (90%) with the support of the nano fertiliser, the number of kernels/row, kernel rows/ear, and 1,000 kernel weight were 105%, 104%, and 106%, respectively, higher than that of the control treatment. All these factors resulted in the grain yield of treatment M3P2 (6.63 MT/ha) being significantly higher than that of the control treatment (5.71 MT/ha) (p<0.05), or 116% higher than the control. This result was wholly derived from the effect of micronano nutrients across three levels of NPK doses and three levels of plant density because there were no significant interaction effects of NPK doses (p=0.92>0.05) and density (p=0.249>0.05) (Table 6).

Table 5. Effects of micronano nutrients across densities and fertiliser applications on yield components of maize hybrid PAC999 in Binh Dinh province, 2017.

Treatment	Ears/ plant	Kernel/rows		Rows/ear		1,000 kernel weight		Shelling percentage		Grain yield	
		Kernel	% check	Row	% check	g	% check	(%)	% check	MT/ha	% check
M1P1	1.00	36	102	12.1ab	100	343ab	104	81	102	6.06bc	106
M1P2	0.98	35	100	12.1ab	100	344ab	104	78	99	5.76c	101
M1P3	0.98	37	105	11.9b	98	342ab	103	79	100	6.25abc	109
M2P1	1.02	35	101	12.0ab	99	334ab	101	79	100	5.75c	101
M2P2	1.02	36	102	11.9b	98	347ab	105	79	100	5.78c	101
M2P3	1.01	36	102	12.1ab	100	350a	106	80	101	6.52ab	114
M3P1	0.98	35	101	12.4ab	102	348a	106	84	105	6.25abc	110
M3P2	1.02	37	105	12.5a	104	351a	106	79	100	6.63a	116
M3P3	0.97	36	103	12.1ab	100	350a	106	79	100	6.04bc	106
Control	0.99	35	100	12.1ab	100	331b	100	79	100	5.71c	100
CV (%)		2.67		3.63		2.75				5.17	
LSD _{0.05}										0.26	

Note: Duncan's multiple range test: similar character in the column (a, or b, c) means no significant difference among the average data.

Table 6. Analysis of variance in grain yield of micronano foliar solution experiment across NPK applications and densities of PAC999 in Binh Dinh province.

Source of variation	DF	Sum of squares	Mean	F ratio	PROB	ER
Replication	2	2.27767	1.13884	18.63	0.000	6
Plant Density	2	.391385	.195692	2.01	0.249	3
Replication-Plant density	4	.390355	.975886E-01	1.60	0.238	6
NPK doses	2	.996476E-02	.498238E-02	0.08	0.922	6
Plant Density-NPK doses	4	1.62549	.406371	6.65	0.005	6
Residual	12	.733629	.611357E-01			
Total (corrected)	26	5.42849	.208788			

Results of the demonstration field applied with micronano foliar fertiliser

Results of the demonstration field application of nano foliar fertiliser to PAC999 maize hybrid in Binh Dinh province: the average plant height of PAC999 (Table 7) was similar in the pilot field with and without nano fertiliser application (209.5-208.1 cm). However, the average grain moisture in the pilot field (30.9%) was much lower than that of the field without nano fertiliser (33.0%). Due to high drying rate of kernels, the average grain weight of 1,000 kernels in the pilot field with nano fertiliser (337 g) was significantly higher than that of the field without the nano application (312.8 g). The grain yield in the pilot field (9.44 MT/ha) was significantly higher than that of the field without nano fertiliser (8.49 MT/ha).

Table 7. Results of the demonstration field applied with micronano foliar fertiliser to PAC999 maize hybrid in Binh Dinh province, 2018.

Plot	Samples	Plant height (cm)	Grain moisture at harvest (%)	1,000 kernel weight (g)	Grain yield (MT/ha)
75% NPK doses with nano application	1	219.8	31.6	335.0	9.23
	2	221.8	28.5	371.6	9.45
	3	224.0	31.2	304.0	9.59
	4	218.3	31.6	329.0	9.68
	5	163.8	31.6	345.7	9.19
	Mean	209.5	30.9	337	9.44
100% NPK doses without nano application	1	219.3	33.4	296.6	8.48
	2	219.0	34.6	312.1	8.17
	3	223.8	30.9	301.8	8.10
	4	217.3	35.9	324.6	8.61
	5	161.5	30.6	328.4	9.09
	Mean	208.1	33.0	312.8	8.49

Grain yield of PAC999 maize hybrid in the demonstration fields in Hau Giang, Long An, and Dong Nai provinces: the data in Table 8 shows that the grain yield of PAC999 maize hybrid with the application of 75% NPK dose (126 kg N - 75 kg P₂O₅ - 75 kg K₂O/ha) with nano fertiliser in Hau Giang, Long An, and Dong Nai provinces (9.2, 9.52, and 8.7 tons/ha, respectively) was 0.28, 0.68, and 0.3 tons higher than the control with 100% of NPK doses (180 kg N - 100 kg P₂O₅ - 100 kg K₂O/ha) (8.92, 8.84, and 8.4 tons/ha, respectively).

Table 8. Grain yield of PAC999 in demonstration plots in Hau Giang, Long An, and Dong Nai provinces, 2017-2018 (MT/ha).

Demo plot	Samples	Hau Giang summer/ autumn 2018	Long An winter/spring 2017-2018	Dong Nai winter/spring 2017-2018
Farmer practice without nano	1	8.59	8.59	8.93
	2	9.26	9.28	8.52
	3	8.85	9.26	8.03
	4	8.84	8.85	8.32
	5	9.05	8.84	8.23
	Mean	8.92	8.96	8.40
75% NPK plots with nano	1	8.61	10.0	8.96
	2	9.22	9.28	8.53
	3	9.34	8.81	8.73
	4	9.05	9.77	9.07
	5	9.8	9.73	8.15
	Mean	9.2	9.52	8.7

Table 9. Efficacy of nano foliar fertiliser on maize production in some provinces of Vietnam, 2017-2018.

Criteria	Binh Dinh	Dong Nai	Long An	Hau Giang
1. Total cost (1,000s VND/ha)				
With nano (75% NPK)	30,675	25,686	27,065	25,836
Without nano (100% NPK)	29,075	25,695	25,355	25,355
Difference	+1,600	-9	+1,710	+481
2. Grain yield (tons/ha)				
With nano (75% NPK)	9.44	8.70	9.52	9.20
Without nano (100% NPK)	8.49	8.40	8.96	8.92
Difference	0.95	0.30	0.56	0.28
3. Income (1,000s VND/ha)				
With nano (75% NPK)	51,920	46,110	50,350	48,760
Without nano (100% NPK)	46,695	44,520	47,435	47,276
Difference	5,220	1,590	2,915	1,484
4. Net benefit (1,000s VND/ha)				
With nano (75% NPK)	21,245	20,424	23,465	22,923
Without nano (100% NPK)	17,620	18,825	22,080	21,921
Difference	3,625	1,599	1,385	1,002
5. Cost of production (VND/kg)				
With nano (75% NPK)	3,249	2,973	2,843	2,806
Without nano (100% NPK)	3,424	3,058	2,727	2,842
Difference	-175	-65	+116	-36

The data in Table 9 shows that treatments with micronano spraying and 75% NPK had the cost of production almost similar to that compared to the treatments without micronano spraying and 100% NPK in Binh Dinh, Dong Nai, Long An, and Hau Giang provinces with the difference only 175, 65, 116 and 36 VND/kg, respectively. However, thanks to the effect of the micronano solution, the grain yields were 0.95, 0.30, 0.56, and 0.28 tons/ha higher than that of treatments without nano fertiliser and 100% NPK in Binh Dinh, Dong Nai, Long An, and Hau Giang provinces, respectively. This effect generated higher income for producers - 5,220,000, 1,590,000, 2,915,000 and 1,484,000 VND/ha - and higher net benefits - 3,625,000, 1,599,000, 1,385,000, and 1,002,000 VND/ha in Binh Dinh, Dong Nai, Long An, and Hau Giang provinces, respectively.

Discussion

In our study, there was no significant difference between treatments for number of days from sowing to emergence and from sowing to pollen-shedding and no clear effect

of the micronano solution across NPK doses and densities on plant height. In a study conducted with maize seeds treated with Fe nano fertiliser, some aflatoxin substrates at harvest could be inhibited and Fe nano fertiliser inhibited disease microorganisms of maize [6, 7]. Nanoparticles were also found to improve the balance between oxidants and antioxidants status of treated plants. Solanki, et al. (2015) and Suriyaprabha, et al. (2012) [8, 9] reported that, for maize seeds treated with SiO nanoparticles, the maize plants had significantly enhanced plant dry weight and enhanced levels of organic compounds such as proteins, chlorophyll, and phenols. The results of our study may be due to soil variation, which was higher than effects of the fertiliser, density, and the nano solution.

Churilov (2010) [10] has found that nano particles stimulate enzyme systems that result in more efficient biological and physiological cycles and stronger antibiotic systems for better seed germination and plant growth. The results of an experiment in Russia [10] have shown the efficacy of micronano fertiliser on maize growth and yields, and the conclusions were that the Katrina CB maize hybrid, which is a susceptible to drought, was micronano more tolerant to drought conditions and produced twice as large a yield when treated with micronano fertiliser, compared to untreated plants. Our study found that, with the support of nano fertiliser, the number of kernels/row and kernel rows/ear and the 1,000 kernel weight were higher than that in the control treatment. In demonstrations, grain moisture in the pilot field was much lower than that of the field without nano fertiliser. The average grain weight of 1,000 kernels in the pilot field with nano fertiliser was also found to be significantly higher than that of the field without the nano application. That is, the grain yield in the pilot field was significantly higher than that in the field without nano fertiliser.

Therefore, our results were similar to those found by Solanki, et al. (2015), Suriyaprabha, et al. (2012), and Churilov (2010) [8-10].

Thanks to the effect of the nano solution, treatments in which 75% NPK doses and micronano foliar fertiliser were applied attained higher yield, higher income, and higher net benefit than the treatments in which 100% NPK doses without nano foliar fertiliser was applied in all four tested provinces. The cost of production per kilogram of grain maize is not much different for treatments with and without the nano application, even in treatments in which nano foliar fertiliser was applied at 75% NPK doses only. This results from the higher cost of spraying three times per season and the high prices of nano foliar fertiliser.

Conclusions and recommendation

Complex micronano particles manufactured by the Institute of Environmental Technology of Vietnam Academy of Science and Technology are effective for maize growth and development in Vietnam. This kind of nano foliar fertiliser helps maize plants better resist abiotic stresses, and attain a faster drying rate during the grain filling period, as well as higher grain weight and higher yields than the control treatments without the application of nano foliar fertiliser. However, it would be advantageous for producers if the use of nano were simpler and the price cheaper.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Melanosis and quality changes of Pacific white shrimps (*Litopenaeus vannamei*) treated with *Houttuynia cordata* extract during cold storage

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Abstract:

Changes in melanosis, microbiology, and fat oxidation in Pacific white shrimps (*Litopenaeus vannamei*) when treated with *Houttuynia cordata* extract (designated E-DC) were monitored during cold storage for seven days at 2°C. Whole shrimps treated with E-DC solution (0.025%, w/v) revealed slow growth of total aerobic microorganisms, *Enterobacteriaceae* and *Pseudomonas aeruginosa*, throughout cold storage in comparison to the control sample treated by water ($p < 0.05$). The changes in pH, grey value, and TBARS value (thiobarbituric acid reactive substances) of shrimp samples treated with 0.025% E-DC solution were lower than those of control samples ($p < 0.05$) and not different significantly in comparison to the 1.25% (w/v) sodium metabisulfite (SMS) samples ($p > 0.05$). These results suggested the potential of using natural compounds from vegetable extraction as a safe and effective alternative for commercial chemical-derived preservatives in shrimp storage.

Keywords: antimicrobial, *Houttuynia cordata*, *Litopenaus vannamei*, melanosis, oxidation.

Classification number: 3.1

Introduction

Pacific white shrimps (*Litopenaeus vannamei*) are an important commercial product and account for 60% of the total shrimp exports in Vietnam [1]. However, shrimps are a highly perishable product because their spoilage begins soon after death and continues in the storage process. The discolouration, melanosis development, oxidation, and microbial spoilage in shrimps are serious problems affecting the organoleptic, nutritional, and economic value of the product [2]. Many studies have focused on preventing melanosis or inhibiting polyphenol oxidase (PPO) using various techniques such as cold or heat treatment or additives [3]. Reducing agents such as sulfiting agents and their derivatives are synthesis chemicals that are widely used for the control of melanosis or browning in the food industry. Melanosis is activated by a biochemical mechanism that oxidizes phenol part in tyrosine to quinones by tyrosinase enzyme [4]. In commerce, sodium metabisulfite (SMS) has been used to retard melanosis development by combining irreversibly with quinones and forming colourless compounds [3]. However, SMS is known to cause allergic reactions and severe disturbances in asthmatic subjects [5]. In recent times, increasing attention has been paid to plant phenolics as potential natural preservatives with antioxidant and antimicrobial activities. Natural antioxidant compounds such as ascorbic acid, citric acid, kojic acid, gallic acid, dodecyl gallate, catechin, and oxalic acid have been substituted for sulfiting agents [2, 3]. Additionally, many studies have reported that extracts prepared from mushrooms and grapeseed can reduce black spots in shrimps [6, 7].

Houttuynia cordata is a well-known, traditionally medicinal material as well as a commonly used spice in

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food. ‘Diếp cá’, ‘lá giấp’, ‘rau giấp’, and ‘giấp cá’ are local names of *H. cordata* in Vietnam. In traditional medicine, *H. cordata* is administered to relieve fever, resolve toxins, reduce swelling, and promote urination [8]. Recently, several studies also provided scientific data about its anti-SARS, anti-inflammatory, anti-allergic, virucidal, antileukemic, anti-oxidant, anti-cancer, and anti-tyrosinase activities [9, 10]. It was noticed that *H. cordata* contains groups of such chemical components as flavones, essential oils, and alkaloids [10-12]. This study aims to investigate the inhibition of melanosis and quality changes of Pacific white shrimps treated with E-DC solution during cold storage to exploit the natural extracts from the spicy vegetables that are rich in phenolic compounds as an initial treatment step in the cold-storage process for shrimps.

Materials and methods

Chemical and raw materials

Malonaldehyde bis (dimethyl acetal) and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (USA). Trichloroacetic acid (TCA) was obtained from Merck (Germany). Other chemicals were of the highest grade available.

H. cordata was collected at supermarkets in July 2018. The voucher samples were preserved at the Department of Food Technology of the Ho Chi Minh city University of Technology and Education.

White shrimps (*L. vannamei*) with the size of 30-40 shrimps/kg were purchased from Thu Duc market, Ho Chi Minh city, Vietnam, in September 2018. The shrimps were kept alive and transported to the laboratory.

Preparation of samples

The dried powder of *H. cordata* leaves (50 g) was extracted with ethanol solvent to yield an ethanol extract via the Soxhlet system. Then, the extract solutions were evaporated under reduced pressure to dry and recover the ethanol extract (E-DC, 15 g).

Preservation of shrimps

At room temperature ($32 \pm 2^\circ\text{C}$), the shrimp (*L. vannamei*) samples were immersed in various solutions, including 0.025% E-DC solution, 1.25% SMS solution, and water (control sample) for five minutes. Then, shrimp samples were taken out and preserved in a plastic box at 2°C . To speed up melanosis development, lipid peroxidation, and bacterial formation, the shrimps were stored at 2°C after

five to seven days. Three shrimps from each treatment were examined in seven days to determine pH, melanosis development, lipid peroxidation, inhibition assays, and microbiological analysis.

pH measurement

The changes in pH values in shrimps during cold storage were determined by the method of Lopez-Caballero with a slight modification [13]. Shrimp meat (2 g) was homogenized by deionized water for one minute, and the homogenate was kept at room temperature for 5 minutes. The pH values in shrimps were measured using a pH-meter (PSI pH 1200, England).

Melanosis evaluation

Fifteen student candidates from the Departments of Chemical Engineering and Food Technology (ages 19-22) were recruited to serve as panellists and were trained carefully in the ability to recognize and describe the common aroma. Control samples and shrimp samples (treated by SMS, E-DC) were evaluated throughout storage and classified according to the level of black-spot development. Grey values in shrimps were detected directly using a modified Montero sensory evaluation and evaluated on the point scale from 0 to 10 based on total surface area of affected shrimps as follows: 0 points = no point; 2 points = light (about 20% of the shrimp's surface affected); 4 points = average (accounting for 20-40% of the shrimp's surface affected); 6 points = significant (40 to 60% of the shrimp's surface affected); 8 points = highly severe (60 to 80% of the shrimp's surface affected); and 10 points = significantly severe (accounting for 80-100% of the shrimp's surface affected) [3].

Lipid peroxidation inhibition assay

Malonaldehyde bis (dimethyl acetal) (MDA) is considered to be the final product of the lipid peroxidation process. MDA forms a pink complex with TBA with the stoichiometry of 1:2, which can be detected at 532 nm using a spectrophotometer (Hitachi UH-530, Japan) [14]. Shrimps were ground by a blender and mixed with 10 ml of 7.5% TCA to produce a mixture. The filtrate was collected and mixed with 0.02 M TBA solution at 100°C for 15 minutes. Absorbance was measured at 532 nm by the spectrophotometer. The lipid peroxidation inhibition ability of the samples was expressed by the TBARS values (thiobarbituric acid reactive substances), which were calculated from a standard curve built at concentrations from 0.01 to 0.05 μM and reported as mg MAD/kg of

shrimp. TBARS values were computed from the mean values of data from three determinations.

Microbiological analysis

Total aerobic microorganisms, *Enterobacteriaceae* and *Pseudomonas aeruginosa*, in shrimp samples after five days preserved at 4°C were determined based on bacterial count method following TCVN 5518-2:2007 (ISO 21528-2:2004) and 3347/2001/QD-BYT at Pasteur Institute in Ho Chi Minh city.

Statistical analysis

Results were calculated to the analysis of variance (ANOVA) whereas the Tukey's test ($p < 0.05$) was applied so that the means could be compared. Statistical calculations were carried out by SPSS 15.0 for Window Evaluation Version (IBM Corporation, USA).

Results and discussion

pH measurement

Changes in pH of white shrimps with and without different treatments during cold storage are illustrated in Fig. 1. The pH values of all shrimp samples increased significantly as the preservation time was extended. On the first day, the pH values of all samples were not significantly different ($p > 0.05$). After seven days of storage, the samples treated with water had the highest pH, followed by those treated with E-DC, and the SMS sample, respectively ($p < 0.05$). At the end of storage (on the seventh day), shrimp samples that were treated with water, 1.25% SMS, and 0.025% E-DC had pH values of 7.96 ± 0.10 , 7.49 ± 0.10 , and 7.43 ± 0.20 , respectively. However, the pH values of the E-DC and SMS samples exhibited no statistical difference ($p > 0.05$). Several reasons could explain the increase in pH value in shrimps during cold storage. It could be due to self-decomposition by endogenous enzymes or it could also be due to the activity of microorganisms that associate with the accumulation of basic compounds leading to a higher pH. Additionally, the hydrolysis of protein produces NH_3 , which is also the cause of growing pH [13, 15]. The lower increase in pH of shrimps treated with E-DC followed the lower microbial count [3, 13]. The pH value is a critical target for shrimp evaluation because it significantly affects the quality of raw shrimps. According to Shamshad (1990), the accepted pH values in shrimps should not exceed 7.6 [16]. Thus, the E-DC sample can be applied to preserve shrimps during cold storage.

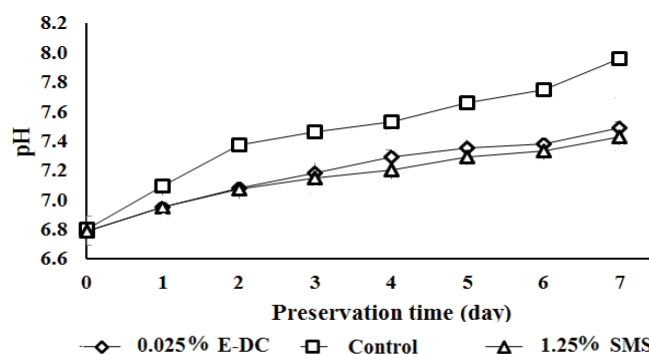


Fig. 1. The changes in pH in shrimps during cold storage at 2°C for seven days. Results are presented as the mean of three determinations (n=3).

Melanosis evaluation

The development of melanosis in shrimp samples is exhibited in Figs. 2 and 3. On day 0, there was no change in all samples (grey value = 0). When storage time increased, the melanosis scores in all samples increased ($p < 0.05$). Shrimp samples treated with SMS and E-DC had grey values lower than the control sample in cold storage and did not display significant difference ($p > 0.05$). In general, grey values occurred significantly after three days of preservation. The considerable differences between the control sample and the E-DC sample suggested that *H. cordata* extract (E-DC) can prevent melanosis development in shrimps during cold processing. In commerce, SMS has been used in shrimps to prevent melanosis development by combining irreversibly with quinones and forming colourless compounds [3]. However, SMS is known to cause allergic reactions and severe disturbances in asthmatic subjects. The ability of *H. cordata* extract to reduce melanosis development can relate to the high content of total polyphenol compounds in this plant. The hydroxyl group can participate in reducing DOPA-chrome to DOPA (L-3,4-dihydroxyphenylalanine), by providing electrons or by cross-linking with PPO via hydrogen bonding [13]. These results suggest the potential of using natural compounds from vegetable extraction as a safe and effective alternative for commercial chemical-derived preservatives in shrimp storage.

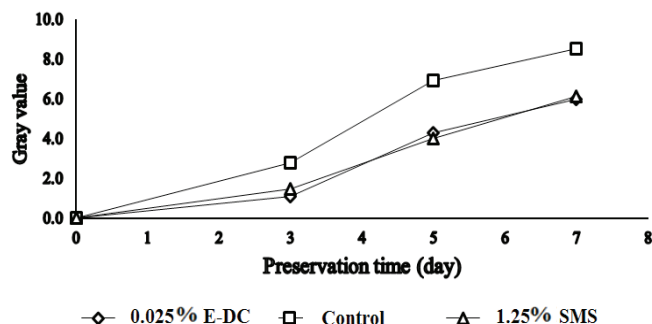


Fig. 2. Changes in the mean grey value in shrimps during cold storage. Results were presented in terms of mean (n=15).

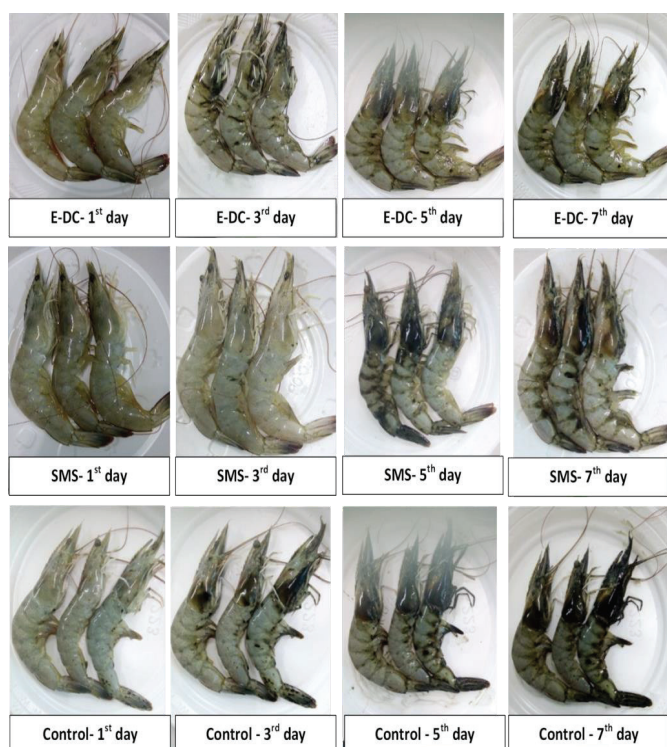


Fig. 3. Development of melanosis in shrimps during cold storage.

Lipid peroxidation inhibition assay

The results of the lipid peroxidation inhibition assay are illustrated in Fig. 4. In general, TBARS values of the SMS sample were the lowest, followed by the E-DC, and the control sample, respectively. During the first two days of preservation, TBARS values did not differ significantly ($p>0.05$). The TBARS values of SMS and E-DC samples were found to be higher from the first day to the fifth day and decreased significantly from the fifth to seventh day of storage at 2°C. Additionally, the TBARS value of the control sample reached a peak value on the fourth day and declined over the next three days. The maximum of the TBARS values after the fifth day for the E-DC sample, the SMS sample, and the control sample were 3.62 ± 0.23 , 3.56 ± 0.24 , and 4.82 ± 0.35 mg MDA/kg of shrimps, respectively. The rising TBARS values (0-5 days) in the first stage were caused by a powerful process of fat oxidation, the product of the fat oxidation such as hydroperoxide that oxidized quickly into secondary products such as aldehyde. Under the effect of enzymes and microorganisms, the secondary oxidation products continue to be converted to all other products, leading to reduced TBARS value [6]. These results revealed that E-DC is able to slow the process oxidation of fat in shrimps during cold processing.

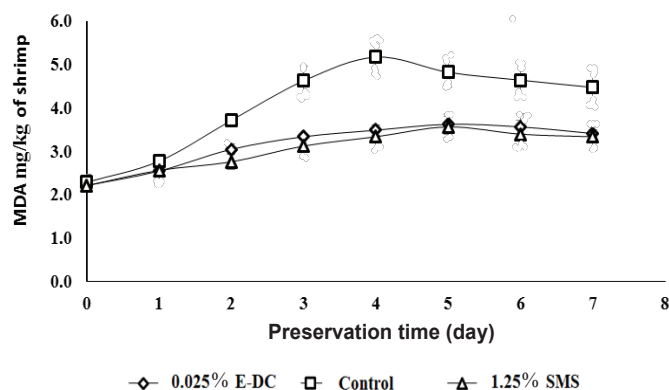


Fig. 4. The TBARS value changes of shrimps during preservation at 2°C for seven days. Results are presented as the mean of three determinations ($n=3$).

Microbiological analysis

Table 1. The result of microbiological analysis of shrimp samples after five days.

Criteria (Cfu/g)	Control		E-DC	
	1 st day	5 th day	1 st day	5 th day
Total aerobic microorganisms	5.7×10^5	1.1×10^6	5.7×10^5	5.8×10^5
<i>Pseudomonas aeruginosa</i>	720	<10	720	<10
<i>Enterobacteriaceae</i>	6000	30	6000	10

<10: not detected.

Results demonstrated that the total aerobic macrobiotic of the control sample (1.1×10^6 Cfu/g) was approximately two times higher than the E-DC sample (5.8×10^5 Cfu/g) after five days (Table 1). Therefore, the shrimps treated with E-DC were able to inhibit the growth of aerobic microorganisms better than those in the control sample. According to TCVN 5289:2006 requirements of frozen seafood, total aerobic bacteria should not exceed 10^6 Cfu/g. Thus, it can be seen that samples treated with E-DC are suitable while the control sample is not acceptable. Additionally, results revealed that the numbers of *P. aeruginosa* and *Enterobacteriaceae* in the E-DC sample were lower than in the SMS sample and declined significantly after five days in the cold preservation. *P. aeruginosa* and *Enterobacteriaceae* are two common pathogenic microorganisms in cryopreservation products [17]. From this, it can be concluded that E-DC can inhibit harmful microorganisms and aerobic bacteria such as *P. aeruginosa* and *Enterobacteriaceae* more effectively than seen in the control samples. Several previous studies have demonstrated that essential oil extracted from *H. cordata* contain antimicrobial compounds such as methyl nonyl ketone, bornyl acetate, myrcene, pinene, acetic acid geraniol ester, camphene, sabinene, decanal, dodecanal, 3-oxo-dodecanal, caryophyllene, limonene, 4-terpineol, terpineol, geraniol, tetradecanoyl-phorbol-acetate, and tetradecanoyl ester [12]. Among them, ketones of methyl

nonyl can inhibit the growth of *Escherichia coli* - an aerobic bacterium often occurs in shrimps. Decanal- β -dicarbonyl compound has also been reported to be a major antimicrobial ingredient in essential oil from *H. cordata* [18].

Conclusions

This study is the first time that a plant was examined for its ability to preserve white shrimps. The ethanolic extract that was prepared from *H. cordata* leaves retarded melanosis development, pH value, and lipid peroxidation, and inhibited harmful microorganisms. Specifically, shrimps treated with E-DC solution (0.025%, w/v) inhibited the growth of the total aerobic microorganisms to two times after five days when compared to the control sample ($p < 0.05$). The development of two pathogenic microorganisms such as *Enterobacteriaceae* and *Pseudomonas aeruginosa* was controlled effectively throughout cold storage at 2°C. The changes in pH, grey value, and TBARS value of shrimp samples treated with 0.025% E-DC solution were lower than those of control samples ($p < 0.05$) and did not differ significantly in comparison to the 1.25% (w/v) SMS samples ($p > 0.05$). The results demonstrated that ethanol extract prepared from *H. cordata* leaves can be used as a natural melanosis inhibitor, an antimicrobial, and an antioxidant agent for cold-stored shrimps. Additionally, the extract from *H. cordata* leaves was applied for the first time as a safe and 'green' additive for shrimp preservation.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Initial characterisation of exosomes released by umbilical cord-derived mesenchymal stem cells and mature dendritic cells, under 'Good Manufacturing Practice' conditions

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Abstract:

Exosomes represent an important mode of intercellular communication and play key roles in many physiological and pathological processes. Exosomes have hitherto exhibited their capacity to modulate biological activities through their carrying of functional molecules such as proteins, lipids, and genetic materials. In the current study, we investigated exosomes released by mature dendritic cells (mDCs) and umbilical cord-derived mesenchymal stem cells (UCMSCs) under Good Manufacturing Practice (GMP) conditions. Ultracentrifugation was used to isolate and purify the exosomes. Additionally, a transmission electron microscope (TEM) and immunoblotting were used to characterise exosomal morphology and markers. The preliminary results showed that both mDCs and UCMSCs secreted exosomes into GMP culture media. Exosomes exhibited a cup-shaped morphology and showed positive for CD63. Additionally, no difference was observed between mDC-derived exosomes and UCMSC-derived exosomes regarding marker expression or morphology. This data indicates the potential for further development of GMP exosomes for clinical application.

Keywords: dendritic cells, exosomes, mesenchymal stem cells.

Classification number: 3.2

Introduction

Research suggests that both normal and tumour cells secrete extracellular vesicles into the extracellular space [1-3]. Extracellular vesicles (EVs) are enclosed by a phospholipid bilayer similar to the cellular membrane, and are known to vary in size. Based on their biogenesis, EVs can be classified into three main classes: (1) apoptotic bodies (1,000-5,000 nm), which are a product of apoptosis upon programmed cell death; (2) microvesicles (100-1,000 nm), formed by direct shedding from the plasma membrane; and (3) exosomes (30-150 nm), which are formed through endocytosis and released into the extracellular milieu through the exocytosis mechanism [1, 3, 4]. Of these, exosomes are the most interesting and best studied in terms of functioning. Exosomes exhibit a cup-shaped morphology under inspection with a transmission electron microscope and are floated at a density of 1.10-1.21 g/ml in a sucrose gradient [5, 6]. During formation, exosomes are enveloped within proteins and genetic materials such as mRNAs, microRNAs, and other non-coding RNAs (ncRNAs) [7]. Such exosomal components can be delivered to both neighbouring and distant cells and modulate the behaviour of recipient cells. The mechanism of component delivery means that EVs, including exosomes, are bioactive and carry a potential for therapeutic use.

Dendritic cells (DCs) arise from progenitor cells in the bone marrow and reside in peripheral tissues in an immature state [8]. Under appropriate stimulation, immature DCs undergo maturation and express stimulatory molecules, secreting cytokines to activate tumour-specific cytotoxic T lymphocytes and B cells [9]. The capacity of DCs to present antigens makes them a candidate of interest in the field of

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cancer immunotherapy, in their potential to stimulate the immune system to eradicate tumours [10]. However, DCs which are generated *in vitro* can be affected by immune-inhibiting factors in the tumour environment [10]. It has been reported that the production of soluble factors by tumour cells might lead to a down-regulation of the entire metabolism of DCs [11].

Moreover, tumour cells secrete cytokines to inhibit tumour antigen presentation by DCs [12]. This can lead to immune tolerance and/or activate regulatory activity, or suppressor T cells, meaning that the immune response then fails to be induced [12]. Thus, the use of DC-derived exosomes in cancer immunotherapy is one way to avoid the inhibition of DC metabolism and DC antigen presentation [13]. Additionally, it has been illustrated that DC-derived exosomes have a longer half-life after injection than *in vivo* DCs [14]. Moreover, DC-derived exosomes are stable for storage for six months at -80°C without being affected either structurally or functionally. This enables them to have a considerable potential for therapeutic use such as DC-derived exosome-based cancer immunotherapy [13].

Mesenchymal stem cells (MSCs) were first discovered in bone marrow in the early 1970s [15]. Currently, MSCs are isolated from a variety of tissues, but the majority are isolated from bone marrow (BM) [16], adipose tissue (AD) [17], dental pulp [18], and the umbilical cord (UC) [19]. MSCs have been shown to differentiate into a number of cell types, thus enabling MSC therapeutics [20]. In fact, the differentiation capacity of MSCs formed the original rationale for their clinical application, with the expectation that they would differentiate and replace damaged cells. However, it is difficult, perhaps impossible, to verify the actual presence of these cells at the site of injury. Thus, the correlation between functional improvement and cell engraftment or differentiation at the site of injury cannot always be recognised. It has been proposed that MSCs express their effects not through their differentiation potential, but rather, through secreted factors, notably enveloped EVs/exosomes [21-23]. Therefore, MSCs could potentially be replaced with MSC-derived EVs/exosomes in order to effect disease treatment.

In the current research, we isolate mDC-derived exosomes and UCMCS-derived exosomes from a GMP cell culture medium, comparing their characteristics in terms of morphology and marker expression.

Materials and methods

Cell lines and cell culture

A tumour cell line A549 (ATCC) was cultured in DMEM (Dulbecco's Modified Eagle's Medium with 1 g/l glucose, L-glutamine, and sodium pyruvate - Corning) with 10% foetal bovine serum and 1% penicillin-streptomycin 100X (Thermo Fisher Scientific, USA), at 37°C, in a humidified atmosphere containing 5% CO₂. The cells were for use as a tumour antigen preparation for loading to DCs.

Tumour antigen extraction from A549 cell lysis and protein quantification

A number of 1×10^7 - 2×10^7 cells were re-suspended in 1 ml phosphate buffered saline (PBS). The cells were then processed in 10 rapid freeze-thaw cycles (in liquid nitrogen) to generate total protein lysate. Trypan blue staining was used to examine live cells. The rate of cell survival was required to reach 0% after the final cycle. Total protein lysate was centrifuged at 2,000xg/10 minutes and the pellets discarded. The collected supernatant was filtered using a 0.2 µm pore filter (Corning, USA). The concentration of purified total protein was determined using a Bradford (Sigma Aldrich) assay, following the manufacturer's instructions. The total protein lysate was stored at -80°C for further use.

Dendritic cell culture and differentiation

Ethical approval for umbilical cord blood collection and cell isolation was obtained from the ethics committee of the Dinh Tien Hoang Institute of Medicine. The pregnant donor was required to be negative for HIV, CMV, HBV, HCV, and TPHA to be selected for umbilical cord blood collection for the research. Umbilical cord blood (UCB) was obtained by venipuncture from the umbilical cord veins immediately after normal full-term delivery, following informed consent. Mononuclear cells (MNCs) were isolated using density gradient separation by Ficoll-Paque/Lymphoprep™ (Stem Cell Technologies, Canada). Monocytes were then isolated by incubating MNCs (2×10^6 cells/ml) with Lymphocyte Serum-Free Medium KBM 551 (Corning, USA) at 37°C for 1.5 hours. Adherent cells (CD14⁺ monocytes) were cultured continuously and floating cells removed. After 24 hours (Day One), 500 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, USA) and 500 U/ml of interleukin 4 (IL-4) (Peprotech, USA) were added to the medium to induce monocyte differentiation into DCs. On Day Three, the culture medium was refreshed with the addition of a half volume of fresh medium with

supplementary cytokines. On Day Five, the tumour antigen extracted from the A549 cell lines was added into the cell culture medium (50 µg tumour antigen/ml medium) in order to load the DCs with the antigen. On Day Six, tumour necrosis alpha (TNFα) 1,000 U/ml was added to the culture medium to induce DC maturation for one day. On Day Seven, the culture medium was harvested for exosome isolation and mDCs were harvested for marker analysis.

Monocyte and dendritic cell marker analysis

Adherent cells (CD14⁺ monocytes) at Day One of the cell culture process and mDCs from Days Six and Seven were collected for cell marker analysis by flow cytometry. Typical surface markers of monocytes (CD14) and DCs (CD40, CD80, CD86, major histocompatibility complex molecule - HLADR, and CD123) were used to examine cell states and phenotypes. Briefly, cells were suspended in phosphate-buffered saline (PBS) and labelled with CD14-PC7, CD40-PE, CD80-PE, CD86-PE, HLADR-FITC (Beckman Coulter, USA), and CD123-APC Vio (Miltenyi Biotech, Germany). Appropriate isotype controls, including IgG1-PE (Beckman Coulter, USA), IgG1-FITC (Beckman Coulter, USA), IgG1-PC7 (Beckman Coulter, USA), IgG1-APC (Beckman Coulter, USA), and IgG1-APC Vio (Miltenyi Biotech, Germany) were included in each experiment. Cell marker analysis was performed using the Flow Cytometry System (Beckman Coulter), equipped with Navios Software.

Umbilical cord-derived MSC culture

Ethical approval for using umbilical cord-derived MSCs was obtained from the ethics committee of Vinmec International General Hospital Joint Stock Company. The umbilical cord (UC) was collected from consenting patients. UCs were cut into 2-3 cm long segments and washed with PBS to discard traces of blood. The cleaned UCs were then minced into small pieces with Collagenase Type I (Gibco, USA) in a gentleMACS™ Dissociator (Miltenyi Biotech, Germany) for 1.5 hours, following which they were washed three times in PBS to remove collagenase and centrifuged to collect pellets. The pellets were transferred into a 25 cm² culture flask (Nunc™ Cell Culture Treated EasYFlasks™, Thermo Fisher Scientific, USA), which had already been coated with a xeno-free substrate (CTS™ CELLstart™ Substrate, Gibco, USA) and which contained a GMP Power Stem medium (Pan Biotech, Germany). The cells were then incubated at 37°C with 5% CO₂ to allow cellular migration from the explants. The culture medium was replaced every

three days. When the cells reached 80% confluence, they were passaged using CTS™ TrypLe™ Select Enzyme (Thermo Fisher Scientific, USA). The passage 1 cells were subsequently seeded in T75 flasks (375,000 cells/flask). The passage 1 UCMSCs were required to reach 80% confluence, at which point the supernatant was harvested for exosome isolation.

Umbilical cord-derived MSC marker analysis

The cells (UCMSCs at passage 1) were harvested following supernatant collection using CTS™ TrypLe™ Select Enzyme (Thermo Fisher Scientific, USA). The UCMSCs then were analysed for markers using the Human MSC Analysis Kit (BD Biosciences, USA), which includes positive markers for human MSCs (CD73, CD90, and CD105) as well as negative markers (CD11b, CD19, CD34, CD45, and HLA-DR). Staining was performed according to the manufacturer's instructions. Markers were detected and analysed using Navios Software equipped with a flow cytometry instrument (Beckman Coulter, USA).

Exosome isolation

The cell culture supernatant (three T75 cell culture flasks for the mDCs and three for the UCMSCs) was centrifuged at 300×g/10 minutes at 4°C to remove cell debris, then at 2,000×g/10 minutes at 4°C to remove apoptotic bodies. The supernatant was then continuously centrifuged at 10,000×g/30 minutes at 4°C to remove microvesicles and at 100,000×g/70 minutes at 4°C (Optima XPN-100 Ultracentrifuge, Beckman Coulter) to obtain exosome pellets. The exosome pellets were washed in PBS and centrifuged again at 100,000×g/70 minutes at 4°C to obtain clean exosomes, which were re-suspended in 50 µl PBS and either used immediately or stored at -80°C for further use.

Analysis of exosome morphology using transmission electron microscopy

Purified exosome pellets were fixed with 4% paraformaldehyde. Following this, a 5 µl drop of the suspension was loaded onto Formvar-carbon coated grids (TED PELLA Inc., CA, USA) and left to dry at room temperature for up to 20 minutes. The grids were subsequently washed with PBS and incubated with 1% glutaraldehyde for five minutes, then washed for 8×2 minutes with distilled water. The sample was then stained with uranyl-oxalate pH 7 for five minutes at room temperature, prior to being incubated with methyl cellulose-uranyl acetate for 10 minutes on ice. Finally, the grids were left

to dry at room temperature and observed with transmission electron microscopy (TEM) at 80 KV (JEM1010-JEOL).

Western blotting analysis

For each sample, exosomal proteins equivalent to 5×10^6 secreted cells were loaded per lane and separated by 4-12% polyacrylamide SDS-PAGE gel (Invitrogen, USA) at 200 V/35 minutes/4°C. Proteins were then transferred to a PVDF membrane (Amersham™) for 200 mA/2 hours at 4°C. The membrane was probed with antibodies to CD63 (Mouse monoclonal IgG, Santa Cruz Biotechnology), followed by incubation with anti-mouse-HRP (Amersham™). Proteins were detected through use of a chemiluminescence substrate (Amersham™) and images captured using ImageQuant LAS 500 (GE Healthcare Life Sciences).

Statistical analysis

A Student's t-test was performed to analyse significance of difference between the mean of the two groups. Error bars indicated $\pm$ SD (Standard Deviation).

Results

Characteristics of morphology and immunophenotype of monocyte-derived DCs

In order to generate mature DCs (mDCs), monocytes were cultured and induced by GM-CSF and IL-4, with observation of immature DCs after four days of monocyte culture. These immature DCs were both adherent and floating cells, expressing some short dendrites (Figs. 1A, 1A1). Mature DCs, which exhibited the specific dendritic cell morphology of long and thin dendrites, were observed at Day Seven (Figs. 1B, 1B1). Analysis of typical monocyte and DC surface markers revealed a significant difference in cell surface marker expression between Day 0 and Day Seven: expression of mDC markers such as CD40, CD80, CD86, and HLA-DR increased markedly at Day Seven of culture. Specifically, CD40 increased from 21.4 to 82.5%, CD80 from 0.42 to 58.7%, CD86 from 16.57 to 74.1%, and HLA-DR from 20.35 to 73.25%. However, expression of CD14, which is the specific marker for monocytes, showed no change between Day 0 and Day Seven. Furthermore, CD123, which is a marker of plasmacytoid DCs, showed very slight expression and did not change between Days 0 and Seven (Fig. 1C). The above information regarding markers and morphology indicates that successful differentiation of monocytes into mDCs occurs after seven days in the acculturation process.

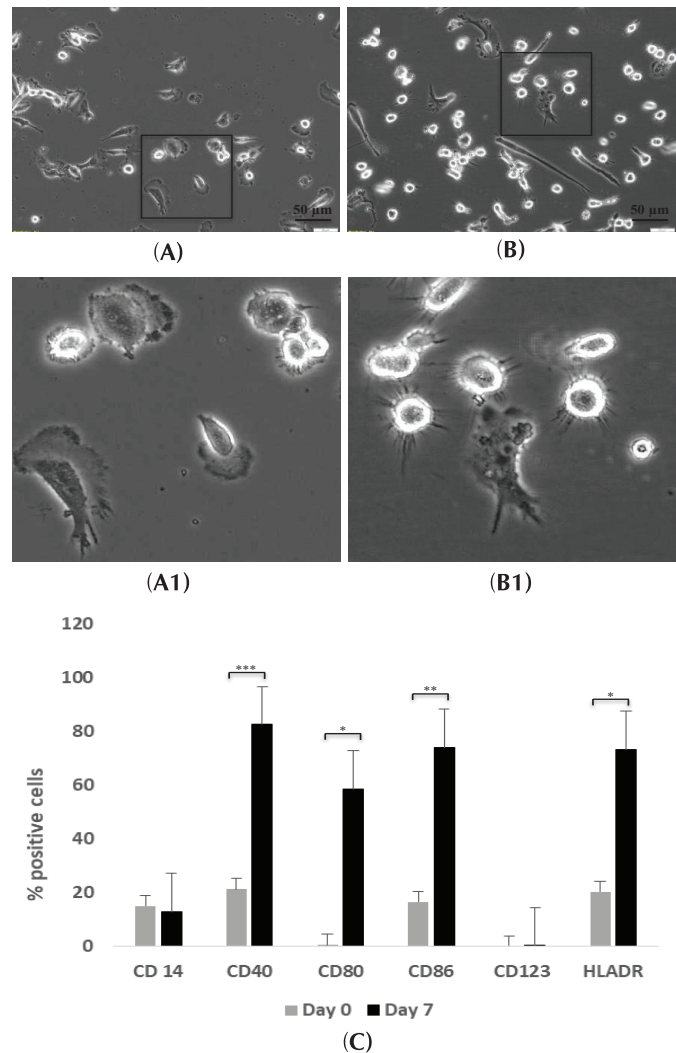


Fig. 1. Morphology and marker expression of immature and mature dendritic cells. (A, and A1) Immature DCs express short dendrites; (B, and B1) mDCs express long and thin dendrites; (C) Immuno-phenotype of monocytes and mDCs: different expression of CD14, CD40, CD86, CD123, and HLADR at Day 0 (monocytes) and Day Seven (mDCs) in cell culture (n=3). *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

Characteristics of morphology and markers of UCMSCs

Cells were captured for morphological analysis at the time point of conditioned media collection. Typical fibroblast morphology of MSCs was observed (Fig. 2A).

After the collection of the conditioned medium for exosome isolation, UCMSCs were harvested for marker analysis. Results showed that UCMSCs under GMP-culture conditions expressed positive markers for MSCs, including CD73, CD90, and CD105, and were negative for CD11b, CD19, CD34, CD45 and HLA-DR at the time point of exosome harvest (Fig. 2B).

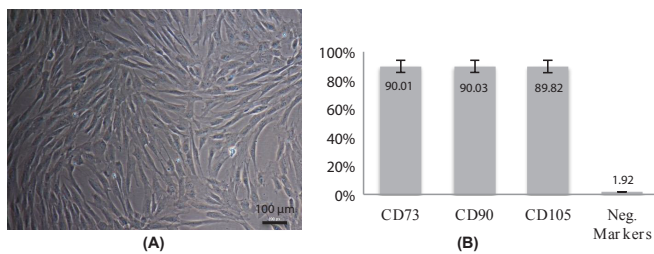


Fig. 2. Morphology and marker expression of UCMSC passage 1 at the time point of conditioned media collection for exosome isolation. (A) UCMSCs at passage 1 adhere to plastic culture flasks and show a fibroblast-like shape; (B) UCMSCs express positive markers of CD73, CD90, and CD105 and do not express MSC negative markers, including CD11b, CD19, CD34, CD45, and HLA-DR.

Exosome characteristics

Exosomes were isolated using conventional differential ultracentrifugation. The isolated exosomes exhibited a cup-shaped morphology under examination with a transmission electron microscope (Figs. 3A, 3B, 3C). It was also noted that exosomes have a size range of 50 nm to 200 nm. There were no differences in morphology or size between UCMSC-derived exosomes and mDC-derived exosomes. The biomolecular analysis of the exosomes revealed that they were expressed with CD63 (Fig. 3D). However, CD 63 was only detected in exosome samples from UCMSCs and mDCs, and not in the parental UCMSCs or mDC lysates. Additionally, there were no differences in CD63 expression between UCMSC-derived and mDC-derived exosomes.

Discussion

Exosomes, which were first observed more than 30 years ago [24, 25], are formed through endocytosis and released into the extracellular environment through the exocytosis mechanism [4]. With recent advances in science and technology, a variety of different techniques and commercial kits have become available for exosome isolation [26]. However, the choice of EV isolation technique depends on sample sources and the downstream application of the EVs [27]. The most common method used to isolate exosomes from cell culture media is differential ultracentrifugation [28]. In the current research, exosomes released by mDCs and UCMSCs were isolated from cell culture supernatant using differential centrifugation [28], allowing for the removal of dead cells, cell debris, aggregated proteins, and other extracellular vesicles (apoptotic bodies and microvesicles) [29]. Data generated from transmission electron microscopy indicated that only the exosome population had been harvested. The exosomes were of typical cup-shaped morphology and were not contaminated with other vesicle populations, which lack this form (Figs. 3A, 3B, and 3C). The cup-shaped morphology is generated by the collapse of the exosomal membrane, due to dehydration during staining [30]. Additionally, there was no contamination by protein aggregation found in the exosome fraction (Figs. 3A, 3B, and 3C). Immunohistochemistry data revealed exosomes expressed CD63 (Fig. 3D), and it is interesting that CD63, which is enriched in exosomes and considered their marker, was detectable only in the exosome fraction and not in

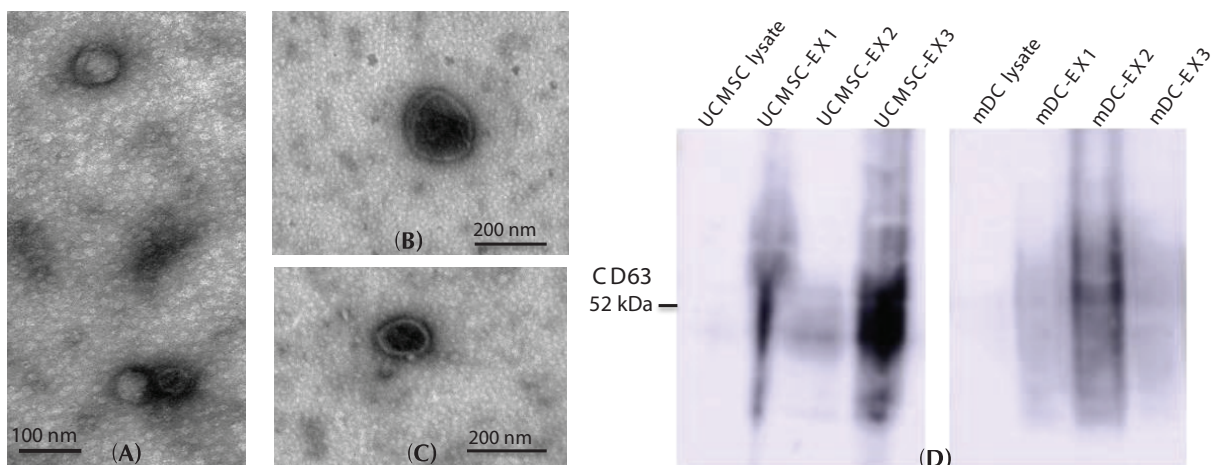


Fig. 3. Exosome morphology and marker expression. (A, B, and C) Exosomes exhibit a cup-shaped morphology and are sized 50 nm to 200 nm under examination with transmission electron microscopy. (A) Exosome representative from mDCs; (B and C) Exosome representatives from UCMSCs; (D) Expression of CD63 exosomal marker. Exosomal proteins were loaded per lane to the equivalent of 5×10^6 parental cells. The same number of cells were lysated and loaded as in the control. Exosomal samples were positive for CD63 but parental cells (UCMSCs and mDCs) were not. UCMSC lysate: parental UCMSC lysate, UCMSC-EX1: UCMSC-derived exosome sample 1, UCMSC-EX2: UCMSC-derived exosome sample 2, UCMSC-EX3: UCMSC-derived exosome sample 3, mDC lysate: parental mature DC lysate, mDC-EX1: mature DC-derived exosome sample 1, mDC-EX2: mature DC-derived exosome sample 2, mDC-EX3: mature DC-derived exosome sample 3.

the cell lysate. This result gives further confirmation that CD63 is highly enriched in exosomes only. These data indicate that differential centrifugation, with the final step of washing exosomes with PBS, is appropriate in harvesting clean exosomes. The washing of exosomes with PBS ensures freedom from contamination with chemicals and other contaminants such as extracellular protein aggregates, which can destroy or alter the functioning of exosomes. This is important information for the future clinical application of exosomes.

The literature suggests that exosomes are released by most cell types both *in vitro* and *in vivo*, including immune cells and stem cells. Importantly, exosomes can deliver their contents to target cells through the mechanisms of ligand/receptor interaction, direct membrane fusion, or internalisation [31]. By such mechanisms of information transmission, exosomes can alter the behaviours of recipient cells. Regarding exosomes released by DCs, Zitvogel, et al. (1998) [32] have reported the novel finding that DCs secrete exosomes bearing functional major histocompatibility complex (MHC) classes I and II, which turned exosomes becoming an antigen presenting agent. Since then, further reports have indicated that DC-derived exosomes might be novel candidates for cell-free vaccines and anti-tumour therapy [13, 33-35]. As examples, DC-derived exosomes could directly activate T cells by presenting a T cell antigen or activate natural killer (NK) cells by presenting the NKG2DL ligand to the NK cell's NKG2D receptor [35]. Additionally, DC-derived exosomes indirectly activate T cells by transferring antigens to DCs or tumour cells, thus turning DCs and tumour cells into antigen-presenters for T cells [35]. Furthermore, human UCMSC-derived exosomes have expressed a capacity for wound-healing via the Wnt-4 pathway [22], as well as for promoting cell proliferation and protection against oxidative stress-induced cell apoptosis, by activation of ERK1/2 and p38 [21]. These exosomes carry a high dose of cytokines such as IL6 and IL8 [21]. Interestingly, human UCMSC-derived exosomes are known to reduce anti-inflammatory factors such as TNF α and IL-1 β but to increase the concentration of anti-inflammatory factor transforming growth factor beta (TGF- β) in the injured brains of Wistar rats [23]. These data indicate that immune regulation may also be an important mechanism in UCMSC-derived exosome tissue regeneration.

Conclusions

Increasing knowledge of exosomes and their capacities provides evidence for their application in disease treatment. This study provides preliminary data on exosomes released by UCMSCs and mDCs under GMP-culture conditions, with the ultimate aim of assessing their clinical application.

Exosomes were isolated successfully from a cell culture supernatant and characterised according to their typical cup-shaped morphology and CD63 marker. Despite the limitations of this study, such as the lack of exosome quantification, exosomes were successfully generated and isolated under serum-free conditions. The exosomes will be further investigated for their role in cellular biological processes in order to further reveal their capacities.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Multiplex polymerase chain reaction (M-PCR) for bacterial vaginosis detection

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Abstract:

Bacterial vaginosis (BV) is one of the most common vaginal infections in women of reproductive age. If not treated promptly, the disease can lead to serious complications affecting the fertility and long-term health of women. Research on BV requires effort from a variety of disciplines and its treatment can only be determined by coordinated actions in research and treatment. Currently, BV diagnostic methods are often based on culture techniques and Gram staining. However, molecular biology research is developing and has proved to more advantageous for identifying key pathogens. Combining both methods, we conducted a study to develop a diagnostic procedure using multiplex polymerase chain reaction (M-PCR) for simultaneous and accurate detection of the bacterial species that cause BV, incurring minimal costs and time for testing.

Keywords: bacterial vaginosis, BV, diagnosis, multiplex PCR, PCR.

Classification numbers: 3.2, 3.5

Introduction

Bacterial vaginosis (BV) is one of the main causes of vaginitis among women of reproductive age (15-44 years) [1]. In most cases, BV causes no complications because it is not harmful when properly treated. Sometimes, it can appear and disappear for no apparent reason. However, untreated BV has been associated with serious health problems that affect women's fertility and long-term health. BV may cause increased susceptibility to sexually transmitted infections in non-pregnant woman [2, 3]. It also increases the risk of pelvic inflammatory disease [4], an infection of the female genital tract that causes the womb, fallopian tubes and ovaries to become swollen, increasing the risk of infertility and ectopic pregnancy [5]. In addition, BV affects patients who undergo assisted reproductive technology as it can reduce the likelihood rate in falling pregnant by in vitro fertilisation (IVF) [6]. Therefore, treatment of and screening for BV is essential, especially for pregnant women and patients undergoing assisted reproduction.

Although BV was first described in 1895, the cause of the BV microbial alteration is still not fully understood. All parts of the body have bacteria, and some are beneficial while others are harmful. When there is an imbalance of naturally occurring bacterial flora, harmful bacteria grow in number, and problems can arise. Determining which organisms are truly pathogenic poses many difficulties because of the complexity and variability of the vaginal microflora. However, unlike a typical infection caused by a specific bacterium agent, BV involves multiple pathogens [7]. This is a limited definition of BV, but it is important to note that BV can be defined as an alteration in the normal vaginal microbial ecosystem. The development of new methods, such as polymerase chain reaction (PCR), to analyse complex bacterial systems that are often difficult to culture enables the discovery of new agents, in addition to the common bacterial species known as *Gardnerella vaginalis*, *Mobiluncus* spp. and *Atopobium vaginae*. Several studies have confirmed that when the population level of

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these organisms drops below a critical level, the harmful bacteria may proliferate to become the dominant species in the vaginal microbial ecosystem [8, 9].

Currently, many BV diagnostic methods are based on culture techniques, such as Amsel's criteria [10] and Gram staining [11]. However, conventional microbiological methods only assess the occurrence of pathogens without precisely identifying each species. The modern approach used in bacterial studies came with the invention of PCR, which led to multiplex PCR (M-PCR), real-time PCR, taxon-directed PCR, broad-range bacterial 16S rDNA PCR and fluorescence in situ hybridization (FISH). These novel methods take advantage of the nature of 16S ribosomal RNA (16S rRNA), a gene that is unique insofar as it is present in almost all bacterial species. Bacterial vaginosis is not caused by one single infectious agent, and exclusion of the responsible agents is difficult in treatment studies. To identify these bacteria, therefore, it is essential to determine their antimicrobial susceptibility pattern.

Single PCR (s-PCR) is used to identify 10 common bacterial species that commonly cause BV: *Gardnerella vaginalis*, *Mobiluncus mulieris*, *Bacteroides fragilis*, *Atopobium vaginae*, *Ureaplasma urealyticum*, *Megasphaera* type I, BVAB 1, BVAB 2, BVAB 3 and *Sneathia sanguinegens*. Polymerase chain reaction primers are designed to target the variable regions of 16S rRNA and allow amplification of the gene in a wide range of different microorganisms. In this study, we establish a clinical method to detect fastidious microorganisms that cause BV using M-PCR. The diagnosis of BV using M-PCR is clinically effective, and the results can be used for treatment selection for patients.

Materials and experimental methods

Sample collection

The study subjects were 10 common bacterial species that cause BV: *Gardnerella vaginalis*, *Mobiluncus mulieris*, *Bacteroides fragilis*, *Atopobium vaginae*, *Ureaplasma urealyticum*, *Megasphaera* type I, BVAB (Clostridia-like BV-associated bacteria) 1, BVAB 2, BVAB 3 and *Sneathia sanguinegens* (Table 1).

The biospecimens collected for this study were vaginal fluids collected via intravaginal tampon from patients of the Modular Center at the Military Medical University and the Assisted Reproductive Health Center at 16A Hospital. These patients have tested as positive with by culture for key pathogens, either for one species or for co-infection with several species.

Materials (Table 2)

Equipment and chemicals used in the study include:

- QIAamp DNA mini kit (QIAGEN, Hilden, Germany);

- GoTaq Green Mastermix 2X (ProOmega, USA);
- 0.5X TBE Buffer, 2% and 3% Agarose gel, ethidium bromide;
- Pipette and pipette tips 1000 µl, 200 µl, 100 µl, 20 µl, 10 µl, Eppendorf tube 1.5 ml, glassware;
- Equipment: Heidich Miko 22R Centrifuge, Rotamixer vortex mixer, Thermomixer shaking, Wealtec E-centrifuge (mini centrifuge machine), PCR cabinet, PCR Fast Thermal Cycles 9800;
- Electrophoresis apparatus: horizontal electrophoresis slab gel apparatus;
- UV and visible light spectrophotometer: Gel Doc™ XR+ System.

Genomic DNA extraction

To collect a sample, the doctor scraped the swab firmly against the surface of each sample more than six times. The swab was kept at room temperature after collection. DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Standardisation of Single PCR technique (Table 3)

Primers: primers were designed based on the specific 16S rRNA specific region of 10 bacterial species, published in NCBI and examined to ensure the optimal conditions for the PCR procedure.

Table 1. Nucleotide sequences of primers used for PCR.

	Symbol	BV agents	Primer (5' - 3')	Amplicon size (bp)
Group G1	BV4	<i>Gardnerella vaginalis</i>	TTACTGGTGTATCACTGTAA CCGTCACAGGCTGAACAGT	330
	BV5	<i>Megasphaera</i> type I	GATGCCAACAGTATCCGTCGG CCTCTCCGACACTCAAGTTCGA	211
	BV6	<i>Bacteroides fragilis</i>	TTCGCTTTTCTGTTTCTGTGT CAGCAACCACCAACATTATT	842
	BV7	<i>Atopobium vaginae</i>	TAGGTCAGGAGTTAAATCTG TCATGGCCAGAAGACCGCC	155
Group G2	BV2	<i>Ureaplasma urealyticum</i>	AGAAGACGTTTAGCTAGAGG ACGACGTCCATAAGCAACT	541
	BV8	BVAB 1	GGAGTGTAGGCGGCACTA CTCTCCGATACCTCCAGCTCTA	90
	BV9	BVAB 2	TTAACCTTGGGGTTTATTACAA GAATACTTATGTGTTAACTGCGC	260
Group G3	BV10	BVAB 3	CATTAGTTGGGCACTCAGGC ACATTTGGGGATTGTCTTCGCC	160
	BV12	<i>Mobiluncus mulieris</i>	ATGGATATGCGTGTGGATGG CCAGGCATGTAAGCCAAA	80
	BV13	<i>Sneathia sanguinegens</i>	AATTATTGGGCTTAAAGGGCATC AGTACTCTAGTTATACAGTTTGTAG	102

Table 2. Components for single PCR.

Component	Volume
GoTaq Green Mastermix 2X	6.25 µl
Deionized water	3.75 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
DNA template	2 µl
Total	12.5 µl

Table 3. PCR thermal cycle.

Temperature	Time	Number of cycles
95°C	5 min	1
94°C	45 sec	40
Optimised temperature range	45 sec	
72°C	45 sec	
72°C	10 min	1
4°C	∞	1

To check the specificity of primers and PCR, s-PCR products were electrophoresed in 2% agarose gel for 30 minutes at 110V, then stained with ethidium bromide for 10 minutes and screened under UV light. After checking all primers, we proceeded to develop multiplex PCR to simultaneously identify 10 bacterial species.

Optimisation of the multiplex PCR technique

Multiplex PCR was used to simultaneously detect 10 BV agents. Using the annealing temperatures of standardized s-PCR, we used multiplex primer pairs in the same reaction tube with the same concentration. We then adjusted the concentration gradient by increasing the concentration of weakly active pairs and reducing the concentration of active pairs based on the amplified signal strength of the PCR products in agarose gel.

The M-PCR products were electrophoresed in 3% agarose gel for 45 minutes at 110V, then stained with ethidium bromide for 10 minutes and screened under UV light.

Results and discussion

Standardisation of the single PCR technique

In this study, s-PCR was used to check the specificity of identification of 10 BV agents. After electrophoresis, the optimum annealing temperatures determined were 55°C (Group G1 and group G2) and 63°C (Group G3).

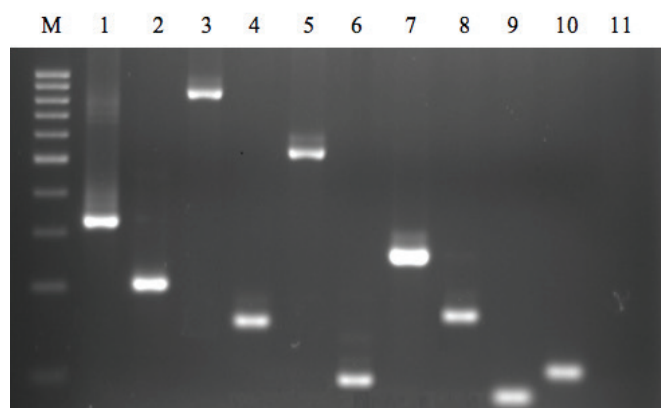


Fig. 1. Electrophoretic analysis of the amplified fragments using s-PCR in 2% agarose gel. Lane M: 100 bp DNA ladder; lane 1: *Gardnerella vaginalis* - BV4 (330 bp); lane 2: *Megasphaera* type 1 - BV5 (211 bp); lane 3: *Bacteroides fragilis* - BV6 (842 bp); lane 4: *Atopobium vaginae* - BV7 (155 bp); lane 5: *Ureaplasma urealyticum* - BV2 (541 bp); lane 6: BVAB 1 - BV8 (90 bp); lane 7: BVAB 2 - BV9 (260 bp); lane 8: BVAB 3 - BV10 (160 bp); lane 9: *Mobiluncus mulieris* - BV12 (80 bp); lane 10: *Sneathia sanguinegens* - BV13 (102 bp); lane 11: negative control.

The results were obtained as a single band corresponding to each bacterial species without by-products, and the gel electrophoresis image was very clear. Therefore, we decided to set the annealing temperature for G1 and G2 at 55°C and for G3 at 63°C.

Currently, there are many traditional methods for determining the presence of BV agents. These include culture, Amsel's criteria, Gram staining and molecular biology techniques. Each method has its advantages and disadvantages. Culture is the classic method but has high requirements regarding the conditions of sample collection and preservation. In addition, some anaerobic species cannot be identified by traditional methods and must be studied using modern molecular biology techniques. With single PCR, we identified 10 common BV agents: BV4, BV5, BV6, BV7, BV2, BV8, BV9, BV10, BV12 and BV13 (Fig. 1). Molecular biology research is developing and has proved advantageous for identifying key pathogens. This successful provides doctors with a basis for treatment with appropriate antibiotics for each pathogen and improves efficiency in testing as well as treatment.

Optimisation of the multiplex PCR technique

After determining the optimum annealing temperature for G1 and G2 at 55°C and for G3 at 63°C, we used multiplex primer pairs in the same reaction tube with the same concentration, then adjusted the concentration of each primer pair for M-PCR (Table 4).

Initially, 10 pairs of primers were divided into three groups based on annealing temperature, product size and

product signal strength. Specifically, the assay G1 consisted of BV4, BV5, BV6, BV7; assay G2 consisted of BV2, BV8, BV9 and assay G3 consisted of BV10, BV12, BV13.

Table 4. Multiplex PCR thermal cycle.

Temperature	Time	Number of cycles
95°C	5 min	1
94°C	45 sec	40
55°C (assay G1 and G2) 63°C (assay G3)	45 sec	
72°C	45 sec	
72°C	10 min	1
4°C	∞	1

Based on the result of single PCR procedure, we adjusted the concentration of all primers by comparing to s-PCR products amplicon band combined with the signal strength of each primer pair. We chose the following concentrations as the optimum concentrations (Table 5).

Table 5. Primer concentrations in multiplex PCR.

	BV agents	Primers concentration	Annealing temperature
Assay G1	BV4	0.3 µl	55°C
	BV5	0.2 µl	
	BV6	0.3 µl	
	BV7	0.2 µl	
Assay G2	BV2	0.3 µl	55°C
	BV8	0.2 µl	
	BV9	0.2 µl	
Assay G3	BV10	0.5 µl	63°C
	BV12	0.2 µl	
	BV13	0.5 µl	

In a recent study by Tosheva-Daskalova, et al. [12], three primers were used for the M-PCR reaction to detect three pathogenic bacteria, *Gardnerella vaginalis* - BV4, *Atopobium vaginae* - BV7 and *Mobiluncus* spp. However, in this study, we used four pairs of primers in assay G1 to detect BV4, BV5, BV6 and BV7. The results showed that this assay simultaneously detected more than two pathogens BV4 and BV7 but still ensured the accuracy and quality of the electrophoretic band, resulting in higher test efficiency (Figs. 2-4).

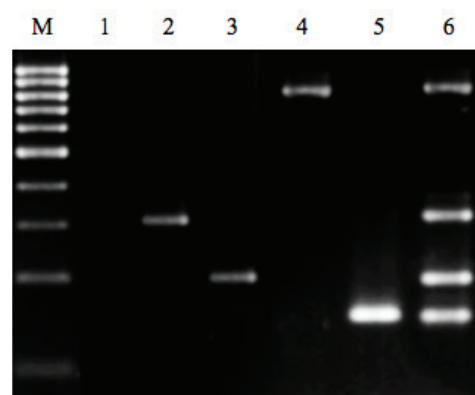


Fig. 2. Electrophoretic analysis of assay G1 in 3% agarose gel. Lane M: 100 bp DNA ladder; lane 1: negative control; lane 2: *Gardnerella vaginalis* - BV4 (330 bp); lane 3: *Megasphaera* type 1 - BV5 (211 bp); lane 4: *Bacteroides fragilis* - BV6 (842 bp); lane 5: *Atopobium vaginae* - BV7 (155 bp); lane 6: group G1.

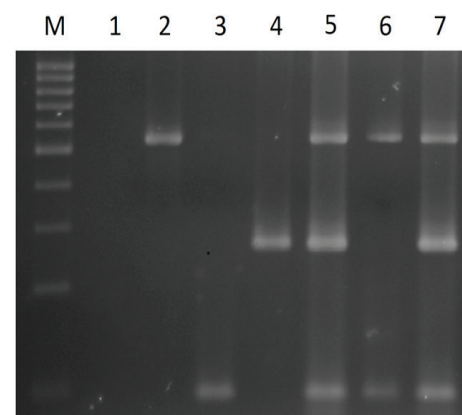


Fig. 3. Electrophoretic analysis of assay G2 in 3% agarose gel. Lane M: 100 bp DNA ladder; lane 1: negative control; lane 2: *Ureaplasma urealyticum* - BV2 (541 bp); lane 3: BVAB 1 - BV8 (90 bp); lane 4: BVAB 2 - BV9 (260 bp); lane 5: group G2.

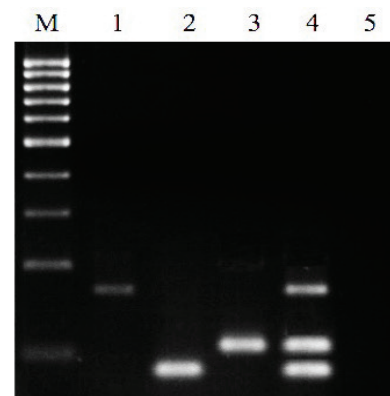


Fig. 4. Electrophoretic analysis of assay G3 in 3% agarose gel. Lane M: 100 bp DNA ladder; lane 1: BVAB 3 - BV10 (160 bp); lane 2: *Mobiluncus mulieris* - BV12 (80 bp); lane 3: *Sneathia sanguinegens* - BV13 (102 bp); lane 4: Group G3, lane 5: negative control.

The M-PCR reaction resulted in a separable band, as in s-PCR, without cross-reactivity between primer pairs. Clinical trials on the biospecimens showed the M-PCR products were successful in identifying bacterial species and can be used to test for BV caused by one species or co-infection with several species. Thus, this research has successfully developed the multiplex PCR process to simultaneously detect 10 common bacterial species that cause BV.

It is not possible to completely replace the classic diagnosis methods. However, this process has many advantages, especially its ability to accurately and simultaneously identify many pathogens as a basis for effective treatment. Besides, about the storage, samples used in PCR only require cold storage, so the conditions are not as strict as those for culture or Gram staining. In the near future, multiplex PCR can be expected to change the way medical laboratories analyse BV samples. This would lead to earlier diagnosis and prevention of possible complications in certain women without the impediments of high cost, long assay times and difficulties in workflow.

Conclusions and future plan

Conclusions

The study has established the success of the multiplex PCR procedure in the simultaneous detection of 10 common bacterial species that cause BV.

It has applied multiplex PCR as a clinical method for diagnosing BV in certain patients and has shown positive results.

Future plan

We plan to continue to optimise the process to create a database for future research on diagnosing BV.

We will use a larger sample size to increase the statistical significance and credibility of the study results.

The authors declare that there is no conflict of interest regarding the publication of this article.

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Two newly recorded species of Urticaceae among the flora of Vietnam used as green foods for H'mông beef cattle

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Abstract:

Oreocnide kwangsiensis Hand.-Mazz. and *Boehmeria japonica* (L. f.) Miq. (Urticaceae) are newly reported among the flora of Vietnam that are used as green foods for beef cattle by local H'Mong people in northern Vietnam in the winter-spring season. The authors provide descriptions, illustrations, notes on utilisation, and a diagnostic comparison of these species with ones that are morphologically similar.

Keywords: *Boehmeria japonica*, green foods, new record, *Oreocnide kwangsiensis*, Vietnam.

Classification number: 3.4

Introduction

Urticaceae Juss. comprises around 2,000 species and is grouped into 54 genera. The family is widely distributed in tropical regions, but also extends to temperate regions [1, 2].

Southern China and northern Vietnam, where high concentrations of limestone karst occur, is considered a biodiversity hotspot [3]. Due to their similar habitats, these two areas share many similar species, among them Urticaceae [4, 5]. Recent investigations in northern Vietnam have repeatedly reported dozens of new records of Urticaceae in the country although its original range of distribution is southern China [6-10].

Urticaceae appears to be of significant economic use, such as for fibre products, traditional folk medicine, food for local people, and feed for grazing [4]. During our extensive fieldwork in northern Vietnam in recent years, we noticed that local H'Mong people graze beef cattle by feeding two hitherto unknown Urticaceae species in the winter-spring season. After a comparison with specimens in major herbaria, such as HITBC, HN, HNU, IBK, IBSC, K, KUN, L, MO, NIMM, NY, P, PE, and VNMN (herbarium code according to <http://sweetgum.nybg.org/science/ih/>), and having consulting the related taxonomic literature dealing with the flora of Vietnam and neighbouring regions [4, 5], we confirm that these two species are *Oreocnide kwangsiensis* Hand.-Mazz. and *Boehmeria japonica* (L. f.) Miq., which have not yet previously been reported in Vietnam [5]. Reporting them as new records in this paper provides useful data for compiling the Flora of Vietnam and for their further economic use.

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Taxonomic treatment

1. *Oreocnide kwangsiensis* Hand.-Mazz., Sinensia 2: 2. 1931; Chen, et al. in Fl. China 5: 184. 2003 - Chéo béo Quảng Tây (in Vietnamese) (Figs. 1A, 1B).

Type: China. Kwangsi: Lin-yen bor.-occ.: Binglu, 800 m., 2 August 1928, R.C. Ching 6630 (holotype: PE, isotype: PE).

Description: shrubs, 1-1.5 m tall. Leaf blade narrowly elliptic or elliptic-lanceolate, 2-11×1-4 cm, stiffly papery, glabrous, base broadly cuneate or subround, apex acuminate or caudate-acuminate, margin entire. Basal veins 3, lateral pair reaching to apex, secondary veins indistinct, 2-3 pairs. Petiole 0.5-2 cm long. Stipules lanceolate, 3-4 mm long, sparsely pubescent. Pistillate inflorescences borne on axils of both young and older branches, often dichotomously branched three times, 5-8 mm long; glomerules 3-4 mm in diam., staminate flower sessile, 1 mm in diam., tepal 3, stamen 3. Pistillate flower coniform, ca. 1.5 mm long. Achene black, ovoid, compressed, 1.5-2 mm.

Ecology: *Oreocnide kwangsiensis* grows on the edges of secondary forest or shrubbery on limestone.

Distribution: China, new to Vietnam.

Specimen examined: Vietnam. Cao Bang: Ha Quang, Ha Thon, 5 March 2016, Do Van Truong, et al., T_CB 21 (VNMN). Ha Giang: Hoang Su Phi, Nam Ty, 22 March 2018, Do Van Truong, et al., VNMN_CIB 168 (VNMN).

Discussion: *Oreocnide kwangsiensis* was a species endemic to China. The type specimen was collected from Guangxi province, China where bordered with northern Vietnam [4]. Due to the similarity of habitats, it is also found in northern Vietnam. This species is morphologically most similar to *Oreocnide trinervis* Miq. from which it can be distinguished by the leaf shape and leaf surface morphology, as summarised in Table 1.

Table 1. Diagnostic comparison of *O. kwangsiensis* and *O. trinervis* [4].

Characteristics	<i>O. kwangsiensis</i>	<i>O. trinervis</i>
Leaf shape	Narrowly elliptic or elliptic-lanceolate	Broadly ovate or oblong-ovate
Leaf surface	Glabrous on both surfaces	Pubescent abaxially

Oreocnide kwangsiensis is popularly used for raising and fattening H'mong beef cattle. In a recent study, Hoang, et al. (2017) [11] determined the nutritional value of *Oreocnide kwangsiensis* and some other plant species by means of the *in vitro* gas production method. The results show that the organic matter digestibility and total digestible nutrients of *O. kwangsiensis* is higher than that of the other species studied. Furthermore, the crude protein values of *O. kwangsiensis* are higher than that of some conventional feedstuffs, such as elephant grass and corn stover [11, 12]. Hence, this new finding will significantly contribute to developing indigenous fodder crops for raising and fattening H'mong beef cattle, especially in the winter-spring season.

Conservation status: *Oreocnide kwangsiensis* is known from more than 120 collections in a wide range of habitats in many limestone locations in North-Western Guangxi and Southern Guizhou in China and Northern Vietnam. Therefore, this species has been assessed as being of least concern.

2. *Boehmeria japonica* (L. f.) Miq., Ann. Mus. Bot. Lugduno-Batavi 3: 131. 1867; Chen, et al. in Fl. China 5: 172. 2003 - Gai lá nhám (in Vietnamese) (Figs. 1C, 1D).

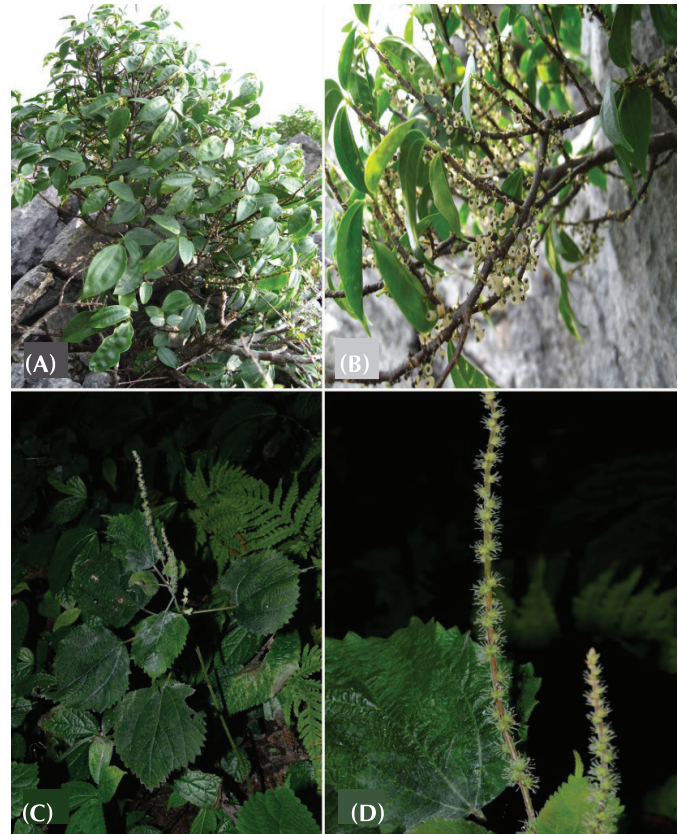


Fig. 1. Photographs of *Oreocnide kwangsiensis* (A, B) and *Boehmeria japonica* (L. f.) Miq. (C, D) (all photos taken by Fu Long-fei).

Table 2. Diagnostic comparison of *Boehmeria japonica*, *B. tricuspis*, *B. silvestrii*, *B. spicata*, and *B. allophylla* [4, 5, 13, 14].

Characteristics	<i>B. japonica</i>	<i>B. tricuspis</i>	<i>B. silvestrii</i>	<i>B. spicata</i>	<i>B. allophylla</i>
Leaf texture	Papery	Papery	Herbaceous	Herbaceous	Herbaceous
Leaf shape	Ovate or broadly ovate	Oblate to 5-angled or oblate to orbicular-ovate	5-angled or orbicular-ovate	Ovate-rhombic or rhombic	Elliptic
Leaf margin	Coarsely 7-14-dentate	Coarsely 8-12-dentate	8-10-dentate	Coarsely 3-9(-13)-dentate	Minutely serrulate

Type: Japan. *Thunberg s.n.* (LINN. no. 1456-5, lectotype selected by Yahara 1984: 133).

Description: subshrubs or perennial herbs, 0.6-1.5 m tall; upper stems and branchlets often densely appressed or patent strigose. Leaves opposite, equal or subequal in size, suborbicular, orbicular-ovate or ovate, 7-17(-26)×5.5-13(-20) cm, papery, adaxial surface roughish, strigillose, abaxial pubescent; base broadly cuneate or truncate, margin coarsely 7-14-dentate, teeth 6-20 mm long; apex cuspidate, sometimes inconspicuously tricuspidate. Petiole 6-8 cm long, appressed or patent strigose. Stipules lanceolate, 0.8-1.2 mm long. Spiciform inflorescence borne on unbranched axillary, or sometimes sparse branches; staminate one 3-15 cm long; pistillate one 7-20(-30) cm long. Staminate flowers 4-merous, sessile; pistillate flower: tepal 1-1.2 mm long. Achene obovoid, ca. 1 mm long, smooth.

Ecology: *Boehmeria japonica* grows in moist and humid limestone sites.

Distribution: China, Japan, and new to Vietnam.

Specimen examined: Vietnam. Cao Bang: Ha Quang, Ha Thon, 5 March 2016, Do Van Truong, et al., T_CB 12 (VNMN).

Discussion: *Boehmeria japonica* is most similar to *B. tricuspis* (Hance) Makino, *B. silvestrii* (Pamp.) W.T. Wang, *B. spicata* Thunb. and *B. allophylla* W.T. Wang. Despite most of these species being widespread in China, none have previously been reported in Vietnam [5]. Observation of morphological characteristics show that the leaf margin of some specimens collected from northern Vietnam has about 14 dentations, which matches very well with *B. japonica*, while others have less than 14 dentations. The detailed

distinguishing characteristics of *B. japonica* and its relatives are summarised in Table 2.

The fibres of *B. japonica* are widely used to make ropes and cloth, while its leaves are used agriculturally as indigenous fodder crops [4]. Our recent finding shows that *B. japonica* is frequently used for raising and fattening H'mông beef cattle during the winter-spring season in northern Vietnam. This new recording of this species in Vietnam will enable local people to develop more choices for its further economic use.

Conservation status: this species has been assessed as being of least concern [13].

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The authors declare that they have no conflict of interest regarding the publication of this article.

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Cultivation of *Haematococcus pluvialis* for astaxanthin production on angled bench-scale and large-scale biofilm-based photobioreactors

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Abstract:

The green microalga, *Haematococcus pluvialis*, is currently cultivated for natural astaxanthin in suspended systems. Immobilised cultivation in a twin-layer (TL) porous substrate bioreactor is a potential revolution in microalgal biotechnology worldwide. For the first time in Vietnam, small-scale (0.05 m²) and large-scale (2 m²) biofilm-based photobioreactor systems arranged at an angle of 15° were successfully designed, assembled, and operated; the temperature, humidity, air, and light conditions for *H. pluvialis* cultivation were successfully controlled. Studies were conducted of both systems to determine the optimal storage time of algae after harvest from suspension before inoculation into the TL system, carbon dioxide supply method, light intensity, and initial cell density. In the 0.05 m² and 2 m² systems, dry biomass productivity reached 12 g m⁻² d⁻¹ (3% astaxanthin content in the dry biomass) and 11.25 g m⁻² d⁻¹ (2.8% astaxanthin) after 10 days of cultivation. The 2 m² biofilm-based photobioreactor system provides many advantages in scaling up astaxanthin production from *H. pluvialis*.

Keywords: astaxanthin production, biofilm-based photobioreactor, *Haematococcus pluvialis*, twin-layer porous, twin-layer system.

Classification number: 3.5

Astaxanthin from *H. pluvialis* and algae suspended cultivation for astaxanthin harvest

Astaxanthin is a keto-carotenoid that is mainly used as a supplementary pigment in feedstock for salmon and shrimp cultivation feedstock; it is sometimes also applied in poultry farming to implant colouration in egg yolks [1]. Recent studies have shown the strong anti-oxidant activity of astaxanthin in a rat model [2] with benefits to the immune system, cardiac muscles, reducing risks of various cancers, and human skin-ageing treatments [3-8].

The green alga *H. pluvialis* is the most common natural astaxanthin producer at the commercial scale. This alga species is able to accumulate astaxanthin pigment up to 5.9% of its dry biomass [1, 9, 10]. The *H. pluvialis* life cycle includes one biflagellate green cell stage, one non-motile green cell (palmella) stage, and one thick-walled cyst (akinetete) stage (Fig. 1). Changes in cell states are driven by environmental conditions. The most notable life-history stage of *H. pluvialis* is the cyst-forming period with its distinctive cell enlargement and increase of astaxanthin production which causes the change in algal color from green to red [11].

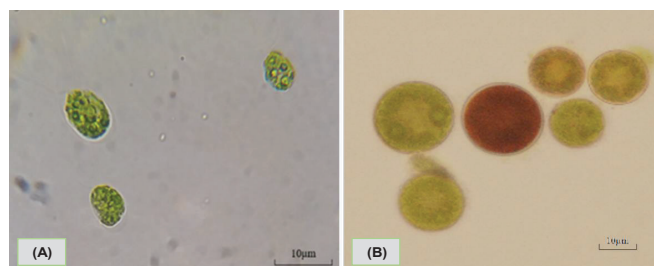


Fig. 1. Microscope image of different *H. pluvialis* life stages: (A) Two-flagellated cells; (B) Immobilized green cells and thickened wall red cysts (x40).

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To attain maximal astaxanthin production, *H. pluvialis* is mainly cultured in two-phase cultivation systems. The first phase, known as the green phase or growth phase, is optimised for vegetative growth to achieve a high cell density. In suspended cultivation, a maximum light intensity of $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ should not be exceeded in order to maintain cell growth and divisions, and environmental parameters such as temperature, carbon dioxide (CO_2) levels, and pH need to be closely monitored [1, 12]. As the required biomass is attained, the second phase, known as the stressed or red phase, is switched on to stimulate astaxanthin accumulation [1, 12].

In the two-phase system, each growth phase requires different cultivation conditions and technologies, high energy consumption, and prolonged cultivation time [13, 14].

Currently, suspended cultivation of *H. pluvialis* is more common for the production of astaxanthin at the commercial scale. Suspended cultivation is applied in open ponds or closed photobioreactors. Open-pond cultivation is utilised only for the stressed phase with a short cultivation time (4-6 days) to minimise contamination and apply stressed conditions [12]. The closed photobioreactor can minimise contamination and control culture parameters better but it has drawbacks such as of high assembly and maintenance cost [15-17]. Moreover, suspended systems have very low biomass concentration (0.05-0.06% of cultivated liquid) and the harvest of algae thus demands additional costs of energy and labour [18].

Previous studies of astaxanthin accumulation in *H. pluvialis* in Vietnam: studies of *H. pluvialis* and astaxanthin production in Vietnam have just been conducted since 2010. The Institute of Biotechnology (Vietnam) managed to select one *H. pluvialis* HB strain (own isolate) with a high astaxanthin accumulation capability (4.8% in dry biomass). This strain's favourable growth conditions include RM culture medium [19], a temperature of 25°C , light intensity of $30 \mu\text{mol photon m}^{-2} \text{s}^{-1}$, and nitrate as a nitrogen source [20]. A maximum cell density of $4.02 \times 10^6 \text{ cells ml}^{-1}$ was obtained by increasing the nitrate concentration in the RM medium four-fold and switching the light cycle from 12 light/12 dark hours to 16 light/8 dark hours with nutrient supply by exchange of the culture medium [21, 22].

To stimulate astaxanthin accumulation, other than the limited nutrient condition, it is important to note that the carbon source is a limiting factor in *H. pluvialis* astaxanthin synthesis [23]. With supplementation with 100 mM bicarbonate, the HB strain switched to the cyst stage

within 3 days and accumulated astaxanthin amounting to 3.96% in the dry biomass [23]; however, this experiment was only conducted at the scale of a 500 ml conical flask containing 350 ml algae liquid cultivated in two separate phases, with sedimentation by gravity and centrifugation to harvest the algal biomass. Cultivation at the 10 l scale resulted in an increase in cell density ($4.12 \times 10^6 \text{ cells ml}^{-1}$) though astaxanthin synthesis at this scale has not been investigated [23].

Trinh, et al. (2017) [24] recently conducted a study using two-phase suspended cultivation. In the algal growth phase, the algal cell density increased by only 3.5 times (from an initial density of $2.10^5 \text{ cells ml}^{-1}$) after 18 days of cultivation. In the astaxanthin synthesis induction phase in a 5 l culture medium bioreactor, cell density did not increase after 10 days of cultivation and the astaxanthin content was very low ($194 \mu\text{g l}^{-1}$).

At a larger scale, there are studies using two-phase suspended cultivation in closed systems of 26, 50, and 100 l with a long cultivation period (~25 days) and a relatively complicated process involving multiple centrifugations to increase algal density and exchange the culture medium [21, 22]. In the 50 and 100 l systems, the cell density did not improve significantly and there was no report of the astaxanthin content in the dry biomass.

Immobilised cultivation of *H. pluvialis* in a vertical TL biofilm photobioreactor

The TL biofilm photobioreactor was invented by Melkonian and coworkers in Cologne [25, 26] for microalgae biomass cultivation. This system is able to hold eight twin-layered modular units (each with a ground size of 1 m^2). The algae growth area is $2 \times 0.67 \text{ m}^2$ for both sides in one unit [27]. The twin-layered structure includes one layer of vertically arranged non-woven glass fiber (80 g m^{-2} , Isola AS Eidanger, Norway) attached to source layers to maintain a continuous medium flow by means of gravity with a flow rate of $6\text{-}10 \text{ l h}^{-1} \text{ m}^{-2}$ using an agriculture drip-irrigation system (Netafim, Frankfurt, Germany) operating at a maximum pressure of 0.8 bar. The prepared culture medium (80-100 l) is stored in closed containers or reservoirs and is distributed all over eight twin-layered structures by two independent pumps (gamma/5b, ProMinent Dosierttechnik GmbH, Germany). After flowing through all these structures, the medium is collected below and directed back into the reservoirs. The medium is exchanged once after 6 days [27].

Above the source layer a substrate layer is attached by self-adhesion (both layers are hydrophilic). The substrate layer can be made of common printing paper ($45\text{-}60 \text{ g m}^{-2}$,

for instance ‘Kölner Stadt-Anzeiger’, Dumont Schauberg, Cologne, Germany) and is used as carrying agent to immobilise algal cells. This substrate layer prevents cells from infiltrating the culture medium and source layer but allows the source layer to control the growth of the immobilised biomass via diffusion of the culture medium [26]. Before inoculation into the TL system, algal cells are harvested from the liquid medium by centrifugation at 1,000 g. The suspended liquid is inoculated into substrate layers using a paint roller at the density of 2 g dried biomass m^{-2} . The roller is also used to transfer algae from one TL module to another [27].

The TL system has been used to cultivate various algal species, including *H. pluvialis* [10, 25, 28-30]. These studies have investigated the influence of many parameters such as the inoculum temperature, light intensity, and nutrient concentration on the immobilised cultivation of *H. pluvialis*; however, these studies were limited by continuous illumination at a maximum intensity of 230 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. The immobilised cultivation in these studies was applied the stressed phase of *H. pluvialis* and not to the whole cultivation process, including cell multiplication [10, 28, 30, 31].

The TL photobioreactor has recently shown a great promise, achieving production of both biomass and astaxanthin of *H. pluvialis* in only a one-phase system at high light intensity was achieved in a TL photobioreactor recently [32]. The algae were cultivated under light intensities ranging from 20 to 1,015 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ with 1-10% CO_2 added in the gas phase. Dried biomass production reached 19.4 $\text{g m}^{-2} \text{d}^{-1}$ and the final dry biomass, 213 g m^{-2} , after 16 days of cultivation. During the whole process, the astaxanthin content increased with light intensity and astaxanthin production reached 0.39 $\text{g m}^{-2} \text{d}^{-1}$, with a final amount of astaxanthin of 3.4 g m^{-2} . The astaxanthin content was 2.5% in the dry biomass. In comparison with two-phase cultivation using the same TL photobioreactor, one-phase cultivation provided a similar amount of total astaxanthin with half of the cultivation time. It was also more convenient than two-phase suspended cultivation [32].

Until recently, immobilised cultivation using the TL system included two set-ups: a bench-scale system and a pilot system. Both systems are vertically oriented which increased aerial efficiency eight-fold. However, the productivity in each unit decreases as mutual shading by the modules decreases the light intensity inside each unit; the investment, maintenance, and harvesting costs also increase per module [27].

Immobilised cultivation of *H. pluvialis* on angled a TL biofilm-based photobioreactor for astaxanthin production in Vietnam

The use of a vertical biofilm-based photobioreactor for *H. pluvialis* immobilised cultivation in Vietnam involves several difficulties, including higher investment and maintenance costs and the unavailability of several materials (stable non-woven fiberglass and high quality paper) in Vietnam. Hanging the modules vertically requires the membranes to be strong enough to withstand gravity. The larger the surface area of the culture, the greater the gravity because the mass of the membranes and the water increase. Therefore, the vertical system is impractical to use in Vietnam, especially when use of ground area is not an issue. Accordingly, in Vietnam, the TL biofilm-based photobioreactor should be angled at 15-20° on a solid surface to support the gravity of the membranes.

The bench-scale TL biofilm-based photobioreactor (0.05 m^2) for *H. pluvialis* immobilised cultivation includes the following components: chamber, supply system, nutrient circulation system, air circulation system (with or without CO_2), steel frame, and light supply system.

The cultivation chamber is made of acrylic glass because this material allows 90% of light to be transmitted (this is determined by measuring light intensity before and after it passes through the acrylic glass). It is also easy to handle and is more durable than silica glass. Each acrylic plate is 5 mm thick and is attached via cyanoacrylate glue and sealed by thermal glue. Fig. 2 presents the technical parameters of the chamber. The cultivation chamber contains supplying elements for immobilised algae: source layer, substrate layer, and air conducts. This chamber minimises contamination from the external environment.

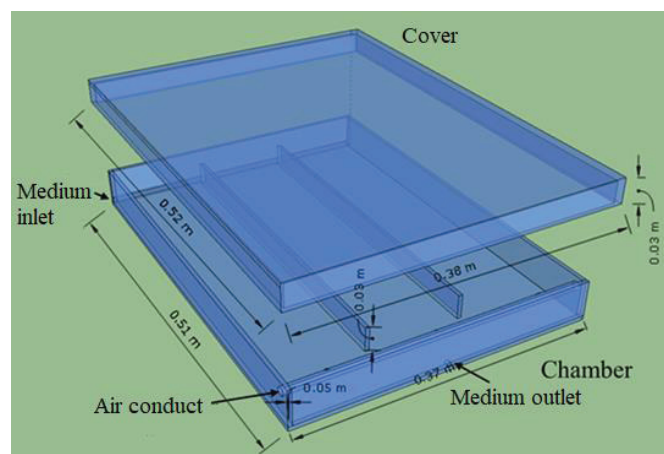


Fig. 2. Design of bench-scaled system.

The provision of nutrients requires a sufficient supply of medium liquid to maintain the wetness of the two layers. The dripping nutrient irrigation system is described in Fig. 3A. The medium is stored in a 20 l container located below the system and is continuously pumped into the dripping system via a pumping system with a flow rate of 1.2 l min^{-1} . The dripping system is assembled from a pressurised Capinet dripper with a flow rate of 5 ml min^{-1} , plastic ducts (outer diameter: 8 mm, thickness: 2 mm), and various joints. The medium flows through the chamber, wets the layers, and is collected in the reservoir via the duct system.

Fresh air (with or without a CO_2 supplement) is supplied via the system depicted in Fig. 3A. The main components include a air pump (160 W, 115 l min^{-1}) and an air filter-air is compressed by the pump to a pressure of 0.033 Mpa and flows through the filter. The CO_2 can be supplemented by

air ducts (outer diameter: 10 mm, thickness: 2 mm) leading into the filter; pressurised valves are used to mediate the air pressure to evenly distribute the air to all the chambers. Fig. 2 indicates the location of the duct system which leads the air into the chambers.

A steel frame is designed and assembled as indicated in the diagram in Fig. 3B. The material used is holed 3x3 cm V-shaped steel of 3 mm thickness with an electrostatic coating. The components are assembled using bolts and screws designed for holed steel assembly.

Light system: the experiment utilises many different light sources; the lamps are assembled as show in Fig. 3C. The lamps are automatically switched on and off by a timer with light cycle of 14 hours light/10 hours dark. The light intensity depends on each experiment and was measured using a Lutron LX-1108 (Taiwan) photometer.

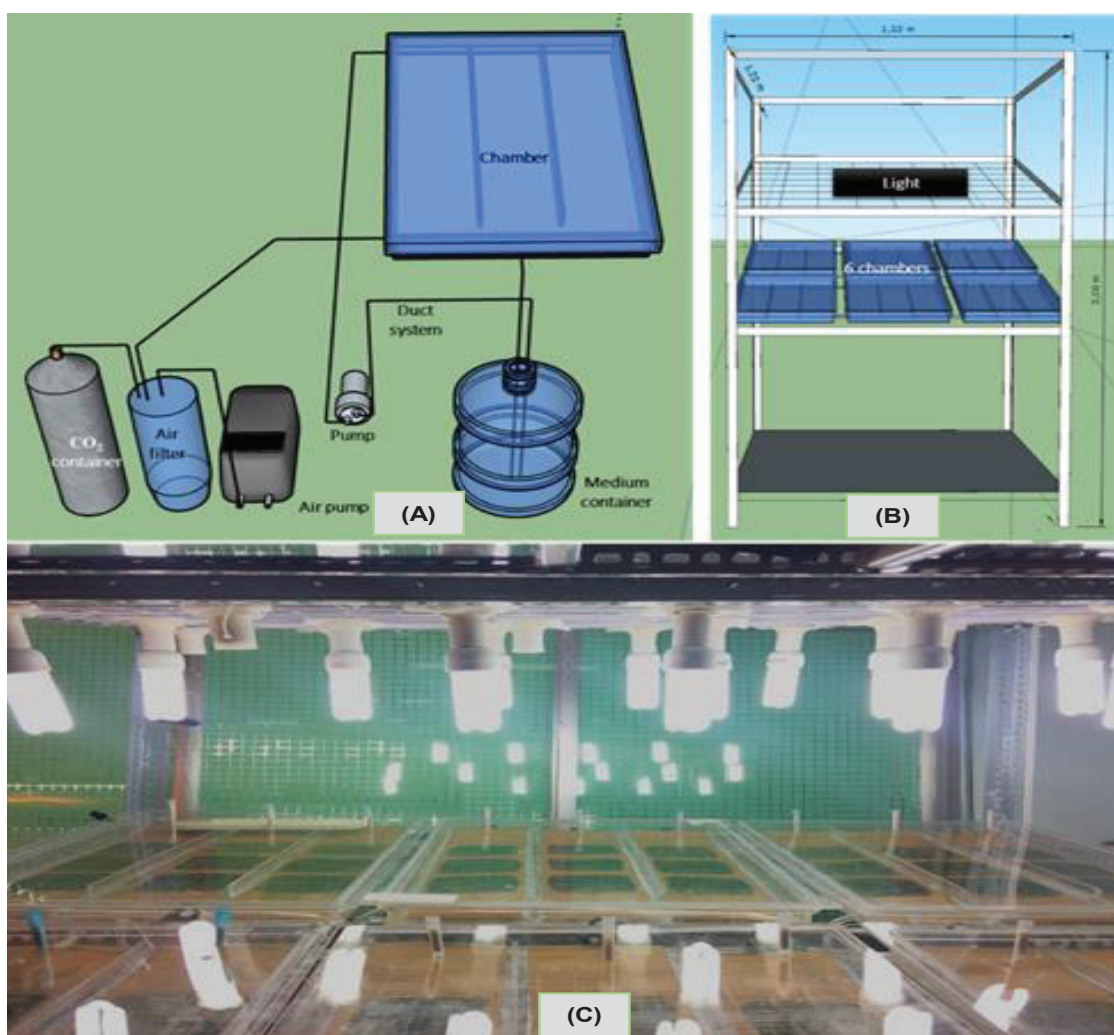


Fig. 3. (A) Nutrient and air supply system for cultivation chamber of bench-scale system; (B) Positioning of chambers and lights in bench-scale system; (C) The bench-scale system in use with *H. pluvialis* on the biofilm.

Suitable layer materials for conditions in Vietnam: the materials for algae attachment need to be durable, inexpensive, widely available, and non-toxic; material which can enhance biofilm yield should be preferred. Non-woven fiberglass and printing paper are most often used as a source layer and substrate layer, respectively, in TL photobioreactor for algae cultivation.

The source layer is made of non-woven fiberglass (0.5x0.1 m). Experiments with substrate layers show that there are only two suitable materials: Whatman filter paper and kraft paper (70 g m⁻², Vietnam). These materials are durable with a suitable pore size for keeping the algae in place after immobilisation. They were then tested in algae cultivation experiments to compare dry biomass growth in order to select the most appropriate material for use in later studies.

The results of the *H. pluvialis* cultivation experiment show that dry biomass growth in filter paper and kraft paper is not significantly different (filter paper: 6.81 g m⁻² d⁻¹, kraft paper: 6.63 g m⁻² d⁻¹, $p > 0.05$) at the same inoculation density of 5 g dry biomass m⁻² after 10 days. The kraft paper was then selected as the substrate layer since (1) it provides

biomass growth similar to that of filter paper, (2) kraft paper is much cheaper than filter paper, (3) kraft paper is widely available in Vietnam, and (4) kraft paper has high physical durability and is easy to handle during cultivation and harvesting (unpublished data).

Large-scale biofilm-based photobioreactor (2 m²): in order to scale up the angled TL photobioreactor system, the biotechnology research team of Nguyen Tat Thanh University successfully designed, assembled, and is optimising the angled biofilm-based biophotoreactor for *H. pluvialis* cultivation at a scale of 2 m².

The 2 m²-scaled biofilm-based photobioreactor for *H. pluvialis* immobilised cultivation uses the same component set as the bench-scaled one. The large-scale photobioreactor utilises four chambers assembled in the same system; each chamber provides a 0.5 m² area for algae growth.

The technical parameters of the large-scale chamber are described in Fig. 4. These are the result of several experiments and modifications to suit real-life conditions: (1) Kraft paper and fiberglass plate size of 1x0.6 m; (2) Size and weight of chamber for convenience in handling; (3)

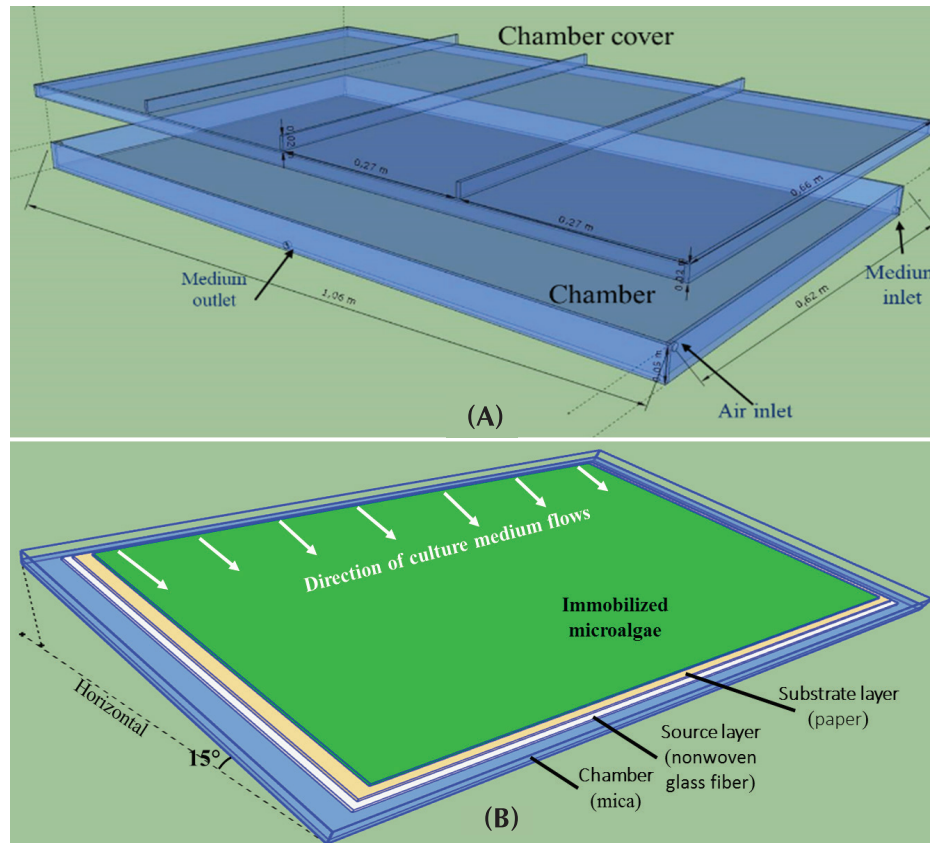


Fig. 4. (A) Design of large-scale system chamber; (B) Components of the TL photobioreactor system.

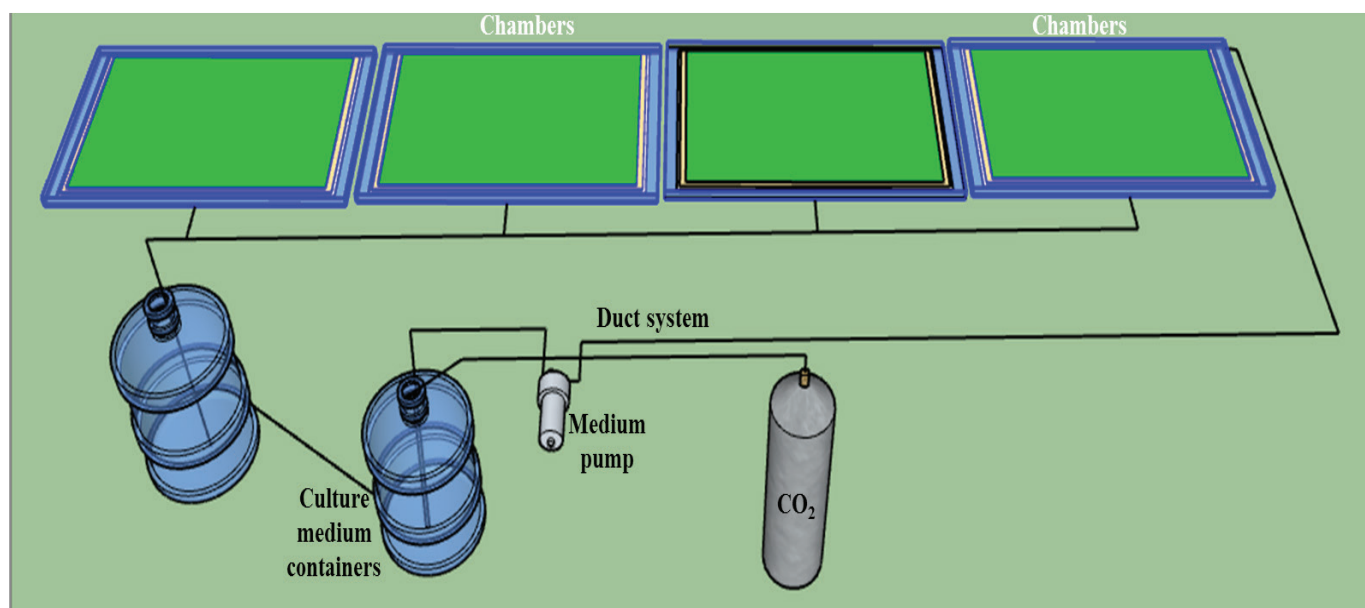


Fig. 5. Design of nutrient and air supply system for 2 m² system chambers.

Suitable size to correspond to the light power of the lamps to achieve maximum efficiency; and (4) An appropriate chamber size for manipulation and maintaining a culture below 28°C inside the chamber.

The nutrient supply system is similar to the bench-scale system (Fig. 5). The large-scale system has its own modifications, for example, a suitable number of drippers in the larger cultivation size (15 drippers/chamber); the drippers are positioned 6 cm away from each other.

A Harwin HP 2500 pump (5-12 W, flow rate: 1.2 l min⁻¹) is used to circulate the medium in the four chambers. The duct system is made of soft polyethylene (PE) 16 mm pipes with a 1.2 mm thickness. Fresh air (with or without CO₂ supplement) is supplied via the system described in Fig. 5. The main components are an air pump: 160 W, 115 l min⁻¹; and an air filter: air is compressed by the pump to a pressure of 0.033 Mpa and flows through the filter. The CO₂ can be supplemented by air ducts (outer diameter: 10 mm, thickness: 2 mm) leading into the filter and pressurised valves.

The steel frame is designed and assembled as in indicated in the diagram in Fig. 6. The material used is holed 3x3 cm V-shaped steel of 3 mm thickness and with electrostatic coating. The components are assembled using bolts and screws designed for holed steel assembly.

The light source for the 2 m² system includes: (1) a light system that provides 300-1,300 μmol photon m⁻² s⁻¹

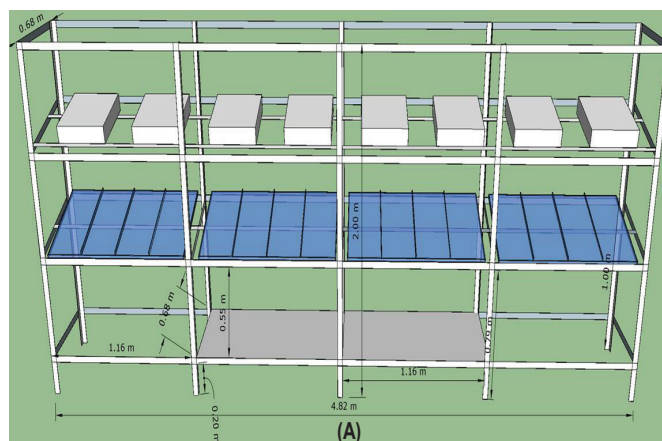


Fig. 6. (A) Diagram of chamber and light source positioning in 2 m² system; (B) The 2 m² system in use with *H. pluvialis* on the biofilm.

intensity (provided by eight 400 W Philips high pressure sodium lamps) or (2) a light system that provides 300-1,150 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ intensity (provided by ten 250 W Philips high pressure sodium lamps). The lamps are assembled according to Fig. 6. The light intensity differed in each experiment and was measured using a Lutron LX-1108 (Taiwan) photometer.

Cultivation of *H. pluvialis* in the astaxanthin accumulation phase on an angled bench-scale TL biofilm-based photobioreactor

Immobilised algae cultivation for astaxanthin harvest was carried out at bench scale to investigate the factors influencing the growth rate and astaxanthin accumulation of *H. pluvialis*.

Experiments on the angled 0.05 m^2 bench-scale system include: (1) Investigation of the most suitable CO_2 supply method; (2) Investigation of the most suitable light intensity (intensities from 200 to 1,150 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ were investigated); (3) Investigation of most suitable initial cell density (2.5, 5, 7.5, 10 g dried biomass m^{-2}); and (4) investigation of the influence of green algal biomass storing time on biomass growth and astaxanthin accumulation (storing algae at 4°C over 1, 3, 5, and 7 days after centrifugation).

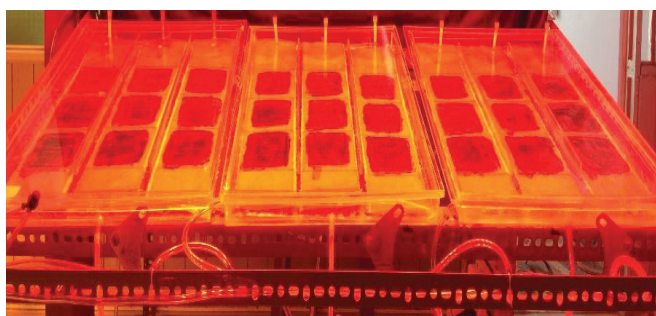


Fig. 7. The microalgae *H. pluvialis* on bench-scale system after 10 days of cultivation at an initial dry biomass density of 7.5 g m^{-2} .

The result shows that the most suitable CO_2 supply method is aerating fresh air with 1% CO_2 supplement into the culture medium to supply dissolved CO_2 and to maintain a pH favourable for algae growth. The most suitable light intensity for dry biomass and astaxanthin accumulation is 600-700 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$. The most suitable storing time is less than 24 hours after centrifugation; a longer storing time

causes a higher cell death rate and decreases algae growth after immobilisation. With an initial density of 7.5 g m^{-2} , average dry biomass production reached 12 g $\text{m}^{-2} \text{ d}^{-1}$ after 10 days of cultivation and the astaxanthin content amounted to 3% of the dry biomass (Fig. 7).

Cultivation of *H. pluvialis* in the astaxanthin accumulation phase on an angled large-scale TL biofilm-based photobioreactor (0.5 $\text{m}^2 \times 4 = 2 \text{ m}^2$)

The experiment was managed to establish the protocol for immobilised high productivity *H. pluvialis* cultivation on an angled large-scale system. The system is designed to maintain a temperature of 24-26°C, and humidity below 80% via a cooling and dehumidifying system to maintain algae growth. The system operated continuously for 10 days with 14 light/10 dark hours cycle.

For experiments on the biofilm, cultures of *H. pluvialis* CCAC 0125 (Culture Collection of Algae at the University of Cologne, Germany) were expanded to 10 l PE bags with 6 l of BG11 medium [19] and placed in 23-25°C. Algae were exposed to a light intensity of 50-60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, a photoperiod of 14/10 hours light/dark cycle and were aerated with fresh air. Microalgae were collected from the logarithmic growth phase after 16 days with a Hettich ROTANA 460 centrifuge (Germany). The percentage of flagellate cells after centrifugation was 85%, and the maximum storage time of the inoculum was 24 hours at 4°C. At the industrial scale, the inoculum of *H. pluvialis* will be cultured in 80-100 l PE bags. The step required to harvest a large number of flagellate cells in suspension is still being solved.

Initial algae density on biofilm was 5-7.5 g dry biomass m^{-2} . The fixation of algae on biofilm has been tested with many different methods. However, using a large brush to fix the algae shows many advantages. On average, the time needed to paint 1 m^2 of biofilm is 5 minutes. The density and quality of the algae are checked immediately during fixation.

An appropriate CO_2 supply method is aerating fresh air with 1% CO_2 supplement into the culture medium to keep pH in 6.5-8. The culture medium used is BG11 [19] (100 l for 10 days) which is diluted daily to keep electrical conductivity value in the range of 1,800-2,000 $\mu\text{S cm}^{-2}$. The light system providing the highest biomass growth and astaxanthin content has an intensity of 300-800 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Optimisation of high productivity *H. pluvialis* cultivation on a large-scale horizontal system produced some results. Average productivity of $11.25 \text{ g m}^{-2} \text{ d}^{-1}$ and an astaxanthin content of 2.8% of the dry biomass was obtained from the 2 m^2 system in the above-described conditions. Contamination was controlled during the cultivation period (Fig. 8). The 2 m^2 system provided slightly lower yields than the 0.05 m^2 system. However, astaxanthin productivity was higher in both suspended and immobilised systems than in most previous studies (Table 1).

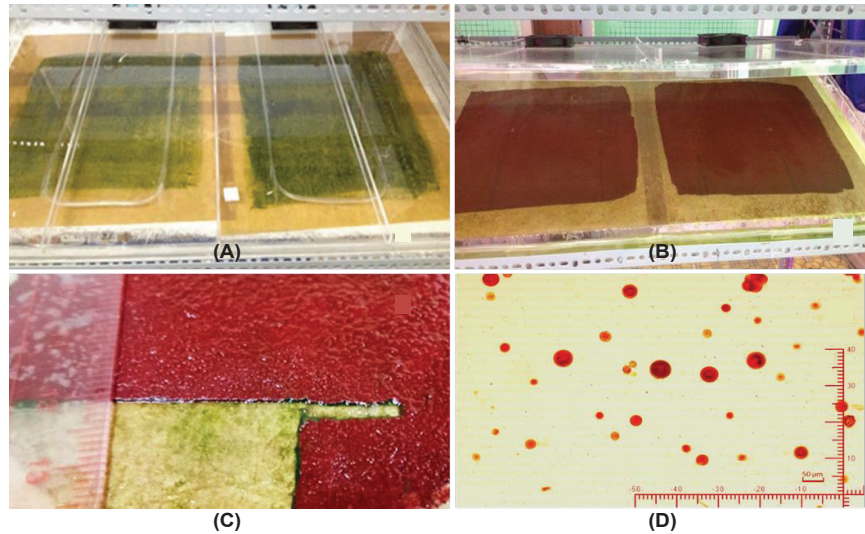


Fig. 8. Surface of *H. pluvialis* biofilm (A) and after 10 days of cultivation (B and C) on a 2 m^2 system; (D) Microscope image of *H. pluvialis* after 10 days of cultivation (x40).

Table 1. Comparison of *H. pluvialis* cultivation results on an angled biofilm-based photobioreactor system with other cultivation system based on surface area.

System	Strain	Medium	Temp (°C)	CO ₂ (%)	Light condition (μmol photon m ⁻² s ⁻¹)	Stress factor	Cultivation period (green phase + red phase) (days)	Astaxanthin content (% dried biomass)	Astaxanthin productivity (mg l ⁻¹ day ⁻¹)	Astaxanthin productivity (mg m ⁻² day ⁻¹)	Dried biomass productivity (g m ⁻² day ⁻¹)	References
Outdoor tube (50 l)	Isolated	BG11	25	For controlling pH	Sunlight 400-1600	Intense light	4 (Red phase)	3.6	7.2	136.8 ^a	3.8 ^a	[33]
Outdoor open pond	ZY-18	NIES-N	28	None	Sunlight Max. 1000	Intense light + N limited	20 (Green phase + red phase)	1.7	-/-	40 ^a	2.34 ^a	[29]
Indoor open pond	26	BG11	20	For controlling pH	20-350 14/10 hour	Intense light	12 (Green phase + red phase)	2.79	4.3	61 ^a	2.2 ^a	[34]
Indoor bubble column	ZY-18	NIES-N	28	None	250 Continuous	Intense light + N limited	12 (Green phase + red phase)	3.6	-/-	237.6 ^a	6.6 ^a	[29]
Indoor bubble column (0.5 l)	K-0084	Modified BG11	25	1.5	350 Continuous	Intense light + N limited	5 (Red phase)	4.0	11.5	528 ^a	13.2 ^a	[13]
Indoor closed container (10 l)	HB (isolated)	Modified RM	25	For controlling pH	85 16/8 hour	Intense light, N limited, high C/N, + bicarbonate	30 (green phase) + 3 (Red phase)	4.88	2.75	92 ^a	1.88 ^a	[23]
Indoor bubble column (5 l)	-/-	RM	25	40 ml/min	60 16/8 hour	N limited, High C/N	22 (Green phase + red phase)	-/-	0.009	0.264 ^a	-/-	[24]
Indoor immobilised biofilm (0.08 m ²)	NIES-144	NIES-N	25	None	150 Continuous	N limited	12 (Green phase + red phase)	1.3	-/-	65.8	3.7	[28]
Indoor immobilised biofilm (0.08 m ²)	SAG 34-1b	BG11	25	1.5	100 Continuous	N limited or exhausted	7 (Green phase + red phase)	2.2	-/-	143	6.5	[10]
Indoor immobilised biofilm (0.05 m ²)	CCAC 0125	Modified BG11	26	1	650 14/10 hour	Intense light + N, P limited	7 (Green phase + red phase)	3.5	-/-	371	10.6	[32]
Indoor angled immobilised biofilm (0.05 m ²)	CCAC 0125	Modified BG11	26	For controlling pH	600-700 14/10 hour	Intense light + N, P limited	10 (Green phase + red phase)	3.0	7.2	360	12	This study
Indoor angled immobilised biofilm (2 m ²)	CCAC 0125	Modified BG11	26	For controlling pH	600-700 14/10 hour	Intense light + N, P limited	10 (Green phase + red phase)	2.8	6.3	315	11.25	This study

^a: the values are converted to 'per surface area'.

Conclusions

Angled immobilised cultivation systems for *H. pluvialis* were successfully designed and operated. The dry biomass productivity and microalgal astaxanthin content of the 2 m² system reached 11.25 g m⁻² d⁻¹ and 2.8%, respectively, which are similar to or higher than that of other systems. Both biomass and astaxanthin production can likely be improved by optimisation of the cultivation process. The data show that these systems can be applied for production at a larger scale. Further studies will be rewarding to improve the dry biomass and astaxanthin productivity of *H. pluvialis* cultivated on an angled TL biofilm-based photobioreactor system.

Angled immobilised cultivation on the TL-biofilm-based system provides remarkable advantages compared with traditional suspended cultivation, such as in term of water, energy, and cultivation time-saving. The angled system is also likely easier to scale up than the vertical TL system and perhaps more cost-efficient (for further discussion of vertical vs horizontal TL systems, see Podola, et al. (2017) [35]). However, understanding the underlying processes (light, nutrient, and air distribution, etc.) in the TL system is still limited relative to suspended systems, although some progress has recently been made [36-39].

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Application of biological methods in the treatment of gaseous ammonia

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Abstract:

In Vietnam, practical applications of biological methods in air pollution control are highly limited. This study evaluated and compared the ammonia removal performance in air of a cow-manure biofilter, commercial compost biofilter, and biotrickling filter with K3 biomedium cultured with attached microorganisms from activated sludge. The results indicated that with an inlet NH_3 concentration of 65-80 mg/m^3 (95-117 ppm), the treatment efficiency was highly promising with an output concentration in the range of 2-5 mg/m^3 (3.0-7.5 ppm) and elimination capacity of 3-9 $\text{gNH}_3/\text{m}^3\cdot\text{h}$. With an inlet concentration below 200 mg/m^3 , all three experimental models could remove ammonia to meet the emission standard (QCVN 19:2009/BTNMT) of 50 mg/m^3 . The study results indicated that the investigated biological technologies have potential for use in removing ammonia and other odorous gases in polluted air.

This study investigated the removal of gaseous ammonia using cow manure, compost, and K3 material as biomedium in biofilters and biotrickling filters.

Keywords: biological methods, biomedium, gaseous ammonia.

Classification number: 3.5

Introduction

Today, air pollution is a serious concern attracting considerable attention from citizens and scientists. Ammonia is one of the most common air pollutants, and is released from various sources such as sewage and wastewater treatment plants, animal-waste decay on livestock farms, organic decomposition in composting processes, as well as many industries such as petrochemical, food, paper pulp, metal, and textiles. Ammonia emissions could have negative impacts on human in terms of comfort because of its bad smell, as well as on the environment because it increases the nitrogen nutrient and acidifies water [1-3]. Traditional technologies have been applied for gaseous ammonia removal, such as condensation at low temperatures and/or high pressures, absorption using water or diluted acidic solutions, adsorption using porous solid materials, and thermal/catalytic oxidation at high temperatures. However, these methods are not particularly efficient, environmentally friendly, or economical, either because they have high costs or are harmful with secondary pollutants [4].

Recently, biological methods have been widely applied for solid waste, wastewater, and even gas treatment. Biofiltration units have been successfully applied to the removal of odorous and toxic air pollutants. They function efficiently and economically when removing low concentrations of pollutants with low installation and operation costs, low energy and maintenance requirements, long life and high durability, environmentally safe operations, and without generating pollutants [5, 6]. Biofiltration units are microbial systems in which microorganisms develop and grow to form a biofilm on a biomedium surface [7]. When polluted gas passes through the biomedium bed, soluble pollutants transfer into the

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liquid phase, and biodegradable pollutants are decomposed by microorganisms in the biofilm. Many industrial and domestic air pollution sources have successfully applied biofiltration to control odours and other air pollutants with high removal efficiencies of >90% and end products of CO₂, water, and microbial biomass [6]. Because of biofiltration's several advantages in terms of cost and the environment, it has become a preferred choice for air pollution control in practical applications. Ammonia emission control through biofiltration with different microorganism media, such as multicultural microbial load on peat and inorganic media [8], compost [9], and agricultural residue media [10] has been investigated by numerous researchers [11, 12].

Biological treatment systems include biofilters (BFs), biotrickling filters (BTFs), bioscrubbers (BSs), and membrane bioreactors (MBRs) [13]. BFs work with biomedica such as compost, activated carbon, peat, perlite, and soil. This traditional technology is widely used and has a long history of development and application. However, it has disadvantages such as media compaction, difficulty in pH and moisture control, biomedica degradation, acidic accumulation, and applicability for low pollutant concentrations. BTFs comprise inert packing materials such as plastic, ceramics, gravel, and wood. This technology works through the recirculation of an aqueous solution distributed from top to bottom of the packed column. The biofilm on packing material surface is the key component in the gas treatment. BTFs have the advantage of liquid phase control, which could provide required nutrients/components and remove acidic/toxic compounds [14]. Therefore, BTFs can avoid the drawbacks associated with BFs and are considered more effective for treating gaseous pollutants. Regarding the other two technologies, BSs are rarely used and MBRs are mainly employed in lab-scale studies. In the biofiltration removal process, ammonia is first converted to nitrite by nitrosomonas, and this nitrite is then converted to nitrate by nitrobacteria [15]. A denitrifying process also exists, where nitrate is converted to nitrogen gas by pseudomonas and clostridium under an anoxic condition.

In Vietnam, such biological methods for air pollutant control have not been widely applied because of limited research and experience. Therefore, the present study investigated the efficiency of two different BFs and a BTF in NH₃ removal. In the lab-scale setup, both BFs and the BTF used local growth microorganisms.

Materials and methods

Materials

NH₃ solution at a concentration of 25% (w/w) was purchased from Xilong (Guangdong, China) and used as the ammonia source in this study. NH₃ removal experiments were conducted using lab-scale BF and BTF models. These models represented improvements over our previous study on the removal of H₂S, which was designed with parameters taken from relevant literature [16, 17]. Table 1 summarises the present study's detailed design and operational parameters. The following three types of biomedica were used: compost and cow manure for the two BF models and K3 inert media for the BTF model (Fig. 1).

Table 1. Configuration and operational parameters of the three models.

Parameters	Unit	BTF	CM-BF	CP-BF
Height×length×width	mm	1100×140×140	1000×110×110	540×150×150
Packing height	mm	400	270	170
Packing volume	l	7.8	3.3	3.8
Gas flowrate	l/min	7.5	7.5	7.5
Empty bed retention time (EBRT)	sec	63	26	30
Liquid flowrate	l/min	0.24	-	-

Cow-manure BF (CM-BF) model: cow manure was incubated for approximately 2 months and then dried under sunlight before being stored in a household in the Mekong delta (Vietnam). This process was to kill weed seeds and insect germs, pathogenic bacteria, and mould, as well as promote organic decomposition and accelerate mineralisation. The dried cow manure was refined and supplied with water before being incubated under an anaerobic condition for approximately 1 month. Its pH after incubation was 7.72 and moisture content was 72.1%. The manure contains humus content and other ingredients that could provide in-situ sources of carbon as well as macro- and micro-nutrients for the microorganisms.

Compost BF (CP-BF) model: this study used commercially available compost (organic fertiliser Agrimartin) in the market (Ho Chi Minh city, Vietnam). Compost-based media are widely used in BFs because of

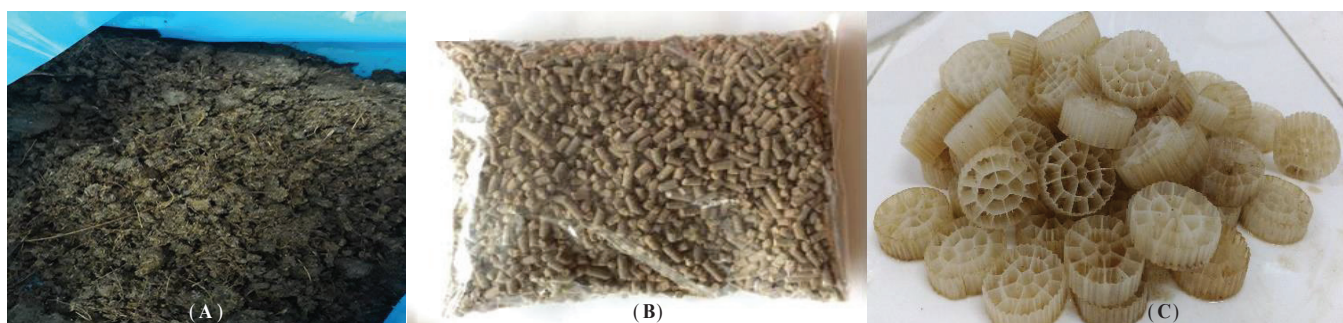


Fig. 1. Biomaterials for NH_3 treatment. (A) cow manure, (B) commercial compost, and (C) K3 biomedium.

their low cost and abundant microbial communities that are ready to decompose various pollutants. Because it would divert the local microbial population, the addition of external microorganisms and enzymes is usually not necessary. The quantitative component of this compost comprised 72% organic (dried) matter, 3.5% N, 2.5% P_2O_5 , and 2.5% K_2O .

BTF model: in this model, K3 medium was used to support microorganism growth. It was made of high density polyethylene in a round shape with a honeycomb structure inside, providing a high surface area through numerous folded wrinkles. First, the K3 medium was placed in an activated sludge wastewater tank under aeration to provide dissolved oxygen and nutrients at a chemical oxygen demand (COD) concentration of 500 mg/l as well as a small amount of NH_4Cl for microorganism adaptation. The concentration of ammonia in the wastewater was initially maintained at 1 mg/l for adaptation and then increased to 10 mg/l for microbial growth. The high organic loading and microorganism content accelerated the development of aerobic and anaerobic microorganisms on the surface of the K3 medium. Subsequently, K3 biomedium (i.e., K3 medium with a biofilm) was placed into the BTF model and operated with wastewater (i.e., domestic wastewater from a student dormitory) containing molasses as carbon sources and nutrients for microorganism growth on the K3 medium.

Ammonia treatment experiments

Figure 2 illustrates the experimental setup for the biological removal of NH_3 in air. The experimental model consisted of three lab-scale models (BTF, CM-BF, and CP-BF) made of acrylic resin. Ammonia-laden air with a high (almost saturated) concentration of ammonia was prepared by passing a clean air flow through a vessel containing 25% ammonia solution for ammonia evaporation. The

ammonia-laden air was then mixed with fresh air at certain ratios to prepare a gaseous mixture with the desired concentrations of ammonia. The mixed gas was then flowed through the three models using a three-way connector split into two lines. One line flowed through an impinger for inlet gas sampling and ammonia concentration analysis, whereas the other line was divided into three lines that flowed directly into the BTF and BF models. In the BTF model, recirculated wastewater was irrigated from top to bottom to provide substrates and nutrients for the microorganisms. Initially, all three models were begun with low ammonia concentrations of 15-30 mg/m^3 for 20 days of adaptation. Subsequently, the ammonia concentration was increased to the required concentration of 50-80 mg/m^3 . During the experiment, the BF models were supplied with water to maintain moisture above 50%. Before sampling, the models were operated and controlled stably for 1 to 2 h. Ammonia gas samples from the inlet and outlet of each model were taken for 1 min at a flow rate of 7 l/min and then sent for concentration analysis using the indophenol method. Ammonia samples were taken and analysed three times/day and the average results were reported.

The ammonia removal efficiency (RE, %) and elimination capacity (EC, amount of ammonia removal per unit volume of biomedium per unit of time, $\text{gNH}_3/\text{m}^3\cdot\text{h}$) were calculated as follows:

$$RE = \frac{C_{in} - C_{out}}{C_{in}} \times 100\% \quad (1)$$

$$EC = \frac{(C_{in} - C_{out})}{V} \times Q \quad (2)$$

where C_{in} and C_{out} (mg/m^3) are the inlet and outlet ammonia concentrations, respectively, and Q (m^3/h) and V (l) are the gas flowrate and packing volume (i.e., cow manure, compost, and K3 biomedium), respectively.

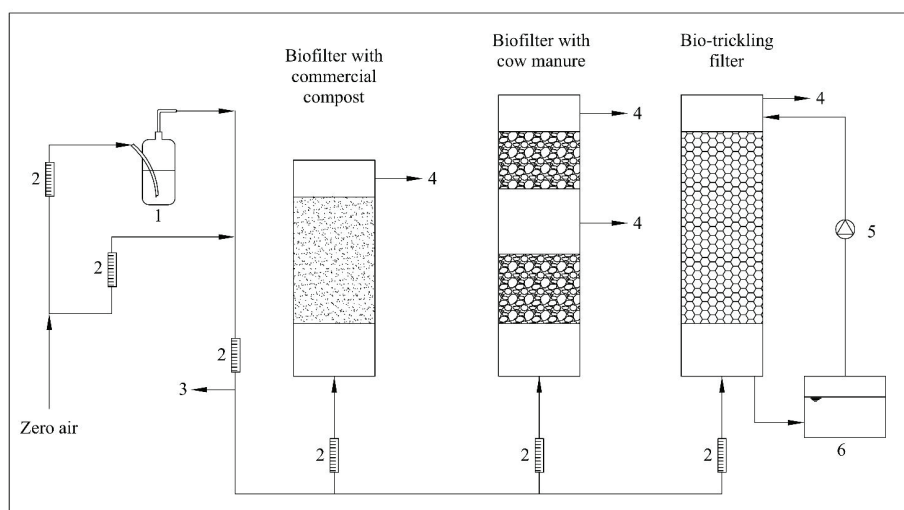


Fig. 2. Diagram of the experimental models: (1) NH_3 solution, (2) flowmeter, (3) inlet sampling location, (4) outlet sampling location, (5) circulation pump, and (6) wastewater tank.

Results and discussion

Evaluation of the ammonia RE of the three models

After 20 days of adaptive operation with low ammonia concentrations, ammonia treatment of the three models was investigated at inlet concentrations of approximately 60 mg/m^3 for 30 days. The treatment efficiency was calculated by measuring the inlet and outlet ammonia concentrations. The results in Fig. 3 show that the performance of the CM-BF model was unstable during the first 12 days, which might have been because the microorganisms in cow manure take longer to adapt and stabilise. The RE of this model was stable at 92% after 26 days of operation and increased to 96% at the end of experiment. A similar trend was observed for the CP-BF model with an RE of 94% after 30 days of operation. The RE of the BTF model was unstable, possibly

because of the characteristics of the model and variation in wastewater recirculation. However, this model could still achieve an ammonia RE of 94% under the experimental conditions after 30 days of operation.

Because the treatment efficiency depended on various factors such as inlet concentration and gas flow rate on the biological bed, this study calculated the ECs of ammonia ($\text{gNH}_3/\text{m}^3\cdot\text{h}$) and the results of which are presented in Fig. 4. The highest EC was achieved by the CM-BF in the range of $6.9\text{--}10.0 \text{ gNH}_3/\text{m}^3\cdot\text{h}$. By contrast, the CP-BF model achieved a lower EC in the range of $5.9\text{--}8.8 \text{ gNH}_3/\text{m}^3\cdot\text{h}$. These results are comparable to relevant studies that have used municipal compost inoculated with thickened municipal activated sludge with ECs of $9.85 \text{ gNH}_3/\text{m}^3\cdot\text{h}$ (three-stage BF) and $8.08 \text{ gNH}_3/\text{m}^3\cdot\text{h}$ (one-stage BF) [18], co-immobilised cells with an EC of $6.8 \text{ gNH}_3/\text{m}^3\cdot\text{h}$ (164 ppm NH_3) [19], and agricultural residue BF medium with ECs of $14 \text{ gNH}_3/\text{m}^3\cdot\text{h}$ (500 ppm NH_3) and $23.5 \text{ gNH}_3/\text{m}^3\cdot\text{h}$ (1000 ppm NH_3) [10].

In this experiment, the BTF had an EC of $3\text{--}4 \text{ gNH}_3/\text{m}^3\cdot\text{h}$, which is rather low compared with the ECs of the BFs. It was also low compared with that reported in a study that used polyurethane foam ($0.9\text{--}21.7 \text{ gNH}_3/\text{m}^3\cdot\text{h}$; 60–1600 ppm NH_3 , EBRT of 150 s) [20]. In terms of stability, the BTF model was more stable than the two BF models, possibly because of the recirculation of liquid and stable attached microorganisms. In addition, the EC for NH_3 in the BTF depended on gas flow rates and bed lengths [21]; thus, further investigation is required to optimise the operation.

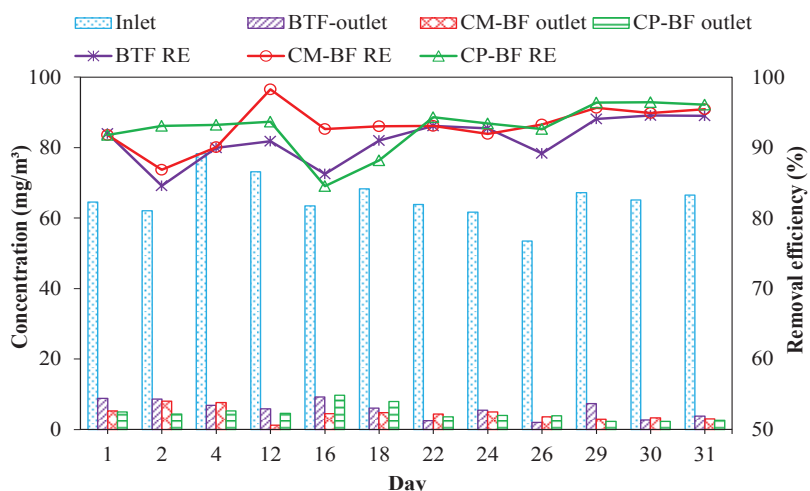


Fig. 3. Comparison of the treatment efficiency of the three models.

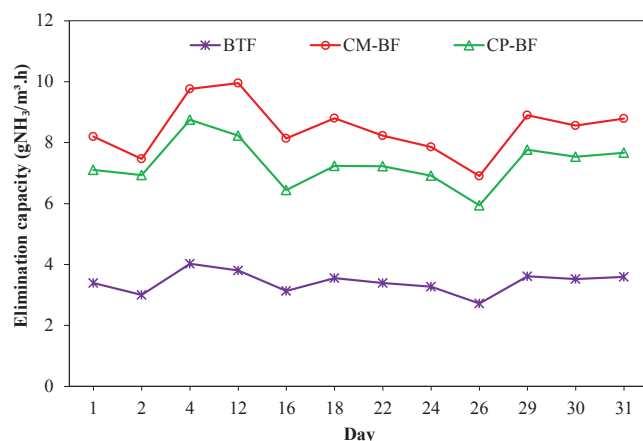


Fig. 4. Comparison of the elimination capacities of the three models.

Performance of three models under high-inlet NH_3 concentration

The aim of the experiment was to determine an NH_3 inlet concentration limit that still met the outlet NH_3 emission standard (QCVN 19:2009/BTNMT) of 50 mg/m^3 . The ammonia concentration and flowrate were increased gradually each day from the values of 8 l/min and 68.4 mg/m^3 , respectively, until the outlet concentration exceeded 50 mg/m^3 . As presented in Fig. 5, under an inlet concentration of $\leq 200 \text{ mg/m}^3$ and flowrate of 16 l/min , the three models were still able to remove ammonia to meet the emission standard. Under the same treatment condition, the CM-BF provided superior treatment, although the performance differences between three models were not significant. The minimum gas retention times of the three models were then calculated, and the results were 63 sec for the BTF, 26 sec for the CM-BF, and 30 sec for the CP-BF.

The NH_3 ECs of the three models were calculated, which is depicted in Fig. 6. The ECs can be observed to continuously increase from 4.2 to $67.7 \text{ gNH}_3/\text{m}^3.\text{h}$ as the inlet concentration increased from 75.1 to 286.9 mg/m^3 . At an inlet concentration of 206 mg/m^3 , the CM-BF, CP-BF, and BTF models had ECs of 47.7 , 38.4 , and $19.0 \text{ gNH}_3/\text{m}^3.\text{h}$, respectively. These EC values for the CM-BF and CP-BF were remarkably high compared with those reported in the abovementioned studies [10, 18, 19], but that of the BTF was still low [10]. These results confirmed the inefficient operation of the BTF and suggested that BFs seem to be a more suitable choice for ammonia removal in practical applications under the current condition.

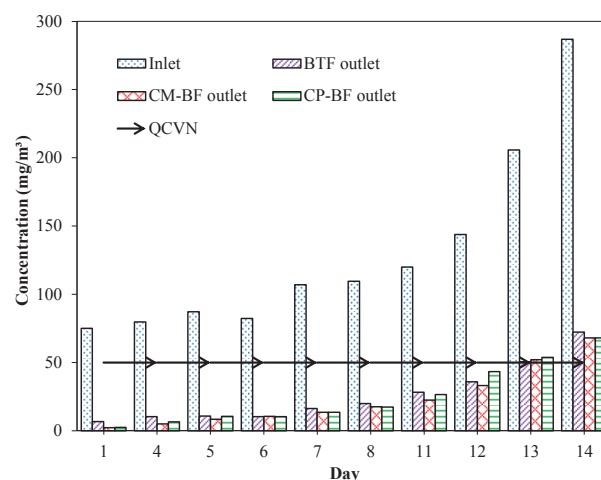


Fig. 5. Inlet and outlet concentrations of the three models.

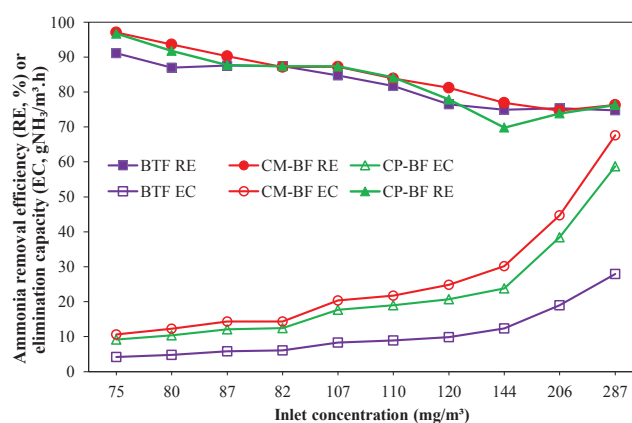


Fig. 6. Removal efficiency (RE) and elimination capacity (EC) of the three models.

Conclusions

The experimental results demonstrated that CM-BFs, CP-BFs, and BTFs with K3 biomedium can be applied to remove ammonia at REs up to 96%, although their optimised conditions have not been investigated. These technologies can remove ammonia in air to meet the National Technical Standard on Industrial Emissions for dust and inorganic substances (QCVN 19:2009/BTNMT) of 50 mg/m^3 if the concentration is below 286 mg/m^3 at a flowrate of 17 l/min . In this study, the stability and efficiency of BFs were higher than those of the BTF, which might have been because of the microorganism attachment and population in the BTF not being well-controlled. This study provides a first attempt at the application of different biological methods to remove ammonia from air. The results indicated that such biological technology could have potential for removing ammonia and

other odorous gases from polluted air. Future studies should focus on investigating and optimising operation parameters (e.g., EBRT, concentration, ammonia loading rate, pH, and temperature), determining microbial strains, applying other media that contain superior microbial strains, and nitrogen balance for circulating wastewater used in BTFs.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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Assessment and prioritisation of low-carbon technology for the waste sector in Vietnam

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Abstract:

This paper presents a multi-criteria assessment approach for the prioritisation of low-carbon technologies for the waste sector in order to enable the implementation of Vietnam's nationally determined contributions (NDCs) in terms of the Paris Agreement. A four-step approach is developed for assessing and prioritising low-carbon technologies for the waste sector using this approach. A set of five criteria and indicators are defined for multi-criteria assessment. Based on mitigation options defined in the NDCs, a shortlist of 26 technological options are defined. The multi-criteria assessment and prioritisation is conducted based on the shortlist. The assessment results in eight prioritised low-carbon technologies. Of the eight technologies prioritised, semi-aerobic landfill, which is a low cost and relatively simpler technology than the other technologies evaluated and can contribute to greenhouse gas emission reduction, is given the highest priority. The technical, financial, and social and environmental feasibility of each of the technologies evaluated is presented.

Keywords: emission reduction, GHG waste sector, low-carbon technology, multi-criteria assessment and prioritisation, NDCs.

Classification number: 5.1

Introduction

In September 2015, the Government of Vietnam submitted its intended nationally determined contribution (INDC), in which the national greenhouse gas (GHG) emission reduction target for the period 2020-2030 is defined, to the secretariat of the United Nations Framework Convention on Climate Change (UNFCCC) [1]. This endeavour was undertaken as part of the global, collective effort to reach a comprehensive, fair, and effective agreement on the post-2020 climate regime.

Following the submissions of country INDCs, the Conference of the Parties (COP21) to the UNFCCC adopted the Paris Agreement in December 2015 to provide an overarching framework and a series of requirements for the post-2020 regime. With the necessary number of instruments for ratification having been submitted, the Paris Agreement successfully entered into force in November 2016 [2]. Subsequently, country INDCs have transformed into nationally determined contributions (NDCs) in anticipation of the submitting countries duly implementing these, starting from 2021. For its part, in October 2016, the Government of Vietnam approved the Paris Agreement implementation plan in which key climate-change mitigation tasks are identified [3].

To implement the plan and especially to meet the mitigation targets, it is crucial to elaborate the NDC into implementable actions in order to achieve the aggregate amount of GHG emission reductions. Such elaboration requires an in-depth technical and realistic assessment of the implementation method for each mitigation option.

Four sectors (Energy/transport; Land use, Land-use Change and Forestry; Waste; and Agriculture) with 45 mitigation options are included in Vietnam's NDC [1]. This paper presents a multi-criteria assessment approach for prioritising low-carbon technologies for enabling mitigation options in the waste sector.

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Methods

Definitions

In this paper, low-carbon technologies for the waste sector are defined as both hardware (e.g. waste collection and transportation infrastructure) and software (e.g. waste management systems) that can contribute to climate change mitigation goals through GHG emission reduction efforts, and that encourage Vietnam to embark on a sustainable and low-carbon development pathway. Not only are hardware, devices, machines, and facilities, which are commonly regarded technological elements considered; techniques, practices, and management tools attached to some of the mitigation options and sectoral attributes are also included in this assessment.

Approach for multi-criteria assessment

A four-step approach is developed in order to conduct a multi-criteria assessment and prioritisation of low-carbon technologies for the waste sector [4]. Details of the steps are shown below.

Step 1: confirmation of progress regarding climate change measures.

Stakeholder interviews and consultations with experts at the Ministry of Construction were conducted to identify suitable technologies to enable four mitigation options for the waste sector. In addition, the need for legislation and standards to enable some of the options were reviewed to capture the enabling conditions and current levels of political appetite for policy reforms.

Step 2: development of low-carbon technologies based on the INDC technology report.

With the current situation and the direction of the climate change measures in Vietnam in mind, technologies applicable to Vietnam's context have been selected from existing technology lists.

Step 3: definition of criteria for evaluation and prioritisation.

Five assessment criteria and indicators are defined for assessing and prioritising low-carbon technology for the waste sector. Consensus on these criteria was reached through consultation with experts at the Ministry of Construction and at a consultation workshop in which multiple stakeholders participated. Details of the defined criteria and indicators are shown in Table 1.

Table 1. Criteria and indicators defined for the prioritisation of low-carbon technologies for the waste sector.

Criteria	Indicators	Evaluation	
<i>Compatibility with policy priorities</i>	Presence of supporting policies and policy tools/ measures	High	Presence of supporting policies and policy tools/measures
		Medium	Presence of supporting policies
		Low	No relevant policy
<i>Economic efficiency</i>	Cost of waste handling/ treatment per ton of waste	The estimated unit cost in US\$ per ton of waste	
<i>GHG emission reduction effect</i>	GHG emission reduction per ton of waste	The estimated unit of GHG emission reduction as ton of CO ₂ eq/ton of waste	
<i>Versatility</i>	Simplicity/ease of application of the technology	High	Already applied or conventional technology in the country
		Medium	Training or technical transfer is required for a certain period
		Low	Regular monitoring and supervision by a technical expert is required
	Limitation of application of the technology in terms of waste amount and/or composition	High	No limitation
		Medium	Limitation in terms of amount of waste or composition
		Low	Limitation in terms of amount of waste and composition
<i>Social and environment impacts</i>	Social impacts	Identify and describe positive and negative impacts on society (qualitatively)	
	Environmental impacts	Identify and describe positive and negative environmental impacts (qualitatively)	
<i>Overall assessment</i>	Identify and describe the extent and conditions of the application of the technology with its priority evaluation.		

Source: [1].

Evaluation was undertaken qualitatively as either high, middle, or low (A, B, or C grading, respectively) for each criterion [5]. What follows is the annotation of the grading letters.

- A: the technology is of relatively higher priority and early deployment is recommended.

- B: the technology can be deployed when barriers are removed by improving the enabling conditions and environment to some extent.

- C: a long lead time for the deployment of the technology in order to arrange an appropriate enabling environment is anticipated; the result is used for the final evaluation.

For the criteria of economic efficiency and GHG reduction effect, the prioritisation of technology is undertaken comparatively based on the available numerical data.

Step 4: selection of the technologies with high priority

Technologies graded with higher priority for selection are selected based on the results of the evaluation in Step 3 and on Vietnam's context, as reflected in the opinions obtained from experts in the waste sector [1].

The sources of GHG emissions in the waste sector mainly comprise:

+ Energy-related CO₂ emissions from waste collection and haulage vehicles [6].

+ CH₄ emissions from organic components in waste arising during the process of their decomposition in an anaerobic condition in the final disposal landfill [7].

+ CO₂ emissions from intermediate waste treatment, such as waste incineration.

Incineration of organic waste before its decomposition may reduce net GHG emissions by the conversion of CH₄

to CO₂ as well as by energy recovery from its thermal treatment. Incineration of plastic waste (fossil fuel-based plastic) can be regarded as a net GHG emission even with heat/energy recovery; however, this is advantageous when its high incineration heat can be utilised effectively [8].

The climate change mitigation technologies in the sector mainly address methane emissions from the decomposition of organic components in municipal solid waste (agricultural waste is not discussed in this chapter but in the agriculture sector), though they also tackle the possible reduction of CO₂ emissions arising from waste collection and haulage vehicles [9].

All the technologies identified are evaluated in terms of their feasible application potential based on five criteria: compatibility with policy priorities; economic efficiency; GHG-emission reduction effect; flexibility; and social and environmental impacts (positive and negative).

Table 2. Results of the multi-criteria assessment of low-carbon technologies for the waste sector.

No.	INDC #	Technology options	Rate*	Evaluation (inc. the extent of and conditions for the application of the technology)
1	W1	Production of organic fertilisers from organic waste (composting)	B	- Conventional technologies in Vietnam - Strict control of waste composition is required to produce good quality compost to compete with chemical fertilisers - A certain area of land is required to handle large amounts of waste (40,000 m²/daily handling of 100 tons)
2	W2	Landfill gas capture/recovery and energy utilisation	B	To effectively introduce the target technology, establishing the appropriate conditions, such as the amount and quality of waste (i.e. sufficient amount of organic waste), and the conditions at the final disposal facility (anaerobic treatment) are vital. The technology is not applicable to facilities that do not meet these conditions. The technology is only applicable if the above conditions are met and if a sufficient amount of methane gas is estimated to be recoverable; hence the opportunity for its application is extremely limited
3	W3	Recycling of solid waste	C	- Although recycling is the win-win technology for both GHG emissions and for the reduction of waste itself, the recyclable and salable materials in the waste have already been collected and recycled using the current market mechanism - Recycling of the remaining materials in the waste is mostly not technologically or financially feasible due to the types of materials or their contamination by or mixture with strange materials - The potential for GHG emissions by recycling is very limited
4	W4	Anaerobic treatment of organic waste with methane recovery for power and heat generation	B	- There is an example of the application of this technology in Ho Chi Minh City - Suitable for areas where a certain amount (50 to 100 tons daily) of food waste is stably generated, such as the wet market (fish meat and vegetable market), hotels, and restaurants - If the above amount of food waste can be regularly obtained, this technology is worth applying for food waste management
5	W5	Semi-aerobic landfill operations	A	Semi-aerobic landfills are a low cost and simpler technology of sanitary landfills and contribute to GHG emission reduction. As a standard for the construction and operation of a sanitary landfill, a semi-aerobic landfill is technologically and economically suitable in Vietnam if proper technological transfer is undertaken
6	W6	Waste to energy (WTE)	B	- There is no full-scale application of WTE technology, though there are some existing waste incineration facilities in operation with no energy recovery - Treatment cost is the highest, though the waste reduction ratio is also the highest - A certain amount of waste is required to reach an economy of scale when applying this technology - A certain heat value is required in waste to maintain its self-sustaining combustion with minimal input of supplementary fuel (The higher the heat value of the waste, the higher the potential for energy recovery from the waste)
7	W7	Conversion to low-carbon fuel trucks for waste collection and haulage vehicles	B	- No application in Vietnam - Low-carbon trucks can contribute to both climate change mitigation and to improving air quality (vehicle-related air pollution) - A supply infrastructure and network needs to be built for low-carbon fuels - The cost of trucks and fuel may increase
8	W8	Construction of a waste transfer station	B	- There are several waste transfer stations that have been built and are currently in operation in Vietnam - It can also mitigate traffic congestion and improve transportation efficiency in waste collection and haulage - The impact of a transfer station upon transportation efficiency depends on the distance between the waste-generation sources and final destination (landfill) and on traffic conditions (traffic volume, road conditions, etc.)

*A: the technology is of relatively higher priority and early deployment is recommended; B: the technology can be deployed when barriers are removed by arranging the deployment environment to some extent; C: long time may be required for technology deployment in order to arrange appropriate environment.

Results and discussion

Results

The qualitative multi-criteria assessment results of each climate change technology in the waste sector are summarised in Table 2. A total of eight low-carbon technologies for waste management and treatment are identified and assessed. The assessment results indicate that semi-aerobic landfill operations are graded highest, with an A grade, the highest priority.

Discussion

In this section, the potential for the deployment of the above-identified technologies, with a focus on three dimensions, namely the technical, financial, and social/environmental aspects, is discussed.

First, as concerns the technical aspect, all of the technologies evaluated and listed above were originally defined to serve different waste management purposes, such as waste treatment to produce reusable products (e.g. composting to produce organic fertiliser), to reduce the amount of waste finally disposed of in landfills, and to prevent environmental pollution arising from the improper management and treatment of waste. Although none of the technologies evaluated were designed to reduce GHG emissions in the waste sector, some of them can significantly reduce the amount of waste and GHG emissions from the anaerobic decomposition of organic waste, such as the composting and incineration of waste to produce electricity. Technically, all of the above technologies are applicable in Vietnam.

Second, initial investment and operation and maintenance costs are the first financial and economic aspects that are taken into consideration when a technology is applied. Some technologies need a large amount of initial investment and have high operation costs even though they have larger positive impacts on waste management, such as waste volume reduction and GHG emission reduction. In this regard, WTE is a typical example of such technology. With WTE, GHG emissions from waste treatment are almost zero, and the amount of waste to be finally disposed of is significantly reduced. However, the initial investment and operation and maintenance costs for this technology are much higher.

Finally, the social and environmental aspect involves public acceptance of the technology and its environmental impacts. Some of the technologies evaluated above have already been used, such as composting and recycling. Other technologies are new to the Vietnamese market, for example, WTE, and have not been used in Vietnam. There has not been any public protest against the technologies that have been used which indicates that they are accepted by society. As concerns the environmental impacts, it can be

said that all of the technologies evaluated that have been designed to treat waste properly do not cause any significant negative impacts.

Conclusions

With the multi-criteria assessment, and based on technology options identified in the NDC, eight low-carbon technologies for the waste sector were evaluated and prioritised. Local contexts have been taken into consideration in the prioritisation of the identified technologies. The semi-aerobic landfill technology option was given the highest priority. This is a low cost and relatively simpler than other technologies and it contributes to GHG emission reduction. As the standard for the construction and operation of sanitary landfills, a semi-aerobic landfill is technologically and economically suitable in Vietnam if proper technological transfer is undertaken.

The authors declare that there is no conflict of interest regarding the publication of this article.

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Quantification of greenhouse gas emissions from different municipal solid waste treatment methods - case study in Ha Noi, Vietnam

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Abstract:

This study focuses on defining the greenhouse gas (GHG) emissions from treatment of municipal solid waste (MSW) in Ha Noi city. Firstly, the MSW samplings at Nam Son and Xuan Son landfills were collected to identify the components. Based on the statistical data on the amount and ratio of MSW collected, the volume of MSW treated by different technologies was estimated. Then, the GHG emissions were quantified by applying the Intergovernmental Panel on Climate Change (IPCC) 2006 model. The annual GHG released from MSW in Ha Noi in 2017 was 1.1 million tons of CO_{2e} from landfilling, 16.3 thousand tons of CO_{2e} from incineration, and 76,100 tons of CO_{2e} from composting. The GHG emission level from landfills is the highest (327 kg of CO_{2e} per ton of treated waste), followed by composting (189 kg of CO_{2e} per ton), and incineration (115 kg of CO_{2e} per ton). The GHG emissions from landfills comprised nearly 90% of GHG emissions from MSW disposal in Ha Noi. The results also revealed that if there are no measures to recover landfill gas for energy generation, the GHG generated from MSW treatment facilities will also contribute significantly to the greenhouse effect and climate change impact. These research results also supply the basis information for decision-makers to select the appropriate MSW treatment technologies for Ha Noi in the context of increasing population pressure and environmental pollution.

Keywords: composting, greenhouse gas (GHG), incineration, landfill, MSW.

Classification number: 5.1

Introduction

Ha Noi is the capital of Vietnam and is the country's economic and political centre. It covers the second largest area of 3,344.6 km². The population in 2017 was 7.65 million people; 49.2% lived in urban areas and 50.8% in suburban areas, distributed among 12 urban districts, 17 suburban districts, and one town [1]. The recent trend toward urbanization has led to a rapid increase in generation of MSW. Statistics reveal that the amount of MSW in Ha Noi city averages 7,500 tons per day and it is growing by an average of 10-16% per year in urban areas [2]. Currently, MSW in Ha Noi is treated mainly by landfills without gas capture, incineration, and composting [3]. Due to the high ratio of organic matter in landfills, anaerobic decomposition creates a huge amount of CH₄ that causes a greenhouse effect 25 times higher than CO₂. According to 2006 statistics from the Intergovernmental Panel on Climate Change (IPCC) [4], CH₄ generated in landfill sites accounted for approximately 27% of the total greenhouse gas (GHG) and approximately 3-4% of total global GHG emissions.

According to the annual report of the Ha Noi People's Committee on the status of MSW generation and management in Ha Noi city, the total collected and treated MSW in 2017 was an estimated 5,300 tons per day [4], including:

i) Landfilling, which is conducted mainly at Nam Son and Xuan Son landfills. These landfills treat approximately 89.5% of waste collected; with a capacity of 4,000-4,500 tons per day, Nam Son is the largest. The MSW is unclassified at these landfills and no gas capture system has been installed.

ii) Composting, which takes place at Cau Dien, Kieu Ky, and Xuan Son composting plants. However, only 0.5% of the total collected MSW all organic waste is treated by

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this method. The output of these systems is organic humus.

iii) Incineration, which is done at Xuan Son, Thanh Cong, and Phuong Dinh waste treatment plants; with a capacity of 700 tons per day, Xuan Son is the largest. This method treats approximately 10% of MSW generated. Additionally, a recycling method is applied but the ratio is tiny and is mainly done by private companies. Emissions generated from combustion are treated to remove pollutant gases before they are discharged into the air.

Recent studies have examined the GHG emissions resulting from various waste treatment technologies; in 2016, Singh, et al. [5] did so at landfills in India. The research evaluated GHG emissions from three different landfills and showed the potential to generate electricity from landfill gas collection systems. In 2019, Zhang, et al. [6] monitored the GHG emissions from a typical limited-controlled landfill according to the guidance of the UK Environment Agency to obtain representative data from the heterogeneous surface of the landfill. The research had identified the CH_4 and CO_2 emission fluxes from the landfill area. This is advisable to devote more attention to and determine potential solutions for reduction of GHG emissions from a limited-controlled landfill. In 2017, Dong, et al. [7] evaluated GHG emissions from the waste sectors in Hong Kong using IPCC 2006 guidelines. The analysis results indicated that the GHG emissions from landfills decreased while total GHG emissions from the entire waste sector increased, mainly due to emissions from the combustion of petroleum for ignition. It revealed that incineration also contributes to the increase of GHGs in waste treatment. In 2009, Manfredi, et al. [8] accounted for GHG emissions from different landfilling technologies in Denmark; these included open dump, conventional landfills with flares and with energy recovery, and landfills receiving low-organic-carbon waste. The results illustrated that GHG emissions from conventional landfills lacking a CH_4 collection system were the major contribution to the total GHGs. This research also concluded that utilization of landfill gases for electricity generation contributed to reduce environmental impacts from landfilling. Additionally, Ritchie, et al. (2009) [9] compared GHG emissions from landfills with waste-to-energy technologies in Vancouver, Canada. The results indicated that GHG emissions from the waste-to-energy facilities were higher than those of the landfills due to plastics remaining in the waste stream. In 2017, Hwang, et al. [10] estimated GHG emissions at nine different technological incineration facilities in Korea by measuring the GHG concentrations in the flue gas samples. The research indicated that the emissions of IPCC default

values were estimated to be higher than those of the plant-specific emission factors. In 2010, Chen, et al. [11] studied estimates of CO_2 emissions from MSW incineration in Taipei city, demonstrating the correlation between GHG emissions and components of waste. Additionally, Marchi, et al. (2017) [12] applied IPCC 2006 guidelines to calculate GHG emissions from different waste treatment methods. The research results helped to orient emission-reduction strategies and environmental impacts of the waste sector in the central Italy.

In 2014 in Vietnam, Ngan, et al. (2004) [13] conducted a study to calculate CH_4 emissions from MSW in Can Tho city. Based on the city's population size and economic development conditions, predictions were made about the total amount of CH_4 gas to be generated from MSW landfills in 2020. In 2015, Tuyen, et al. [14] estimated CH_4 emissions from municipal waste landfills in Thu Dau Mot city, Binh Duong province. Based on the different scenarios of the MSW management and treatment master plan of the province, the research results assessed the potential for reclaiming and reusing CH_4 gas from waste disposal activities to 2030. In 2014 in Hue city, Tuan, et al. [15] estimated the reduction potential of CH_4 emissions from the landfill and from composting. Based on different scenarios, the study revealed that CH_4 can be reduced by changing from landfilling to composting. Giang, et al. (2013) [16] also applied IPCC 2006 guidelines to evaluate the GHG mitigation potential from MSW treatment in Vietnam via landfilling and composting systems by creating various management scenarios. This research illustrated that GHG emissions from waste treatment can be reduced if energy-recovery methods are applied.

The above-mentioned studies estimated GHG emissions from MSW treatment. However, the authors used only statistical data on MSW proportion or default values from IPCC 2006 without identifying the true data from study areas. In addition, further studies on GHG emissions from the composting method have not been conducted. According to the Vietnamese Prime Minister's Decision No. 609/QĐ-TTg on 25 April 2014, approving a master plan for solid waste disposal in Ha Noi to 2030, with a vision to 2050, the estimate of GHG emissions from various MSW treatment technologies is one of the most important objectives. Therefore, this study was conducted to identify the current MSW components in Ha Noi city and estimate the amount of GHG emissions from different MSW treatment methods. The research results will update the GHG emissions data from the waste sector to help decision-makers select suitable technologies for MSW treatment.

Methodology

Method to determine the MSW composition

The composition of MSW at Nam Son and Xuan Son landfills was determined to provide the input data for calculating GHG emissions from different MSW treatment methods in Ha Noi instead of using the default values from the IPCC 2006 guidelines. The two samplings at each landfill were taken at the same time each day at the burial cells after the trucks dumped their garbage loads and before the garbage was compressed. In this research, the coning and quartering method was applied. The MSW samples were placed in a conical heap. This heap was then divided vertically into four equal parts by two lines at right angles to each other. Two opposite quarters were then mixed with each other into one sample. The two other quarters were discarded. This procedure was repeated until the established sample size reached 150 kg as in the guideline of TCVN 9461:2012, the standard test method for determining the composition of unprocessed municipal solid waste in Vietnam [17]. Large or long objects were cut into smaller pieces (5-10 cm) before the sample was taken for sorting. The samples were manually classified and separated into 11 components (food, biodegradable organic matter, garden waste, paper, cardboard, wood chips...) according to the classification of IPCC 2006 [5] and the Vietnamese system [18].

Methods to qualify GHG emissions

Landfill:

The CH_4 and CO_2 emissions in landfills derive mainly from the decomposition of organic components. In this study, the IPCC 2006 method was selected for calculating the amount of CH_4 generated. This method assumes that the degradable organic carbon (DOC) composition will decompose slowly over many years (approximately 10), and that CH_4 is formed during that period. In stable conditions, the CH_4 produced depends mainly on the amount of carbon accumulated in burial cells. CO_2 emission was not included in the IPCC 2006 method because it had been calculated in the Agriculture, Forest, and Land Use Sector (AFOLU). According to the IPCC 2006 (Chapter 3, Volume 5) [19], CH_4 emission from landfills after one year is calculated as in Equation (1):

$$\text{CH}_{4\text{emission}} = \left\{ \sum_x \left[\text{MSW}_x \times L_0(x) \times \left((1 - e^{-kt}) \times e^{-k(t-x)} \right) \right] - R(t) \right\} \times (1 - \text{OX}) \quad (1)$$

where $\text{CH}_{4\text{emission}}$ is CH_4 emitted in year t (tons/year), MSW_x

is mass of waste deposited in year t (tons/year), L_0 is the CH_4 generation potential (tons) (calculated by Equation 2), t is inventory year, x is opening year of disposal site or first year of data available, k is reaction constant ($k = \ln(2)/t_{1/2}$ (year⁻¹), $t_{1/2}$ is half-life time (y), $R(t)$ is recovered CH_4 in year t (tons/year), and OX is oxidation factor in year t .

Data on the amount of MSW in landfills from 2007 to 2017 were collected from the annual report of the Ha Noi People's Committee [20] and the Ha Noi GHG emission inventory report in 2015 [21]; they are illustrated in Fig. 1. The amount of MSW gradually increased over the years in line with the growing population.

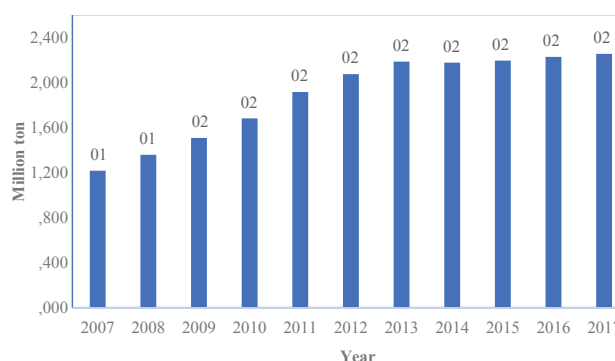


Fig. 1. Amount of MSW treated in landfills from 2007 to 2017 [20, 21].

CH_4 generation potential (L_0) is calculated by Equation (2):

$$L_0 = \text{MCF} \times \text{DOC} \times \text{DOC}_f \times F \times \frac{16}{12} \quad (2)$$

where MCF (Methane correction Factor) is the CH_4 correction factor for aerobic decomposition in the year of deposition, DOC is degradable organic carbon in the year of deposition (tons C/tons waste) (calculated by Equation 3), DOC_f is the fraction of DOC that can decompose, F is the fraction of CH_4 in generated landfill gases, and $16/12$ is the molecular weight ratio of CH_4/C .

$$\text{DOC} = 0.15A + 0.2B + 0.4C + 0.43D + 0.24E + 0.15F \quad (3)$$

where A is food waste (%); B is garden waste (%); C is pulp, paper, and cardboard (%); D is wood and wood products (%); E is rags (%); F is diapers (%). These above ratios are used from the true survey data.

Other fractions (DOC_f , F , MCF , OX , and k) are used from the default value of IPCC 2006 for unclarified MSW, in which: $\text{DOC}_f = 0.5$, $F = 0.5$, $\text{MCF} = 0.6$, $\text{OX} = 0.1$, and k is shown in Table 1.

Table 1. Reaction constant (k) [19].

Symbol	Composition	Used value	Symbol	Composition	Used value
A	Food, organic matters	0.4	D	Milled wood	0.035
B	Garden garbage (leaves, twigs, grass...)	0.17	E	Rags	0.7
C	Paper, cartons	0.07	F	Diapers	0.17

Composting:

CO_2 , CH_4 , and N_2O are all by-products of the composting process. As mentioned above, CO_2 emissions from composting were not included in the IPCC 2006 method. CH_4 and N_2O emissions from composting can be estimated by using the default method of IPCC 2006 (Chapter 4, Volume 5) [22] and given in Equations (4) and (5) below:

$$\text{CH}_{4\text{Emission}} = \sum_i (M_i \times \text{EF_CH}_{4i}) \times 10^{-3} - R \quad (4)$$

$$\text{N}_2\text{O}_{\text{Emission}} = \sum_i (M_i \times \text{EF_N}_2\text{O}_i) \times 10^{-3} \quad (5)$$

where $\text{CH}_{4\text{Emission}}$ is total CH_4 emissions in the inventory year (tons/year), $\text{N}_2\text{O}_{\text{Emission}}$ is total N_2O emissions in the inventory year (tons/year), M_i is mass of organic waste treated by biological treatment type i (tons/year), EF_CH_{4i} is the emissions factor for treatment i (gCH_4/kg waste treated), $\text{EF_N}_2\text{O}_i$ is the emissions factor for treatment i ($\text{gN}_2\text{O}/\text{kg}$ waste treated), 10^{-3} is the conversion factor from kilogram to ton, i is composting or anaerobic digestion, and R is total amount of CH_4 recovered in the inventory year (tons/year).

Currently, Ha Noi conducts composting only as a biological treatment method. The composted waste is organic matter with a certain moisture content; it is necessary to ensure proper moisture for microorganisms. Therefore, the default factors of IPCC 2006 guidelines are used for calculation.

Because the MSW treated at the composting plants is moist, the default values of wet weight were chosen for calculation ($\text{CH}_4 = 4$ (gCH_4/kg wet waste) and $\text{N}_2\text{O} = 0.3$ ($\text{gN}_2\text{O}/\text{kg}$ wet waste)). Ha Noi city has no biogas recovery facilities, so the total amount of CH_4 recovered in an inventory year (R) is irrelevant. Due to the lack of statistical data on MSW treated by composting before 2014, this study estimated GHG generation only from composting methods during 2014-2017. The MSW treated by this method is illustrated in Fig. 2. The amount of MSW treated

by composting technology gradually decreased over time due to the unstable fertilizer quality. This, in turn, led to inadequate funds for operation, so private enterprises did not prioritize investment.

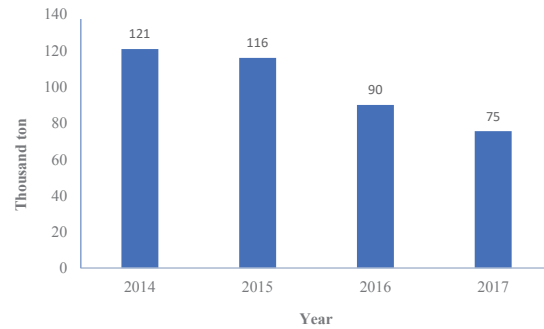


Fig. 2. Amount of MSW treated by composting [21].

Incineration:

In this research, the GHG emissions deriving from incineration are only estimated. The emissions from open burning are not known due to the lack of data. CO_2 , CH_4 , and N_2O emissions from waste incineration are calculated as in Equations (6), (7), and (8), respectively (IPCC 2006, Chapter 5, Volume 5) [23].

$$\text{CO}_{2\text{Emission}} = \text{MSW} \times \sum (\text{WF}_i \times \text{dm}_i \times \text{CF}_i \times \text{FCF}_i \times \text{OF}_i) \times \frac{44}{12} \quad (6)$$

where $\text{CO}_{2\text{Emission}}$ is CO_2 emission in the inventory year (tons/year); MSW is the total amount of MSW as wet weight incinerated (tons/year); WF_i is the fraction of waste type/material of component i in the MSW (as wet weight incinerated); dm_i is dry matter content in the component i of the MSW incinerated; CF_i is the fraction of carbon in the dry matter of component i ; FCF_i is the fraction of fossil carbon in the total carbon of component i ; OF_i is the oxidation factor; $44/12$ is the conversion factor from C to CO_2 (with: $\sum \text{WF}_i = 1$); and i is the component of the MSW incinerated such as paper/cardboard, textiles, food waste, wood, garden (yard) and park waste, disposable nappies, rubber and leather, plastics, metal, glass, and other inert waste.

$$\text{CH}_{4\text{Emission}} = \sum_i (\text{IW}_i \times \text{EF_CH}_{4i}) \times 10^{-6} \quad (7)$$

$$\text{N}_2\text{O}_{\text{Emission}} = \sum_i (\text{IW}_i \times \text{EF_N}_2\text{O}_i) \times 10^{-6} \quad (8)$$

where $\text{CH}_{4\text{Emission}}$ is CH_4 emissions in the inventory year (tons/year), IW_i is the amount of solid waste of type i incinerated (tons/year), EF_CH_{4i} is the aggregate CH_4 emission factor ($\text{g CH}_4/\text{ton}$ of waste), $\text{EF_N}_2\text{O}_i$ is the aggregate N_2O emission factor ($\text{g N}_2\text{O}/\text{ton}$ of waste), 10^{-6} is the conversion

factor from kilogram to ton, and i is the category or type of waste incinerated. Due to the lack of data on CH_4 and N_2O emission factors for each type of waste incinerated, the emission factors from the IPCC 2006 default values ($\text{EF}_{\text{CH}_4} = 0.2 \text{ g/ton MSW}$ and $\text{EF}_{\text{N}_2\text{O}} = 50 \text{ g/ton MSW}$) are used for calculation.

In this study, the Global Warming Potentials (GWPs) from IPCC 2006 [5] are used to change CH_4 and N_2O to CO_2e in which the GWP of $\text{CH}_4 = 25 \text{ CO}_2$ and $\text{N}_2\text{O} = 298 \text{ CO}_2$. These numbers are calculated for a 100-year time horizon. As in the composting case, GHG emissions from incineration were estimated from 2014 to 2017. The amount of MSW treated by this method was collected from the National Environmental Thematic Report in 2017 [2] and the Maintenance Committee of the Technical Infrastructure Works, Ha Noi Department of Construction (Fig. 3). The amount of MSW incinerated increased annually, except in 2017, because the Phuong Dinh and Thanh Cong plants were closed for maintenance.

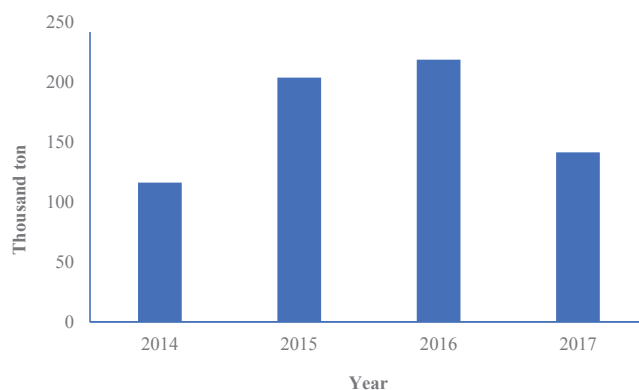


Fig. 3. Amount of MSW treated by incinerators [2].

Because combustion technology applied at incineration plants in Ha Noi (incinerator includes 01 primary combustion chamber, 01 secondary combustion chamber, 02 heat chambers and dust settlements, primary furnace temperature reaches: $800\text{-}900^\circ\text{C}$; secondary furnace temperature reaches $1,200^\circ\text{C}$) is similar to that from the IPCC default, other fractions such as dry matter content in the component i of the MSW incinerated (dm_i), carbon in the dry matter of component i (CF_i), fossil carbon in the total carbon of component i (FCF_i), and oxidation factor ($\text{OF}_i = 100$) from the IPCC 2006 default values are used for calculation (Table 2).

Table 2. Fractions of dm_i , CF_i , FCF_i , WF_i [23].

MSW composition	$\text{dm}_i(\%)$	$\text{CF}_i(\%)$	$\text{FCF}_i(\%)$	WF_i
Food, organic matter	40	38	0	64.2
Garden garbage (leaves, twigs) grass...	40	49	0	6.2
Paper, cartons	90	46	1	3.2
Milled wood	85	50	0	3.4
Rags	80	50	20	2.6
Diapers	40	70	10	1.8
Plastic	100	75	100	2.6
Rubber, leather	84	67	20	2.3
Metals	100	NA	NA	1.6
Glass and porcelain	100	NA	NA	2.5
Other types	90	3	100	9.6

Results and discussion

Composition of MSW in Ha Noi

Table 3 illustrates that the components of MSW are somewhat different between Xuan Son and Nam Son. The proportion of organic waste in Xuan Son (64.2%) is more than that Nam Son (58.4%). This result is consistent with MSW components in Ha Noi reported in the 2016 National Environmental Status Report (54-77%) [24]. It is lower than that of Thu Dau Mot city, Binh Duong province (78.5%) [14], and Can Tho city (80%), while recyclable components are the same [13]. These results may depend on the collected sources; Nam Son landfill receives MSW from metropolitan areas Nam Tu Liem, Bac Tu Liem, Soc Son, Dong Anh, Me Linh, and Thanh Tri districts while Xuan Son treats MSW from Son Tay town and remaining suburban districts. In the urban areas, residents more frequently buy food from supermarkets that has been pre-processed to remove unused parts while suburban residents can harvest directly from the garden. As a result, the garden garbage rate in Xuan Son is twice as high as that in Nam Son, while the rate of recyclable substances such as paper and cartons in Nam Son (6%) is higher than that in Xuan Son (3%). The results tend to be similar for other inorganic waste components. It probably relies on keeping garbage for sale to recycling facilities of suburban residents. Generally, the proportion of MSW components depends on living habits, standards, economic conditions, and the civilization of each region.

Table 3. Composition of MSW in Nam Son and Xuan Son landfills.

No.	Composition	Nam Son (%)	Xuan Son (%)	Average
1	Food, organic matter	58.8	64.2	61.5
2	Garden garbage (leaves, twigs, grass...)	2.8	6.2	4.5
3	Paper, cartons	6.0	3.2	4.6
4	Milled wood	3.6	3.4	3.5
5	Rags	2.9	2.6	2.8
6	Diapers	2.3	1.8	2.1
7	Plastic	3.2	2.6	2.9
8	Rubber, leather	2.3	2.3	2.3
9	Metals	2.0	1.6	1.8
10	Glass and porcelain	3.9	2.5	3.2
11	Sludge	0.2	0.4	0.3
12	Other types	12.0	9.3	10.7

Quantification of GHG emissions from different MSW treatment methods

GHG emissions from landfills:

CH₄ emissions at landfills in Ha Noi city are calculated according to Equations (1), (2), and (3) in which the Degradable Organic Carbon (DOC) values (Table 4) were calculated based on the average rate of each component of MSW in Xuan Son and Nam Son landfills and the default coefficient values in the IPCC 2006 [19]. Because of the lack of data on MSW composition in the past, the field survey results in the research are used to calculate CH₄ emission from landfills in 2007-2017.

Table 4. DOC value.

No.	Symbol	Composition	DOC (%)
1	A	Food, organic matter	8.8
2	B	Garden garbage (leaves, twigs, grass...)	0.6
3	C	Paper, cartons	2.4
4	D	Milled wood	1.6
5	E	Rags	0.7
6	F	Diapers	0.5
DOC = 0.15A + 0.2B + 0.4C + 0.43D + 0.24E + 0.24F			14.6

With the input parameters of the IPCC 2006 model determined, the calculation results revealed that CH₄ emissions increase with time and amount of MSW buried.

CH₄ in year t is generated from the biodegradation of organic ingredients that existed in landfills in previous years. With the calculation starting from 2007, the results are presented in Table 5.

Table 5. CH₄ generated at landfills from 2008-2017 (in tons).

Year	CH ₄	CO _{2eq}
2008	7,626	190,650
2009	13,692	342,300
2010	18,750	468,750
2011	23,302	582,550
2012	26,884	672,100
2013	30,467	761,675
2014	33,692	842,300
2015	36,894	922,350
2016	39,244	981,100
2017	41,100	1,027,500
Total	271,651	6,791,275

The results revealed that, in 2008, approximately 7,626 tons of CH₄ was emitted per year, equivalent to 190,650 tons of CO_{2e} per year. In 2017, the amount of CH₄ emission was 41,100 tons per year, equivalent to 1,027,500 tons of CO_{2e} per year. The total amount of CO_{2e} emissions in the period 2007-2017 was 6,791,275 tons. The calculation result reveals that food waste was the main source of CO_{2e} emissions accounting for 90% of total CO_{2e} emissions into the environment. The remaining waste components such as paper, wood, and cloth accounted for only 10% of total CO_{2e} emissions. If no gas recovery methods or measures to minimize GHGs generated from landfills are implemented, these emissions will increase the greenhouse effect and exacerbate climate change.

Based on the total amount of MSW landfilled [20, 21] and the total estimated GHG amount from 2008 to 2017, GHG emissions from the landfills in Ha Noi would be 327 kg of CO_{2e} per ton of MSW treated. This value is nearly same as the case study of conventional landfills in Denmark (300 kg of CO_{2e} per ton) [6]; is lower than that from the Vancouver landfill (382 kgCO_{2e} per ton) in Canada [25]; and is higher than that in China (259.5 kg of CO_{2e} per ton) with a biodegradable fraction (almost 60-70%) [26]. This difference is due to the waste properties, weather

characteristics, and various infrastructures in these research areas.

GHG emissions from composting:

Composting is an aerobic process in which a large fraction of DOC in the waste components is converted into CO₂ [22]. CH₄ is formed because anaerobic digestion takes place in the compost pile when not enough oxygen is supplied. Composting releases CH₄ from 1% to a few percent of the initial carbon content and N₂O from 0.5% to 5% of the initial nitrogen content. Poor composting is likely to produce more of both CH₄ and N₂O [14]. By applying Equations (4) and (5), CH₄ and N₂O generated by composting are displayed in Table 6.

Table 6. Total amount of CH₄ and N₂O generated by composting (in tons).

Year	CH ₄	CO _{2e} from CH ₄	N ₂ O	CO _{2e} from N ₂ O	Total CO _{2e}
2014	483	12,075	36.22	10,794	22,869
2015	463	11,575	34.72	10,347	21,922
2016	360	9,000	26.97	8,037	17,037
2017	302	7,550	22.63	6,744	14,294
Total	1,608	40,200	120.54	35,922	76,122

Table 6 illustrates that total CH₄ emissions from 2014 to 2017 amounted to 1,608 tons (equivalent to 40,200 tons of CO_{2e}); N₂O emissions amounted to 120.54 tons (equivalent to 35,922 tons of CO_{2e}). The total amount of CO_{2e} generated in 2017 decreased by 37% compared to 2014. The reason is that the MSW treated by composting decreased due to high investment and operational costs but low income from the sale of composting fertilizer. Based on the total amount MSW composted and the total GHG generated annually from 2014 to 2017, the GHG emissions resulting from composting facilities in Ha Noi would be 189 kg of CO_{2e} per ton of MSW treated. This value is within the GHG emissions range (3.2-262 kg of CO_{2e} per ton of MSW) from the research results of Melissa, et al. (2017) [25] in Panama with the same composting technology and waste humidity.

GHG emissions from incineration:

Equation (6) was used to estimate CO₂ generated from incinerators. Because incineration is mainly implemented in Xuan Son, the clarification results from the Xuan Son landfill are used for calculations in this case. The CO₂ emissions from MSW incineration are presented in Table 7.

Table 7. CO₂ emissions from incineration (in tons).

MSW composition	2014	2015	2016	2017
Food, organic matter	-	-	-	-
Garden garbage (leaves) twigs, grass...	-	-	-	-
Paper, cartons	56	99	106	69
Milled wood	-	-	-	-
Rags	884	1,549	1,664	1,076
Diapers	214	375	403	261
Plastic	8,288	14,522	15,596	10,085
Rubber, leather	1,100	1,928	2,071	1,339
Metals	-	-	-	-
Glass and porcelain	-	-	-	-
Other types	1,102	1,930	2,073	1,340
Total	11,645	20,404	21,912	14,169

Total CO₂ emissions from incinerators during the period 2014-2017 were 68,000 tons, with the highest in 2016 (21,912) and the lowest in 2014 (11,645). In the comparison of different MSW components, burnt plastic generates the highest CO₂ emissions by years; the total CO₂ emission from plastic in four years was 48,500 tons, which accounted for 71% of total CO_{2e} emissions.

Equations (7) and (8) were applied to estimate CH₄ and N₂O emissions from incineration. The results are displayed in Table 8.

Table 8. CH₄ and N₂O emissions from MSW incineration (in tons).

Year	CH ₄	CO _{2e} from CH ₄	N ₂ O	CO _{2e} from N ₂ O	Total CO _{2e}
2014	0.023	0.580	5.796	1,727	1,728
2015	0.041	1.016	10.155	3,026	3,027
2016	0.044	1.091	10.906	3,250	3,251
2017	0.028	0.705	7.052	2,102	2,102
Total	0.136	3.392	33.909	10,105	10,108

From 2014 to 2017, total emissions of CH₄ and N₂O were 136 kg of CH₄ (~3.4 tons of CO_{2e}) and 3.391 tons of N₂O (~10,105 tons of CO_{2e}); the total CO_{2e} generated was 10,109 tons. The amount of CO₂ is the main GHG emission from incineration; it accounts for 87% of total GHG

emissions from incineration. On average, CO_{2e} emitted from this treatment method is 115 kg of CO_{2e} per ton of waste. This value in Korea is 134±17 kg of CO₂ per ton of waste [11]. It is a bit larger than that in the Ha Noi case. The GHG emissions at incineration plants are different due to operational systems (i.e., stoker, fluidized bed, moving grate, rotary kiln, and kiln and stoker), therefore, this result is valid only for the current case. If, in the future, Hanoi invests new waste incinerator systems with other technologies, the GHG generated on the volume of waste treated should be re-estimated to avoid errors.

The GHG emission levels of the different MSW treatment methods in Ha Noi are presented in Fig. 4. The figure illustrates that landfills generate the highest amount of GHG emissions, 1.94 times higher than composting and 3.19 times higher than incineration. The reason is that the anaerobic process at landfills continues to happen after the cell is fully filled. In addition, the MSW is usually treated immediately after it is transported to the composting and incineration facilities. These data illustrate that landfills will contribute more significantly to long-term environmental impacts than other MSW disposal methods.

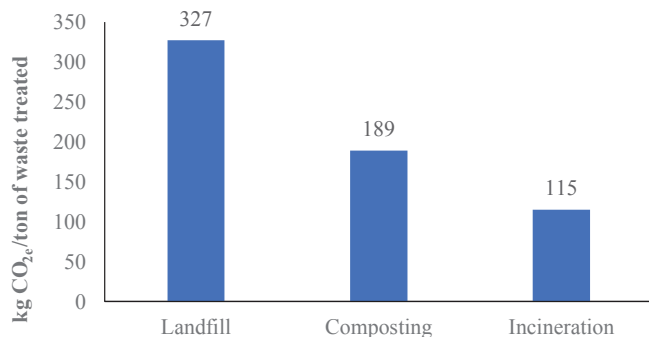


Fig. 4. CO_{2e} emissions from the different treatment methods.

Conclusions

The research results demonstrate that organic waste is the main component of MSW in Ha Noi (61.5%). Currently, Ha Noi has three main MSW treatment methods; landfilling accounts for approximately 89.5% of the total amount of waste collected, followed by incineration (10%), and finally composting (0.5%). The GHG released from MSW treatment in Ha Noi city in 2017 was 6.7 million tons of CO_{2e} from landfills, 16,300 tons of CO_{2e} from incineration, and 76,100 tons of CO_{2e} from composting. The GHG emissions from landfills is the highest (367 kg of CO_{2e} per ton of waste treated), 1.94 times higher than that from composting (189

kg of CO_{2e} per ton) and 3.19 times higher than that from incineration (115 kg of CO_{2e} per ton). The GHG emissions from landfills comprise nearly 90% of GHG emissions from MSW disposal activities in Ha Noi. The results also indicate that if no gas-recovery measures (especially on CH₄) are introduced for energy production, the GHG generated from MSW treatment facilities will contribute significantly to the greenhouse effect and exacerbate climate change. These research results provide the basis information for decision-makers to consider when determining appropriate MSW treatment technology for Ha Noi in the context of increasing population pressure and environmental pollution.

The author declares that there is no conflict of interest regarding the publication of this article.

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The current state of domestic water consumption and the feasibility of implementing a rainwater harvesting system in the coastal zone of the Vietnamese Mekong delta: the case study of the Vinh Chau town, Soc Trang province

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Abstract:

The research was conducted to assess the feasibility of the installation of a rainwater harvesting system for households facing difficulties in using tap water and groundwater for domestic purposes in a coastal area of the Vietnamese Mekong delta - the case study of the Vinh Chau town, Soc Trang province. Direct household interviews and literature review were executed to comprehend the current state of domestic water consumption. According to the research results, Vinh Chau was among the towns seeing moderate rainfall in the delta (~ 1,776 mm/year), and rainwater was used frequently by the local people. However, local households did not employ proper harvesting techniques, hence the harvested rainwater did not meet the required quality for domestic use. Additionally, difficulties in using tap water, groundwater, and surface water were notable. The quality of tap water was not suitable for household consumption because of the presence of aluminum, and the price was high relative to the household's income. Salinization of surface and groundwater, as well as degradation in quantity of ground water, made water of acceptable quality scarce. The research results also indicated that the use of rainwater harvested by a proper system demonstrated a higher cost efficiency than that of other water resources. Therefore, the installation of a rainwater harvesting system for water supply should be encouraged in order to provide a safe and efficient alternative water source for domestic use, and to contribute to the relief of domestic water-related issues and pressure on groundwater extraction in the study area.

Keywords: domestic water use, rainwater, rainwater harvesting system, Vinh Chau town.

Classification number: 5.2

Introduction

The Vietnamese Mekong delta (VMD) is one of the regions in the world projected to be seriously affected by climate change (CC), especially in its coastal areas [1-3]. Under conditions of CC, sea-level rise - and the resulting effects of saline intrusion, tide-induced flood, drought, and degradation of water quality - shortages in water for domestic use have become more commonplace in coastal areas of the VMD. According to the prediction of [4], the percentage of the population in the rural area impacted by saline intrusion will increase from 39.5% in 2012 to 41.4, 45.3, and 47.6% in 2020, 2030, and 2050, respectively.

Not only does the VMD face the degradation of groundwater - both in quality and quantity - but it also suffers from surface water pollution. Several water quality indicators revealed that physical, chemical, and biological pollutants exceeded the allowed standard. In the VMD's coastal regions (e.g., Soc Trang, Bac Lieu, Ca Mau, and Kien Giang provinces), groundwater has been exploited for various purposes, leading to significant drops in groundwater tables in recent decades [5]. According to [6], the groundwater table of Tra Vinh and Soc Trang provinces declines on average from 4 to 9 meters in the dry season. Moreover, groundwater exploitation has resulted in land subsidence in the delta. It is estimated that groundwater withdrawal has caused the VMD to sink approximately 18 cm over the past 25 years, with some areas exceeding 30 cm [7].

Conversely, rainwater is abundant in quantity and is of suitable quality for domestic use in the VMD [8]. While

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researching rainwater quality at the College of Environment and Natural Resources (CENRes) and the Institute Research Institute for Climate Change, scientists from Can Tho University indicated that the quality of rainwater is good, meeting the national standard (QCVN 02:2009/BYT) in physio-chemical indicators, except for turbidity and coliforms. Consequently, rainwater can be an acceptable alternative water source if it is properly harvested and treated.

Nevertheless, most people in the rural areas of the VMD have not collected rainwater properly (without first flushing* or filtering), and therefore have not met the requirement for domestic water quality [4]. According to the research of [9], 4.3% of the population in the VMD harvested standard rainwater for domestic use in 2012. In short, harvesting and using rainwater should be given more attention in order to meet current water demands, adapt to CC, and mitigate the impacts of groundwater extraction in the VMD's coastal areas [7]. For these reasons, the research was aimed at assessing the feasibility of implementing rainwater harvesting systems in the Vinh Chau town, Soc Trang province, that offer opportunities to use alternative water sources to meet the increasing water demands under the impacts of CC.

Materials and methods

Data collection

Secondary data: monthly rainfall data over the period between 2010 and 2015, the current state of utilization and extraction of domestic water in the Vinh Chau town collected from the Vinh Chau census data, reports from the Department of Natural Resources and Environment in the Soc Trang province, and scientific articles on water resources in the Soc Trang province and the Vinh Chau town (e.g., [10, 11]).

Primary data: the current state of the use of tap water, groundwater, surface water, and rainwater for domestic activities was collected from interviews with 35 households in the Hoa Dong commune, Vinh Chau town. The criteria for selecting the study area and interviewees are listed in Table 1. The map of the study area is illustrated in Fig. 1.

*The action of flushing initial runoff water. In this phase, rainwater could be significantly polluted by contaminants on the roof.

Table 1. The criteria to select studied area and interviewees.

No.	Contents	Criteria	Households
1	Location	Commune and households that: • Are located far from the center of the Vinh Chau town • Have difficulties in domestic water use	
2	Water resources for domestic use	• Groundwater • Tap water • Surface water • Rainwater	35
3	Economic conditions	• Belong to various social groups (low, fair, and high)	

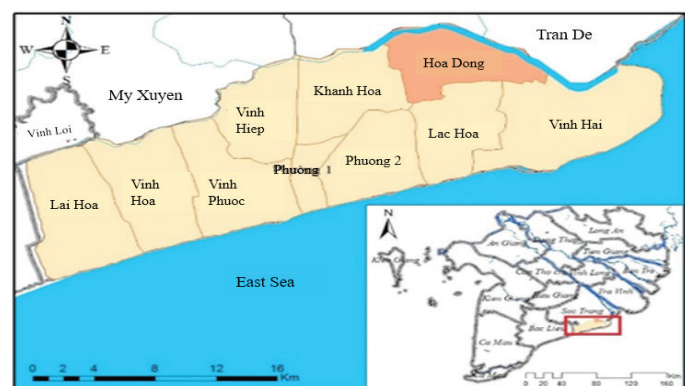


Fig. 1. Studied area in the Hoa Dong commune, Vinh Chau town, Soc Trang province.

Analytical methods

Rainfall data were gathered and plotted on a graph to reflect general trends. The reports were cited for a general evaluation of the current state of water resources for domestic use in the Vinh Chau town.

Primary data were coded and analyzed by descriptive statistics via the Microsoft Excel software to conduct the analysis and assessment of the current situation, needs, and difficulties in the use of water and economic efficiency of the rainwater harvesting system. Calculated figures were indicated in average and percentage values or graphs and tables.

The rainwater harvesting system

Rainwater harvesting systems for domestic water supply were piloted in two households in the Hoa Dong commune, Vinh Chau town, Soc Trang province, and the criteria to select these two households are listed in Table 2. Selected households had representative characteristics of roof and water usage patterns in the study area; therefore, results from the pilot systems were representative for the entire study area.

Table 2. The criteria to select the two household case studies.

No.	Contents	Criteria
1	Location	In areas facing qualitative and quantitative difficulties in domestic water use.
2	Conditions of roof	According to [8], suitable roofs for rainwater collection were made from: Corrugated iron Tile New thatched-roof (a thatched roof over one year old was not suitable for rainwater catchment for drinking)
3	Willingness	Willing to support the research team in installing the systems and providing the necessary information.

Research of the rainwater quality of [8] revealed that the main indicators for not meeting the water quality standard when rainwater flows over rooftops were turbidity and microorganisms. Therefore, the rainwater harvesting system was designed to treat collected rainwater by a physical mechanism. This design is based on the guideline document

of CENRes and the Research Institute for Climate Change, Can Tho University [8], including (Fig. 2A): gutter(s) (1), primary filter(s) (2), a first flush (3), primary container(s) (4), a filter (5), and secondary container(s) (6). Harvested rainwater flows through these parts respectively.

The primary filter removes litter and leaves from the rooftop and gutter. In the first flush (Fig. 2B), rainwater that is polluted with ambient air and rooftop contaminants is retained in the storage chamber equipped with a floating ball inside. If the chamber is filled with water, the floating ball rises to lock the chamber and diverts rainwater from entering the primary container.

The filter (Fig. 2C) is a plastic barrel of 460 mm in diameter, including a 100 mm layer of gravel, a 200 mm layer of sand, a 200 mm layer of charcoal, and a 100 mm

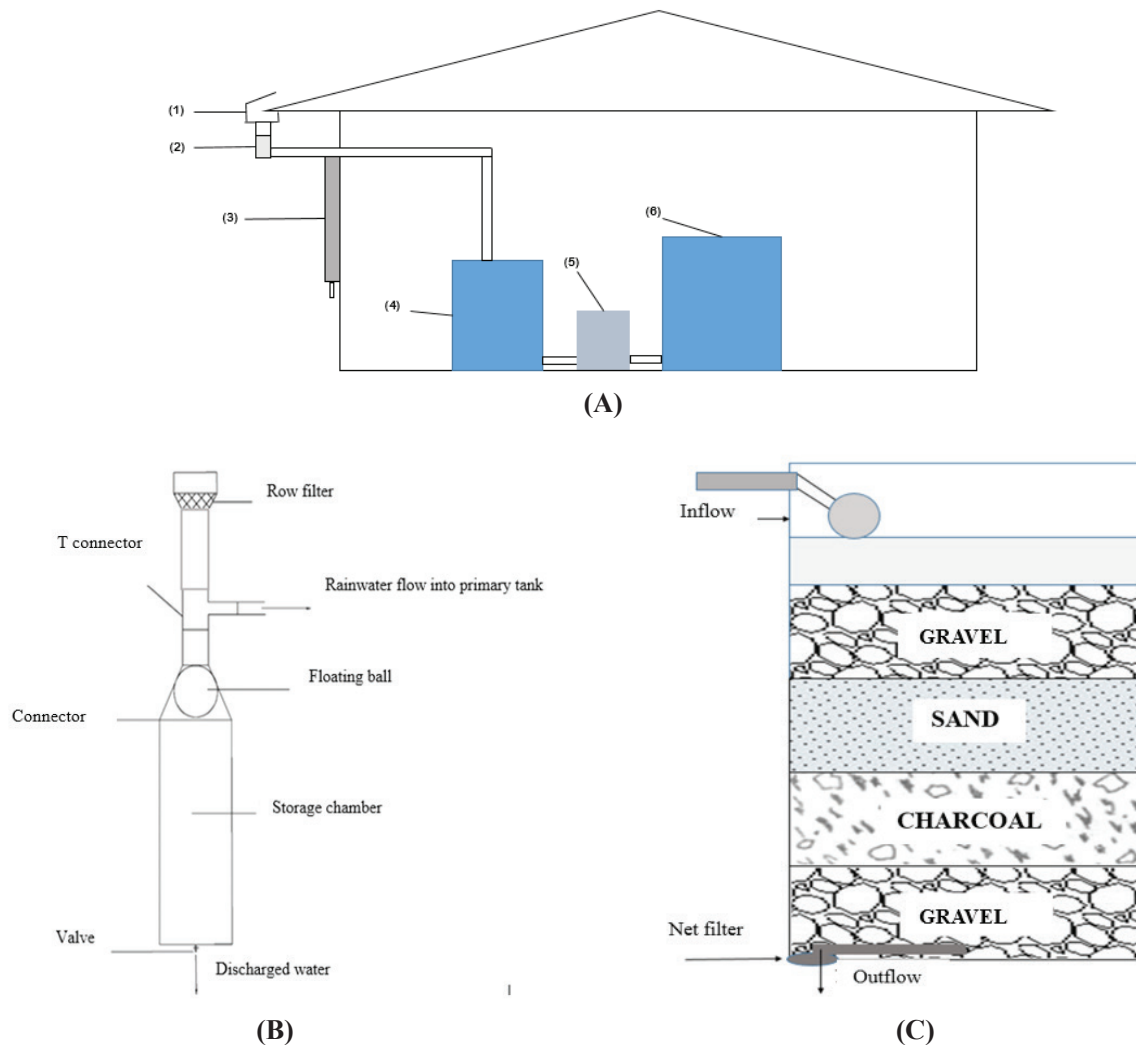


Fig. 2. The rainwater harvesting system (A), the first flush (B), and the filter (C).

layer of gravel. When rainwater flows through spaces between these filtering materials, suspended solids and microorganisms in rainwater are filtered out.

The harvested rainwater can be used for domestic purposes requiring high water quality. It is encouraged that harvested rainwater should be boiled for drinking purposes. Households should harvest rainwater after the first four to six rains of the rainy season. In terms of management, operation, and maintenance, hygiene of the parts and rainwater quality should be checked regularly. The rooftop and gutter require cleaning before the rainy season as well as regular checks during the harvesting season to ensure that rainwater quality is not affected. Additionally, the primary filter, pipes, and the first flush also need to be checked to ensure proper water circulation. The filtering material in the filter should be washed before harvesting by letting sufficient water go through it; these material should be renewed every six months to two years. Containers should be closed and washed when they are not in use to ensure the quality of rainwater. Moreover, users should take water by the tap to avoid infection.



Fig. 3. The 2 pilot rainwater harvesting system in Hoa Dong commune, Vinh Chau town.

According to [8], values of indicators in the rainwater such as turbidity, suspended solids, total coliform, and *E. coli* were significantly reduced after the water was filtered through the system. Harvested rainwater could be used for domestic activities such as food preparation, dishwashing, or other activities requiring high water quality [8]. Therefore, this research focused on assessing the system's economic efficiency and ability to meet the water demands as well as the satisfaction of households. The assessment was based on interviews with 35 households about the installation and consumptive cost of groundwater and tap water and the actual

installation cost of the rainwater harvesting system. The pilot rainwater harvesting systems are illustrated in Fig. 3.

Results and discussion

Rainfall and rainwater quality in the Vinh Chau town

Vinh Chau, a coastal town in the Soc Trang province, has a tropical monsoon climate with two distinct seasons - a rainy season from May to November and a dry season from December to April. Vinh Chau is among the towns in the VMD that experience a moderate amount of rainfall. The annual rainfall from 2010 to 2015 was 1,776 mm/year [12]. The monthly average rainfall in the 2010-2015 period is illustrated in Fig. 4.

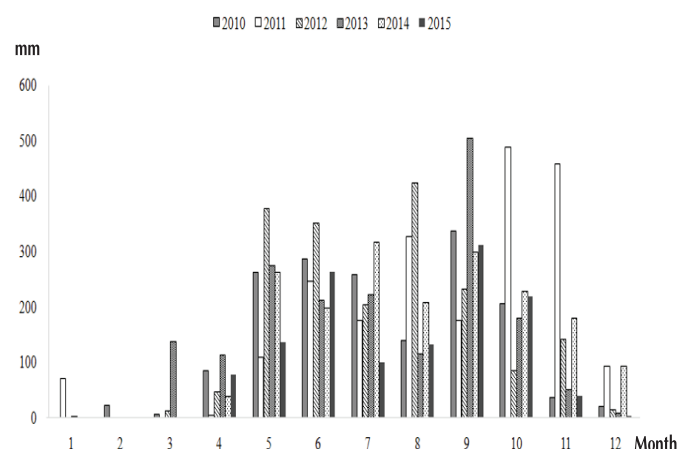


Fig. 4. Monthly average rainfall in the Vinh Chau town from 2010 to 2015.

Limited data about rainwater quality was available for the Vinh Chau town, therefore, we used available data from another province in the VMD with a similar climate but more activities affecting rainwater quality (transportation and industry) - the Can Tho city. The research into rainwater quality conducted by CENRes and the Research Institute for Climate Change from 2011 to 2013 revealed that rainwater in the Can Tho city was of rather good and stable quality. Moreover, the quality of directly harvested rainwater nearly met the QCVN 02:2009/BYT standard for domestic use (except for turbidity and coliforms indicators) [8] (Table 3). The research of [8] demonstrates analyzed results of rainwater quality. A significant proportion of respondents believed that the rainwater quality was good while a small number doubted the rainwater quality (Fig. 5). This led to the conclusion that the conditions of rainfall and rainwater quality in the Vinh Chau town were suitable for domestic use if rainwater was harvested properly.

Table 3. Rainwater quality indicators in Can Tho.

Indicators	Unit	Results	Rainwater quality compared with	
			Drinking water (QCVN 01/2009-BYT)	Domestic water (QCVN 02/2009-BYT)
pH		6.26	Meet	Meet
Turbidity	NTU	2.86	Not meet	Meet
TDS	mg/l	3.94	Meet	
SS	mg/l	2.11		
Microorganism				
Coliforms	MPN/100 ml	39	Not meet	Meet
E. coli	MPN/100 ml	0	Meet	Meet
Nitrate (NO_3^-)	mg/l	0.08	Meet	Meet
Nitrite (NO_2^-)	mg/l	0.01	Meet	Meet
Ammonia (NH_4^+)	mg/l	0.1	Meet	Meet
Heavy metal: As, Cr, Cu, Cd, Al, Hg, Ni, Mn	mg/l	Not found	Meet	Meet
Heavy metal: Pb, Fe, Zn	mg/l	Very low concentration	Meet	Meet
Total organic substance: Benzene, PAH	$\mu\text{g/l}$	Not found	Meet	Meet
Radioactive substance	pCi/l	Not found	Meet	Meet

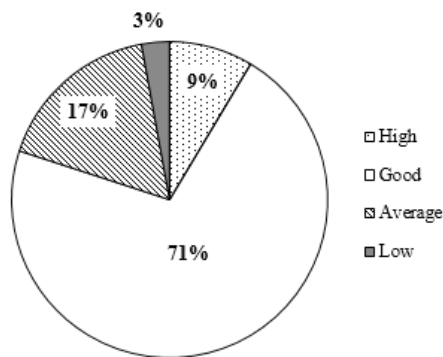


Fig. 5. Rainwater quality assessment.

Current state of domestic water consumption

Tap water (83%), rainwater (94%), groundwater (29%), and surface water (3%) are the main water sources for local domestic activities (Fig. 6). Most households use two or more water sources to ensure that there is sufficient water for their needs and to reduce water consumption expenditures, mostly coming from tap water. On average, there are about four to five members of each household in the Hoa Dong commune with daily water consumption of approximately 117 liters/person/day. More than half of the respondents said their domestic water demands have increased over the past five years. In the next five years, the majority believe

their water demands will remain stable while 17% expect them to increase for various reasons. These reasons include, among others, more family members, warmer weather, and more convenient use of tap water (Fig. 7). The findings strengthen the need to find a sustainable source of water to meet the increasing household water demand, particularly in the context of the degradation of groundwater and salinity intrusion.

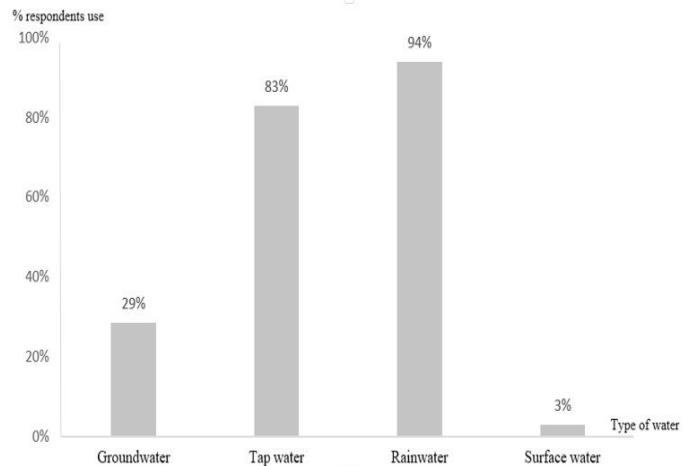


Fig. 6. Water sources for domestic water use.

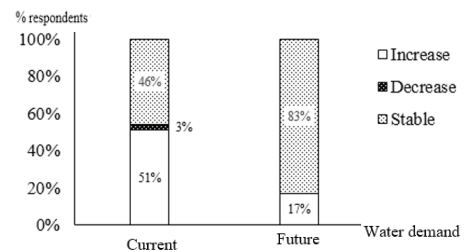


Fig. 7. Current and future water demands.

Households in the Hoa Dong commune face considerable difficulties in the use of domestic water. The market price of tap water is considered high relative to the low income of the local people. However, the quality of the tap water does not meet the proper standards for domestic use - 62% of surveyed households indicate that the aluminum characteristic of the tap water affected their water use. Instead, the households use rainwater for drinking purposes over tap water due to their concerns about health. Local water consumers must measure alum settlement or discharge aluminum water to ensure water quality, causing additional financial cost and wasting time. Moreover, loss of water use entirely occurs frequently in the dry season, leading to a discontinuity in domestic activities. Each water outage lasts five to 30 days, from several hours to all day.

The groundwater in the Hoa Dong commune is not suitable for domestic water consumption because of its salinity. Although the quality remains stable, the quantity of this groundwater resource has declined in recent years [11]. Most households (86%) reported that the flow rate of the pumped water was much lower than it was five years ago, especially in the dry season. These problems cause difficulties for households as they must increase the pumping time, use supporting equipment, and buy fresh water - causing a rise in living expenses.

Surface water in almost all canals in the Vinh Chau town are polluted by saline intrusion, alum affection, and wastewater from domestic activities, livestock farming, and aquaculture [11]. Although the quality of surface water does not meet the standard for domestic use, households in remote areas that are not served by the water supply system - especially the poor - continue to use surface water. They usually use it directly or simply take an aluminum settlement treatment, which is considered not safe for health.

In the study area, most households use rainwater as another domestic water source during the rainy season and store it for drinking purposes during the dry season. There are several reasons that households use rainwater, such as cost savings (82% of respondents), relative good quality (76% of respondents), high rainfall (45% of respondents), and not having any other water resources (3% of respondents). In general, the principal reasons for rainwater harvesting are the cost and the quality of rainwater. In contrast to tap water and groundwater, rainwater is a low-cost water resource used in the rainy season to save expenses. As explained above, the quality of rainwater in the study area is relatively good. However, the reasons that households do not use rainwater are the lack of storage facilities, the presence of mosquitoes in the rainwater container, and the lack of necessity because of the ready availability of tap water.

In contrast to the 18% of households who do not experience any difficulties in using rainwater, the majority of households do face such problems for domestic activities. These include an inadequate amount of rainwater to use in the dry season (48%), the time and effort in preparation for consuming (18%), low rainfall (15%), a lack of storage facilities (9%), and the presence of mosquitoes in the tank (3%).

Rainwater is usually harvested from July to November every year. Therefore, most households only use rainwater

during the rainy season because of a lack of storage facilities to meet the entire family's water demands year-round. Many households find that it takes much time and effort to harvest rainwater by the traditional techniques as they must wait for heavy rain to collect water or take time to discharge rainwater at the beginning of each rain. Additionally, they must place a sheet on the storage tanks to filter litter and dust, then wait for the suspended solid settlement and transfer the fresh water to another tank for use.

In general, the collecting techniques of local people are simple but they take time and effort. Although the directly collected rainwater is of rather good quality, the rainwater is contaminated in indicators such as turbidity, suspended solids, dissolved solids, and microorganisms after flowing over the roof. The causes of pollution can be dust, moss, cat feces, or bird droppings from the roof or gutter. Most of the interviewed households (74%) said they would like to have a more reliable and effective rainwater harvesting system than the current traditional methods.

The efficiency of the rainwater harvesting system

The economic efficiency of rainwater use is higher than that of tap water and groundwater in certain aspects because of the low installation cost and the lack of consumptive cost (Table 4). The cost for installing groundwater wells, including drilling wells and pumping facilities, ranges from 400,000 VND to 6,000,000 VND (depending on the time of installation and depth). Conversely, this cost for tap water ranges from 200,000 VND to 2,000,000 VND (depending on the time of installation and incentive policies). The investment cost for a rainwater harvesting system is 310,000 VND for the first flush and the filter and 2,000,000 VND for the storage tank (plastic, 2 m³). The monthly consumptive cost of tap water is between 10,000 VND and 200,000 VND (over 5,000 VND/m³) and the monthly consumptive cost for groundwater is from 40,000 VND to 200,000 VND. However, there is no cost at all when using rainwater. These figures illustrate that if households implement a rainwater harvesting system, they can save on water consumptive expenses. However, the use of rainwater is seasonal and depends on the weather. If households wish to use rainwater year-round, they must ensure sufficient storage capacity to meet the demand. However, large reservoirs occupy a significant amount of space and come with high investment costs. Therefore, there should be consideration of tank volume for economic efficiency.

Table 4. Installation and use cost of the three water resources.

Water sources	Installation cost (VND)	Use cost (VND/month)
Groundwater	400,000-6,000,000	40,000-200,000
Tap water	200,000-2,000,000	10,000-200,000
Rainwater	2,310,000	0

The pilot households are located in the area with saline-affected groundwater. Therefore, tap water is their main water resource. However, the acidic nature of tap water also causes many difficulties for domestic activities. The total water demand of the households is approximately 127.75 m³/year and the total amount of rainwater collected meeting the demand for use is approximately 90 m³/year. In short, a rainwater harvesting system can meet 70% of a household's water demand. According to the research results, this system provides convenience and safety for rainwater use. Moreover, the first flush and the filter method help households avoid wasting time and effort in collecting rainwater as with the traditional methods. After it is filtered through the system, rainwater can be used directly. The system provides an alternative water source to households, decreasing the demand for and the cost of using tap water and groundwater. Because the first flush and the final water container are close in proximity, dust or litter contamination is minimized. Harvested rainwater using this system results in a better quality than with traditional rainwater harvesting techniques.

Conclusions and recommendation

The Vinh Chau town experiences moderate rainfall in the VMD (about 1,776 mm/year) and relatively good rainwater quality, suitable for harvesting for domestic purposes. In the study area, difficulties in the use of tap water, groundwater, and surface water are remarkable. The quality of tap water is not suitable for household consumption because of the aluminum effect and the price is high relative to the typical household's income. Salinization of surface water and ground water and degradation in quantity of groundwater also pose difficulties for the local people. Rainwater is also used for domestic activities, especially for drinking water purposes. However, local households do not employ harvesting techniques properly, hence the harvested rainwater does not meet the required quality for domestic use purposes.

This rainwater harvesting system is proposed to be widely installed in other areas with similar socioeconomic development and physical settings due to its safety and economic efficiency. This system helps households save time, money, and efforts in harvesting the rainwater. Moreover, it can remove contaminants and microorganisms, thereby significantly improving the quality of harvested

rainwater. Therefore, the installation of this system offers an alternative water source for local households facing difficulties in accessing other water sources - tap water and groundwater in particular - and for households in the coastal area facing salinization of groundwater in general. A larger pilot study in different areas in the VMD should be conducted to provide higher validity on the outcomes, water quality, consumption, and cost comparison. In the short term, more research is needed on rainwater harvesting methods in areas that are threatened; in the longer term, the goal is to provide access to fresh water resources for domestic use and contribute to the relief of domestic water-related issues and pressure on groundwater extraction.

The authors declare that there is no conflict of interest regarding the publication of this article.

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