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Single port laparoscopic colectomy for colonic cancer

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1. Mathematics and Computer Science

- 1.1. Mathematics
- 1.2. Computer Science
- 1.3. Computational Science

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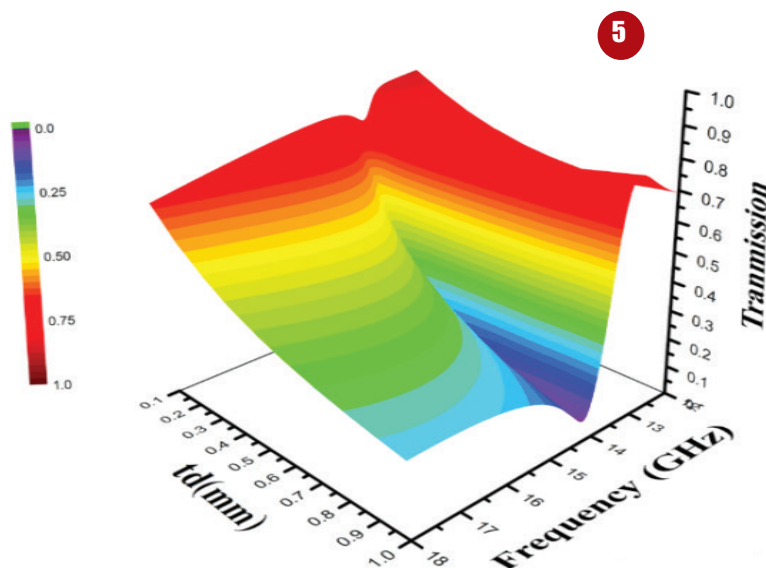
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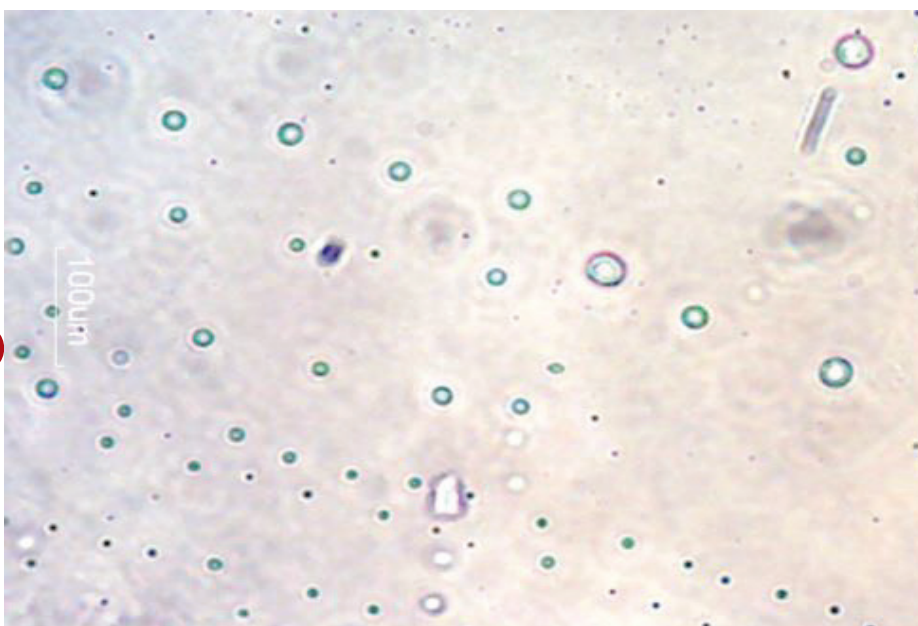
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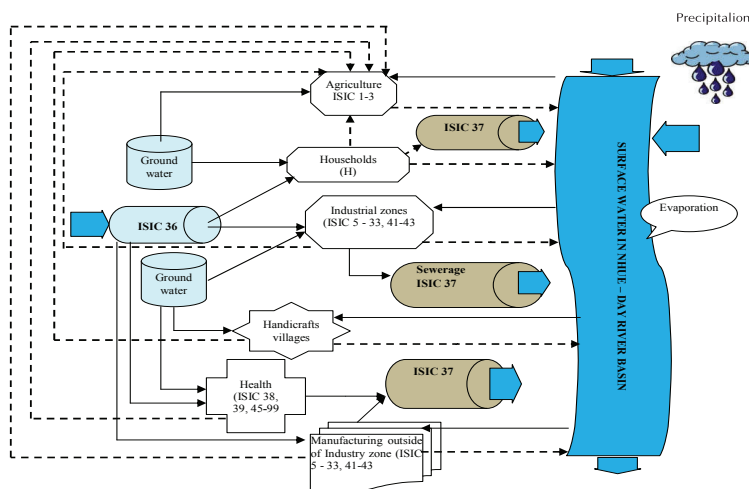
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The effect of the dielectric layer thickness on the negative permeability in metamaterials

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Abstract:

In this work, we investigate the influences of the dielectric layer on the magnetic resonance of the cut-wire pair structure (CWP). The interaction between the cut-wires is modeled through a LC circuit, based on which, the magnetic resonant frequency is calculated. Furthermore, the dependence of the resonant bandwidth on the structure parameters is also determined. By tuning the dielectric layer thickness, we obtained a noticeable broadening of the negative permeability regime of 17%, which represents the enhancement of the magnetic resonance. A good agreement between the theory, simulation, and practical experiment has been demonstrated. We believe that our results should be consequential with regard to the determination of the mechanism behind the wave-matter interaction in the GHz frequency regime.

Keywords: dielectric layer thickness, metamaterials, negative permeability broadening.

Classification number: 2.1

Introduction

In recent years, the revolution in science and technology with regard to seeking novel materials has gained tremendous popularity throughout the world. Metamaterials (MMs) have been one of the most prominent candidates in this regard due to their extraordinary properties. Numerous potential applications of MMs have been proposed and demonstrated, such as biological sensor [1], superlens [2], hi-low pass filtering [3], antennas [4], invisible cloaking [5], and wireless power transfer [6]. Most of these applications are based on the unique optical property of negative refractive index in MMs

[7-9]. Whereas, the permeability and permittivity of MMs are simultaneously negative in a common frequency regime [10-13]. This unique property of MMs is known to be arbitrarily tuned in terms of the arrangement or design of their compositions. In fact, the negative permittivity region can be obtained on a wide scale through the periodic continuous-wire structure [14], but the negative permeability region is restricted due to the resonant conditions. Therefore, the realistic applications of MMs are limited by the narrow negative permeability bandwidth. From the large amount of efforts made to expand the negative permeability of MMs [15-17],

the thickness of the dielectric layer has been concluded to be extremely significant. However, no systematic study on the influences of the dielectric layer thickness currently exists.

In this work, we report the effects of dielectric layer thickness on the negative permeability bandwidth of the conventional cut-wire pair (CWP) structure from 12 to 18 GHz. The negative permeability region is observed to be broadened as a result of the increased dielectric layer thickness. The phenomenon is interpreted theoretically by calculations on the LC circuit model and verified by simulations and experiments with considerable consistency.

Theoretical model

The CWP structure includes two metal patterns on two sides of the dielectric layer (Fig. 1A). Due to Zhou, et al.'s great work [18], the CWP structure can be modeled by an equivalent LC circuit (Fig. 1B) by considering the CWs as the inductors and the spaces between the ends of the CW as the capacitors. Furthermore, the resonant frequency can be theoretically predicted as shown below:

$$f_m = \frac{c}{\pi l \sqrt{2\epsilon c_1}} \quad (1)$$

Since Eq. 1, the resonant frequency depends on the CW length and the dielectric constant of the middle layer. However, some experimental results

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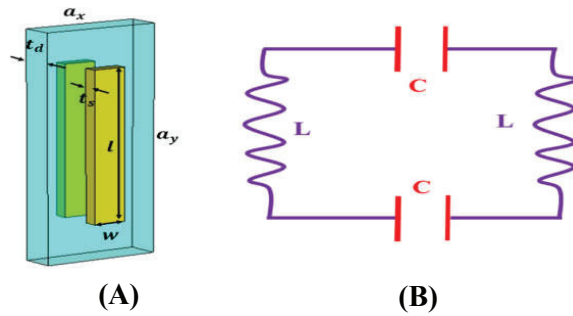


Fig. 1. (A) Unit cell and (B) the corresponding LC circuit of the CWP structure.

reveal that dielectric thickness also influences the resonant frequency [19, 20]. In our study, we propose a new equation to calculate the inductance and capacitance in the equivalent LC circuit, in which the dielectric thickness is considered according to the hybridization model:

$$L = \frac{\mu_0 l w}{2(w + t_s)^2} (t_d - 2t_s) \quad C = \frac{\epsilon \epsilon_0 c_1 w}{t_d} \quad (2)$$

where: l is the length, w constitutes the width, and t_s forms the thickness of a CW; t_d represents the dielectric layer thickness and c_1 is a constant $0.2 \leq c_1 \leq 0.3$.

Hence, the resonant frequency of the equivalent LC circuit becomes.

$$f = \frac{c}{\pi l \sqrt{2\epsilon c_1 \left(1 - \frac{2t_s}{t_d}\right) / \left(1 + \frac{t_s}{w}\right)}} \quad (3)$$

In Eq. 3, the resonant frequency is shown to depend on the the width of the CW and the dielectric layer thickness. It is worth noting that in the case where ($t_s \ll w$) and ($t_s \ll t_d$), Eq. 3 assumes the same form as Eq. 1 of J. Zhou, et al. [18]. In other words, the mutual coupling effect between the CWs has not been included in the calculations of J. Zhou, et al. [18]. Therefore, the fractional bandwidth of the negative permeability can be expressed as follows:

$$\frac{\Delta f}{f_0} = \frac{1}{\sqrt{1 - F}} - 1 \quad (4)$$

where: F is defined as:

$$F = \frac{l}{a_x a_y a_z} \frac{(w + t_s)^2}{w} \frac{t_d^2}{t_d - 2t_s} \quad (5)$$

Since t_d is considered to fall in the range between 0.2 to 1.0 mm, F is always greater than 0 and smaller than 1. Hence, Eq. 4 is positive and identifiable. The relation between the dielectric layer thickness and the negative permeability bandwidth can be realized in Eq. 4 and Eq. 5 or more clearly in Fig. 2, where the evolution of $\Delta f/f_0$ according to t_d is presented.

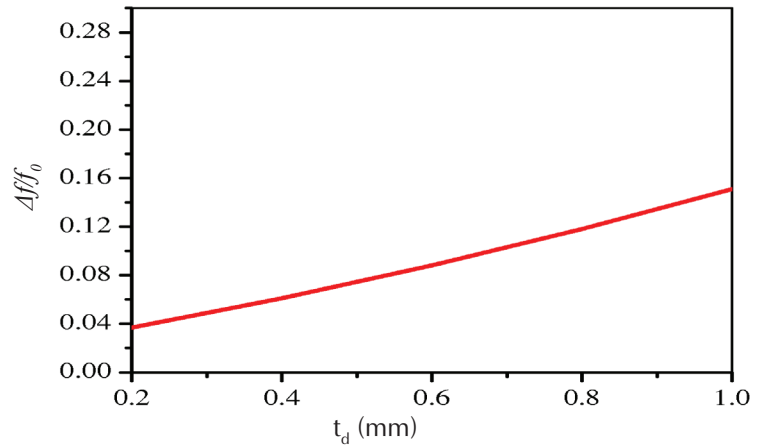


Fig. 2. The dependence of the negative permeability fractional bandwidth on the dielectric layer thickness presented by theoretical model.

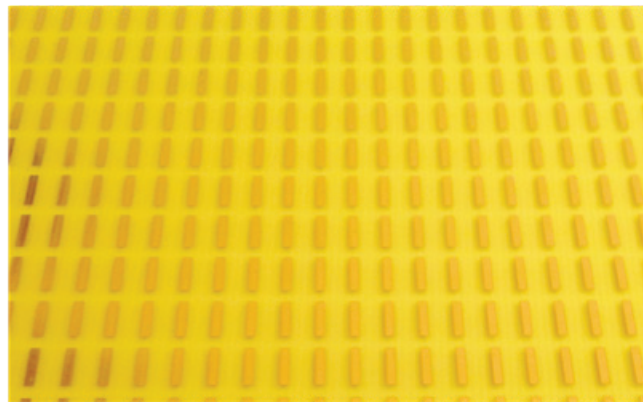


Fig. 3. Fabricated sample with the presented structure parameters.

Simulation and experiment

The proposed CWP structure is composed of a dielectric layer FR4 with the dielectric constant as 4.3 and copper patterns with $t_s = 0.036$ mm, $l = 5.5$ mm, $w = 1$ mm. The structure is periodic along the x and y axis; the lattice constant for each direction is $a_x = 3.6$ mm and $a_y = 7.2$ mm respectively. The thickness of the dielectric layer is tuned from 0.2 mm to 1.0 mm by a step of 0.2 mm. The samples are prepared on the standard printed circuit boards (PCB) through the application of the conventional photolithography method (Fig. 3). The simulations are operated on the simulation program CST [21], and the measurements are performed by the vector network analyzer system to obtain the scattering parameters.

Results and discussions

Figures 4A, 4B present the simulated and measured transmission spectra of the CWP structure at various dielectric layer thicknesses. As expected in Eq. 3, the resonant frequency shifted to the higher frequency regime when we increased the dielectric layer thickness from 0.4 to 1.0 mm.

The results with regard to magnetic resonant frequencies are listed in Table 1 with a comparison between the simulated, experimental, and theoretical calculations from this work and Zhou's work. The influence of the mutual coupling between the two cut-wires in a unit cell should be noticed when t_d is small, as discussed above. Moreover, the resonant peak is observed to demonstrate the blue shift in correspondence to the increase in the thickness of the dielectric layer. The rate of increase presents a good agreement between our calculations and data provided in Fig. 3 and a slightly higher correspondence with J. Zhou, et al.'s [18] work, confirming the improvement in the accuracy of our calculation in comparison to the reference.

In addition to the frequency shift along with the increasing thickness of the dielectric layer, the negative permeability regime is also expected to broaden. Fig. 5 presents numerical results of the transition spectra as a function of dielectric layer thickness from 0.1 to 1.0 mm. We observe that the abandon gaps of the transmission spectra are very narrow and shallow with the thin dielectric layer. Regardless, by increasing the dielectric layer thickness to 1.0 mm, the transmission gap is significantly widened and deepened. Since the magnetic resonance in the structure presents the electromagnetic waves propagating through the MM, the enlargement of the transmission regime may correspond to wider negative permeability.

Thanks to X. Chen, et al.'s work [22], from the scattering parameters obtained by simulation, the permeability spectra are extracted in Fig. 6 A

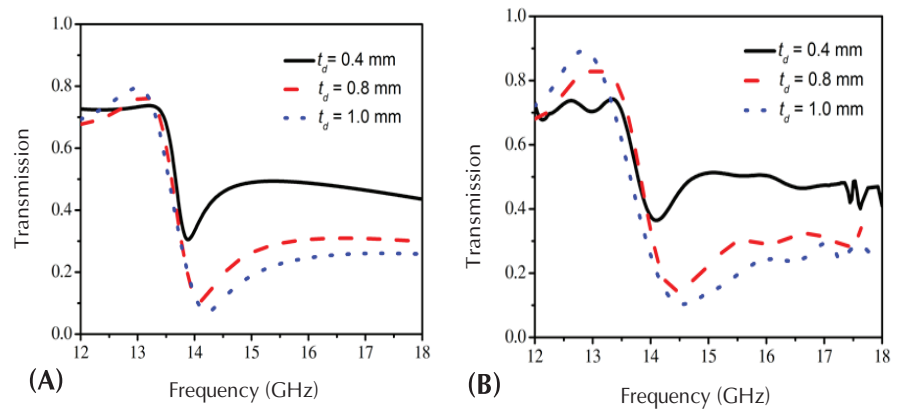


Fig. 4. The (A) simulated and (B) experimental transmission spectra of the CWP with $t_d=0.4, 0.8, 1.0$ mm.

Table 1. The magnetic resonant frequencies obtained in simulation, experiment and calculations in this work and reference [18].

t_d (mm) \ f_m (GHz)	This work	Zhou's work	Simulation	experiment
0.4	14.02	12.41	13.968	14.076
0.8	14.18	13.01	14.172	14.139
1	14.33	13.28	14.268	14.51

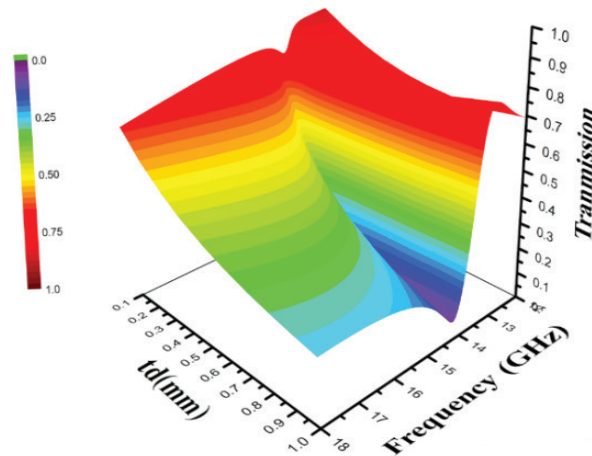


Fig. 5. The dependence of the transmission spectra on the dielectric layer thickness.

at $t_d = 0.2, 0.4, 0.8$, and 1.0 mm. A significant enhancement of the negative permeability can be observed. In fact, at 0.2 mm, the fractional bandwidth is only 4% (bandwidth of 0.55 GHz at the center frequency of 13.79 GHz) and at 1.0 mm, the frictional bandwidth is four times larger, up to 17% (bandwidth of 2.42 GHz at the center frequency

of 14.27 GHz). This implies a good agreement with the simulation results. Our calculations following equation 2.8 exhibit an increase of frictional bandwidth from 3.6% at $t_d = 0.2$ mm to 14.4% at $t_d = 1.0$ mm as depicted in Fig. 6B. Our theoretical calculations also show good correspondence with the simulation results displayed in Fig. 6B.

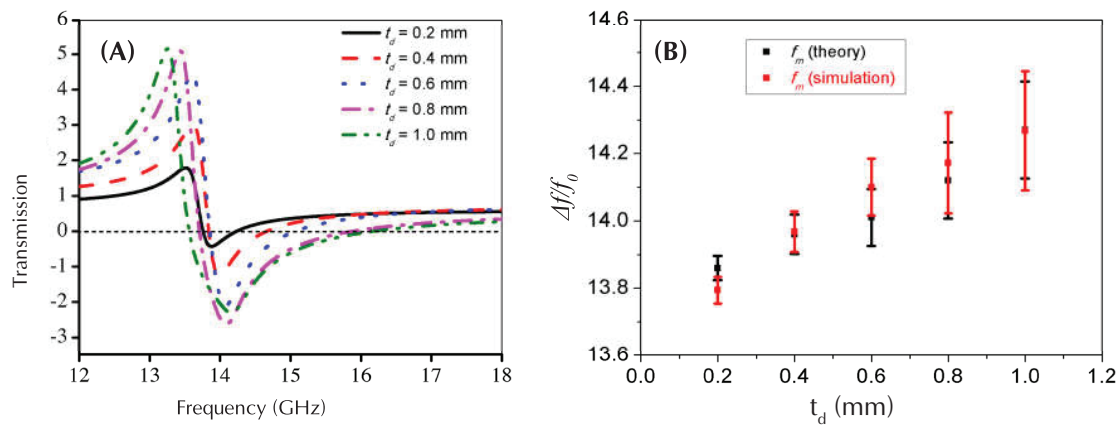


Fig. 6. (A) The dependence of the permeability on dielectric layer thickness at $t_d = 0.2, 0.4, 0.8, 1$ mm and (B) The fractional bandwidth of the negative permeability in theory and simulation.

Conclusions

We have investigated the influence of dielectric layer thickness on the negative permeability of the conventional CWP structure. The negative permeability region exhibits a blue shift and a broadening with increase in dielectric layer thickness. The LC circuit model was employed to interpret this behavior. A good agreement with simulations and experiments was obtained that confirms the validity of our analysis. The results will be useful in understanding the mechanism of wave-matter interaction in MMs in GHz frequency regime.

ACKNOWLEDGEMENTS

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Removal of nickel and methylene blue from aqueous solutions by steel slag as a low cost adsorbent

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Abstract:

Nowadays, wastewater from various industries contains a large number of harmful heavy metals and coloring agents, which have to be removed to restore the quality of the environment. In this study, the removal of nickel ions (Ni^{2+}) and methylene blue (MB) from the aqueous solution using steel slag as a low cost adsorbent was investigated. The chemical and mineralogical compositions, as well as the surface area of slag, were analyzed by using X-ray fluorescence spectroscopy, X-ray diffraction, and the Brunauer-Emmett-Teller method (BET). The effect of several important parameters such as contact time, adsorbent dose, pH, temperature, and initial adsorbate concentration on the adsorption process was studied systematically by batch experiments. The adsorption data were well correlated with the Langmuir isotherm model by all samples. The maximum adsorption capacity of the raw slag samples was 36.49 mg/g for Ni^{2+} and increased from 0.68 to 1.98 mg/g for MB after being acid-activated. The determined thermodynamic parameters indicate that the adsorption of Ni^{2+} and MB on steel slag is spontaneous in nature, endothermic (for Ni^{2+}), and exothermic (for MB).

Keywords: adsorption, dye, heavy metals, low cost adsorbent, steel slag, water treatment.

Classification number: 2.2

Introduction

It is well known that water is a precious and irreplaceable resource for human and animals' life [1]. However, water pollution of heavy metals and dyes, which are major contributors to the contamination of water streams, has been a serious environmental problem in the recent years. The increasing water contamination by heavy metal ions and dyes has become a significant concern for ecological systems and public health because of their nonbiodegradable property, bioaccumulation, and toxicity, even at low concentrations [2]. Nickel is

one of the important toxic heavy metals that is widely used in electroplating, printing, storage-battery industries, silver refineries, and production of some alloys. High concentration of nickel causes poisoning effects like lung, nose, bone cancers, headaches, dizziness, nausea, cyanosis, and extreme weakness [3]. One of the high consuming materials in the dye industry is MB, which is the most commonly used substance for dyeing cotton, wool, and silk [4]. The MB can cause eye burns, nausea, vomiting, diarrhea, dyspnea, tachycardia, cyanosis, methemoglobinemia, and convulsions if

inhaled [5, 6]. Therefore, the treatment of effluent, containing heavy metal ions, and dyes such as nickel and MB, is necessary due to their harmful effects on humans.

Among the various methods currently applied for removing heavy metals and dyes from the water industry, adsorption is the most widely used method due to its merits of efficiency, economy, and simple operation [7]. Different adsorbents have been used for the removal of MB and nickel ions from aqueous solutions, including graphene [8, 9], bentonite [10], activated carbon [4], perlite [11], pumice [12], and hydroxyapatite [13, 14]. However, these adsorbents are relatively expensive, and this has restricted their application at times.

Steel slag is the main by-product of the iron and steel industry. A huge amount of it is accumulated in the environment and causes numerous ecological problems. Therefore, determining the sustainable usage of accumulated steel slag for other purposes will bring economic and environmental benefits. In the recent years, steel slag has been reported as potential adsorbent to remove pollutants from waste water [15-19]. In this study, steel slag was chosen as a low cost adsorbent to remove nickel ions and methylene blue dye. The main objective of this work was to evaluate the removal ability of steel slag and its activated form for Ni^{2+} and MB under different experimental conditions.

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Materials and methods

Materials and chemicals

The experiment material used in this study was electric arc furnace (EAF) steel slag obtained from a steelmaking plant (Danang, Vietnam). The collected steel slag was crushed and sieved to obtain particles. Activated slag was obtained by soaking raw crushed steel slag with 2 M HCl for 24 hours at room temperature. After that, the acid suspension was filtered by vacuum filter and then, the residue was washed with distilled water. Finally, the sample was dried at 100°C for 12 hours and ground to a powder state. Nickel sulfate hexahydrate and methylen blue were purchased from Merck. The reagents dimethylglyoxime (99.00%), NaOH (99.95%), and HCl (36.5%) were provided by Sigma Aldrich. All other reagents used in this study were analytical grade, and distilled or double distilled water was used in the preparation of all solutions.

Characterization of adsorbent

The chemical composition of the slag was determined by X-ray fluorescence spectroscopy (XRF) using a Philips PW 2404 instrument. The morphologies of the samples were investigated by scanning electron microscopy (SEM, Hitachi S4800). The mineralogical composition was analyzed by an X-ray diffractometer Rigaku Ultima IV (Japan), operating at 45 kV and 40 mA, using Cu-K α radiation of $\lambda = 0.15418$ nm, 2θ ranging from 5 to 60°, and step size 0.1°. Phase identification was carried out by comparing the peak positions of the diffraction patterns with ICDD (JCPDS) standards. Surface area of the slag was measured by the BET on Micromeritics TriStar 3000 instrument.

Batch studies

Batch adsorption studies were performed at different doses of adsorbent, initial pH, contact time, concentrations of Ni²⁺ and MB. For each experiments, three replicates of the adsorbents (0.5 g) were mixed with 50 ml solutions of Ni²⁺/MB in 150 ml conical flasks at different

initial concentrations and a certain temperature. The pH of the solutions was adjusted by adding 0.1 M aqueous solutions of NaOH or HCl using a pH meter InoLab Multi 9310. To ensure homogeneous mixing, an orbital shaker with an agitation speed of 150 rpm was used throughout the experiment. Then, the samples were centrifuged at 5000 rpm for 10 min. The concentrations of the nickel ions and the MB dye before and after adsorption were estimated using an UV-visible spectrophotometer (UV-VIS Ultrospec 8000). The effect of the adsorbent dose was conducted using 2.5-20 g/l of adsorbent. The effect of pH was investigated over a pH range of 2-8. The effect of contact time was studied under different given contact time between 5 min and 120 min.

The percentage removal (R%) and the amount of adsorbed nickel ion and MB dye were calculated using the following equations:

$$R = \frac{C_o - C_e}{C_o} \cdot 100 \quad (1)$$

$$Q_e = \frac{(C_o - C_e) \cdot V}{m} \quad (2)$$

where: C_o and C_e are the initial and final concentrations of Ni²⁺ ions and MB before and after the adsorption in aqueous solution (mg/l); Q_e is the amount of Ni²⁺ and MB dye adsorbed by the slag (mg/g); V is the volume of solution (l); m is the mass of adsorbent (g).

Results and discussions

Characterization of sorbent

The chemical and physical characteristics of the steel slag are presented in Table 1. The result showed that the steel slag of this study mainly contains calcium, iron, silicon, magnesium, aluminum, manganese, and phosphorus compounds. The joint presence of calcium oxide and alumina silicate compounds could facilitate the provision of negatively charged sites for cation exchange reactions with metal ions in the aqueous solution [20]. The

surface area and density of the steel slag are 4.23 m²/g and 3.025 g/cm³, respectively. Aqueous suspensions of the slag have a high pH value (10.52) because of great content of calcium hydroxide (30.23 w%) and basic oxides in it, which lead to a high capacity of the slag to neutralize strong acidic media.

The mineralogical compositions of slag samples were determined by the XRD analysis, and obtained results are given in Fig. 1. The analysis of the diffraction patterns showed that both slag samples are heterogeneous materials consisting the following major crystalline phases: larnite (Ca₂SiO₄), wuestite (FeO), gehlertite (Ca₂Al(AlSiO₇)), mullite (3Al₂O₃·2SiO₂), and quartz (SiO₂). Other minor constituent phases in the analyzed samples are very difficult to identify because of the complexity of the diffractograms. Additionally, XRD patterns show that the peaks of the activated slag are more intense and clearer compared to the raw steel slag. This may be because some impurities in the raw slag have been removed when it was soaked in the acid solution.

Morphology study

Figure 2 shows the morphologies of the slag samples before and after adsorption, as characterized by SEM. It can be seen from the SEM images that there is no significant change in morphology of the slag samples before and after adsorption of Ni²⁺ and MB. Under SEM, the slag particles showed irregular shapes with sharp edges about 0.5 μ m to 2 μ m in size.

Effect of initial solution pH

The initial pH value of the adsorbate solution is one of the most important factors influencing the adsorption process due to its strong effect on the surface charge, the surface binding sites of the adsorbent, and the degree of ionization and species of adsorbate [21]. The effect of initial pH on the adsorption process was studied in the range from 2 to 8 at 25 $\pm$ 2°C, the adsorbent dosage of 0.5 g, contact time of 60 min, 50 ml

Table 1. The chemical and physical characteristics of the slag.

Chemical composition (w%)							BET surface area (m ² /g)	Density (g/cm ³)	pH
CaO	Fe ₂ O ₃	SiO ₂	Al ₂ O ₃	MnO	MgO	P ₂ O ₅			
30.23	28.57	22.19	6.68	4.17	2.39	2.20	4.23	3.025	10.52

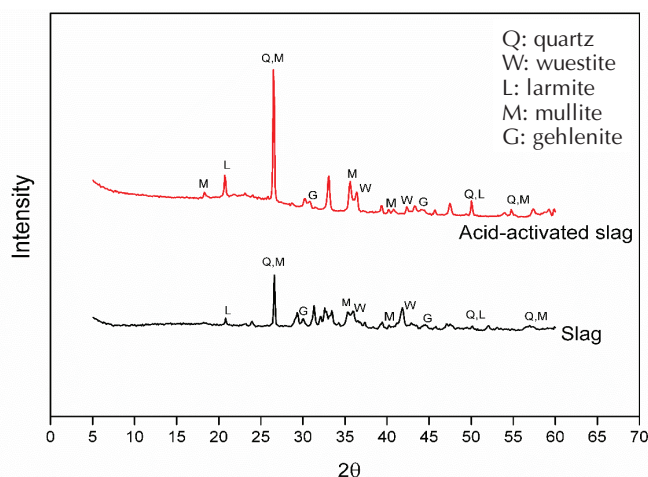
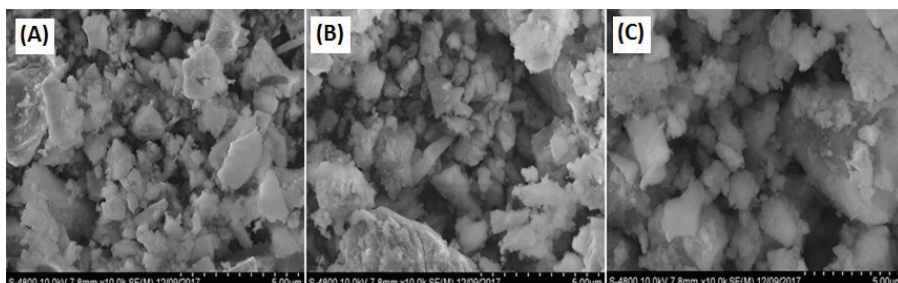
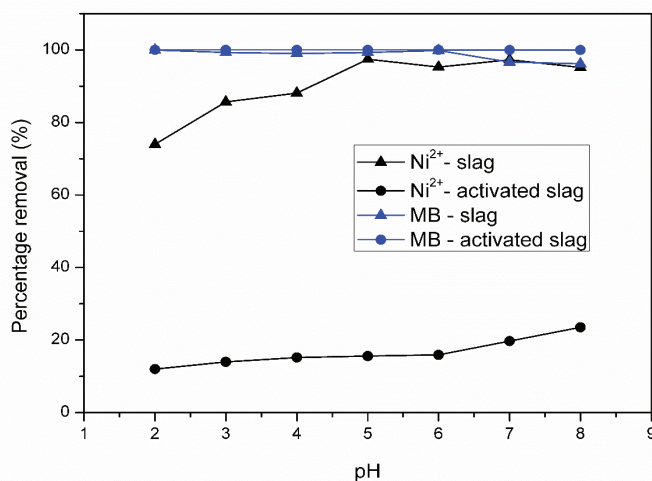


Fig. 1. XRD patterns of slag samples.

Fig. 2. SEM images of slag samples: (A) before adsorption, (B) after Ni²⁺ adsorption, and (C) after MB adsorption.Fig. 3. Effect of pH on Ni²⁺ and MB adsorption onto slags.

of initial Ni²⁺ and MB solutions with a concentration of 200 mg/l and 5 mg/l, respectively. Fig. 3 shows the effect of the initial pH on the adsorption of Ni²⁺ ions and MB onto different forms of slag. It can be seen that the MB removal percentage of both slag samples had no significant change and reached 99.9% for the activated slag sample at the whole pH range. While the raw slag can remove 98.6-99.9% of MB from the aqueous solution at pH 2-6 and its removal ability insignificantly decreased to 96.1% at pH 7-8. For Ni²⁺, the percentage removal generally increased with increasing the pH of the solution. It can reach 77.5% at pH 2 and increase to 98.3% at the pH range of 5 to 8 for raw slag sample. The removal percentage of Ni²⁺ by activated slag in the range of pH 2-8 was 12.3-23.5%, which is about 4-5 times lower than that by raw slag. This can be due to the decreased alkalinity of the slag when it was activated by acid. Therefore, the experiments of nickel adsorption onto the activated slag were not conducted any further.

At a lower pH than 5.0, the Ni²⁺ ion adsorption capacity of slag was low due to the increased concentration of hydronium (H₃O⁺) ions, which compete for Ni²⁺ binding sites on the slag surface. The increase in pH resulted in a reduction in H₃O⁺ ions; the adsorption sites were available to be occupied by more Ni²⁺ ions than H₃O⁺ ions. At an alkaline pH, there was hydroxyl group abundance on the slag surface, which promoted the adsorption of metal cations. However, at pH values higher than 7.0, precipitation usually occurs simultaneously between Ni²⁺ ions and hydroxide ions, and could lead to inaccurate interpretation of adsorption [22]. Based on the above results, the optimum pH values of 5.0 and 6.0 were selected for the adsorption study regarding the removal of Ni²⁺ and MB dye, respectively.

Effect of equilibrium time

The determination of the contact time between adsorbent and adsorbate required for the system to reach

equilibrium is important to determine the possible discrimination order in the behavior of the slag for Ni^{2+} and MB dye removal. The effect of contact time on adsorption of Ni^{2+} and MB onto slag samples was carried out at different contact times ranging from 5 min to 120 min at $25 \pm 2^\circ\text{C}$, adsorbent dosage of 0.5 g, the selected optimum pH values, and 50 ml of adsorbate solutions (200 mg/l for Ni^{2+} and 5 mg/l for MB). As shown in Fig. 4, over 97% of Ni^{2+} ions and MB was adsorbed on the slag phase after only 10 min of the equilibrium periods. The adsorption of the Ni^{2+} and MB dye by the raw slag exhibits a similar trend. The percentage removal increased with a lapse of contact time, and equilibrium was reached after 30 min. Moreover, it can be seen that the adsorption of Ni^{2+} and dye on raw slag had taken place in two stages where the first stage is faster than the second stage. This phenomenon can be confirmed by the slope of adsorption line which had value in the first step of the adsorption process higher than the second. The initial rapid stage can be associated with the presence of the large number of binding sites at exterior surface, which are being fully available at the initial stage of adsorption process. When the exterior adsorption sites become filled with adsorbates, the adsorbate ions then moved with slow rate from the exterior to the interior.

The adsorption of MB on activated slag occurred relatively quickly because there were more active sites on the surface compared to the nonactivated slag. The amount of MB dye removed by the activated slag also rose up to more than 99% after 5 min, and remained invariable until the adsorption attained a balanced status.

Effect of adsorbent dose

To select the best-required dose for the scale-up and to design large-scale equipments, the effect of adsorbent dose on MB dye and Ni^{2+} removal was investigated. The effect of the dose on the adsorption was studied by varying the amount of adsorbent from 0.25 to 1 g in 50 ml of adsorbate solutions with

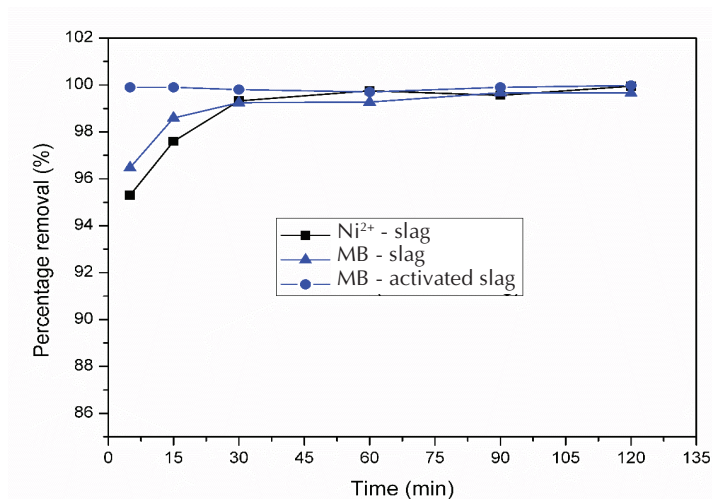


Fig. 4. Effect of contact time on adsorption of nickel and MB onto slag.

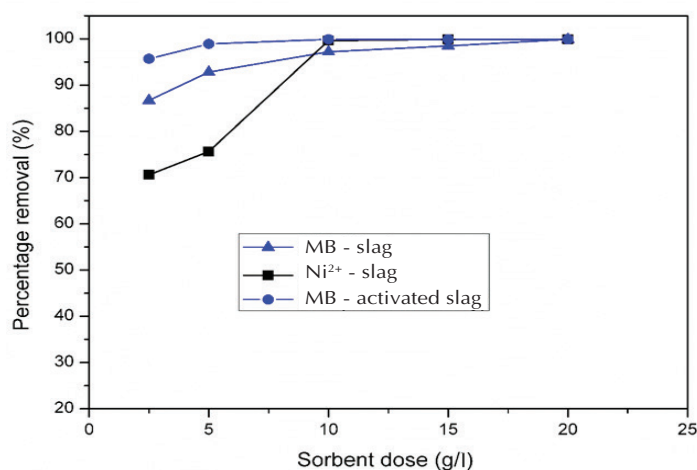


Fig. 5. Effect of sorbent dose on adsorption of Ni^{2+} and MB onto slag.

initial concentrations of 200 mg/l (for Ni^{2+}) and 5 mg/l (for MB) at a room temperature of 25°C , optimum pH, and a contact time of 30 min. The relationship between the removal percentage of Ni^{2+} ions and MB and the adsorbent dose is shown in Fig. 5.

It is shown that the removal efficiency of Ni^{2+} ions and MB increased with increasing the adsorbent dose for all the slag samples due to the availability of more surface area on the adsorbent. This means that there was a small surface area for the attachment of the adsorbate ions at a low adsorbent dose, resulting in the low efficiency of Ni^{2+} ions and MB removal. However, as the adsorbent amount increased, more sites became available for the attachment; hence, the removal

capacity of the adsorbent increased. The increase in the slag dose from 2.5 to 5 g/l resulted in an increase in the adsorption of Ni^{2+} from 70.6 to 75.7% and of MB from 86.7 to 92.8% for the raw slag samples and from 95.7 to 98.9% for the activated slag samples. When the adsorbent dose was sufficient (> 10 g/l), over 99% of adsorbates could be removed. Thus, the complete removal was possible with an adsorbent dose of 10 g/l.

Effect of initial concentration

The effect of the initial concentration on the removal efficiency and adsorption capacity of Ni^{2+} ions and MB on the slag samples at room temperature of 25°C , an adsorbent dose of 10 g/l, optimum pH, and contact time of 30 min is shown in Fig. 6.

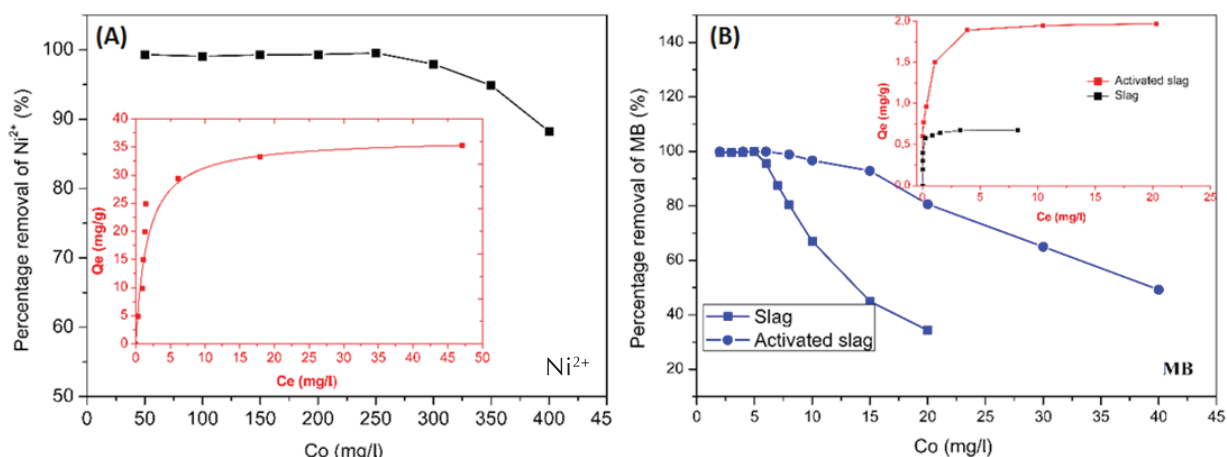


Fig. 6. Effect of initial concentration on (A) Ni^{2+} and (B) MB adsorption.

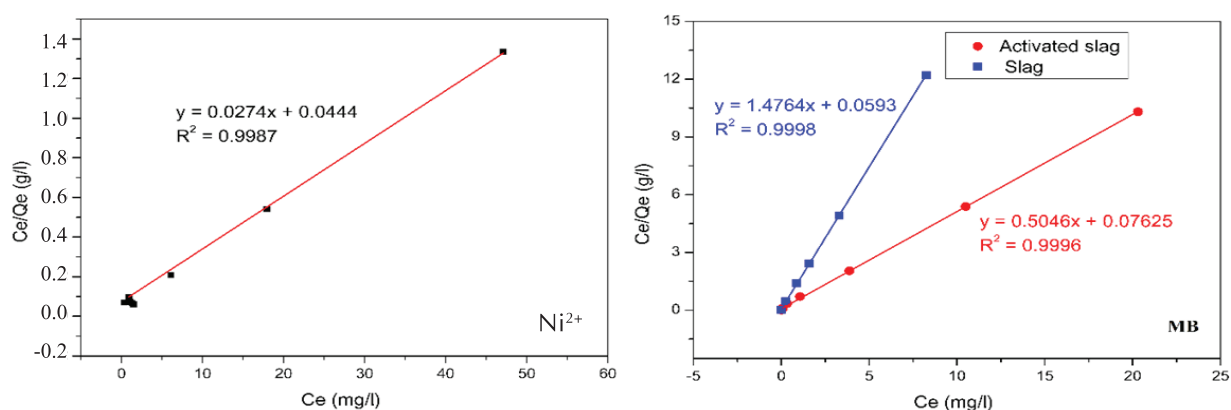


Fig. 7. Langmuir plots for Ni^{2+} and MB adsorption on slag samples.

It is shown that the effect of the initial concentration of Ni^{2+} and MB on their removal efficiency has a similar tendency. The percentage of removal was nearly 100% at a low concentration of Ni^{2+} (< 250 mg/l) and MB dye (< 6 mg/l). With the increase of the initial concentration of Ni^{2+} ions from 50 to 400 mg/l and of MB from 2 to 40 mg/l, the removal efficiency decreased from about 100 to 88.2, 34.5, and 50.02% for Ni^{2+} , MB (on the raw slag) and MB (on the activated slag), respectively. The adsorption capacity increased from about 4.8 to 35.3 mg/g for Ni^{2+} and from 0.2 to 1.97 mg/g for MB. At low initial concentrations, molecules of the adsorbates had more chance to react with the available active sites on the slag samples, resulting in an increase in the percentage removal. The decrease in the removal percentage at high concentrations of adsorbates can be

explained that all the slag samples were limited by the adsorption sites; thus, the adsorption of Ni^{2+} and MB becomes restricted by the saturation of these adsorption sites [23].

Adsorption isotherms

The determination of the adsorption isotherm is important to indicate how adsorbent molecules were distributed between the liquid and the solid phase, and could be accurately used for design purposes and optimization of economical equipments. In this study, the Langmuir and Freundlich isotherm models were used to interpret equilibrium data of Ni^{2+} and MB(II) adsorption on the slag by utilizing the adsorption data obtained from the effect of initial concentrations. However, the experimental data were not correlated to the Freundlich model. Hence, only the results of the equilibrium data analysis using the Langmuir model

are presented. The Langmuir isotherm considers the adsorbent surface as homogeneous with identical sites in terms of energy, and can be described by the following equation [24]:

$$\frac{C_e}{Q_e} = \frac{1}{Q_m \cdot K} + \frac{C_e}{Q_m} \quad (3)$$

where: C_e is the equilibrium concentration of Ni^{2+} /MB (mg/l), Q_e is the amount of Ni^{2+} or MB adsorbed at equilibrium (mg/g), Q_m is the maximum adsorption capacity of Ni^{2+} /MB (mg/g), and K is the Langmuir constant (l/mg). The value of Q_e was calculated using Equation 2. Q_m and K were calculated from a linear plot of C_e/Q_e against C_e with a slope and an intercept equal to $1/Q_m$ and $1/(Q_m \cdot K)$, respectively. The constant separation factor R_L , whose value indicates the shape of the Langmuir isotherm, and predicts if an adsorption system is

favourable or unfavourable, is calculated by the following equation [25]:

$$R_L = \frac{1}{1 + K.C_0} \quad (4)$$

where: C_0 is the initial concentration of adsorbate (mg/l), and K is the Langmuir adsorption constant (l/mg).

Figure 6 shows the adsorption isotherm of Ni^{2+} and MB dye on the slag samples. The Langmuir (C_e/Q_e vs. C_e) plots and the fitting parameters of the Langmuir isotherm for Ni^{2+} and MB adsorption are shown in Fig. 7 and Table 2, respectively.

As a result, the correlation coefficients obtained from the Langmuir equation for the adsorption of Ni^{2+} and MB dye on all slag samples were found to be from 0.9996 to 0.9998, indicating that the experimental data were well correlated with the Langmuir model. This means that the adsorption process was mainly monolayer on a homogeneous adsorbent surface. The Q_m values for Ni^{2+} and MB adsorption on the non-activated slag were 36.49 mg/g and 0.68 mg/g, respectively. While the maximum adsorption capacity of MB on the activated slag was 1.98 mg/g, about 2 times higher than that of the non-activated slag sample. The enhancement in the adsorption capacity of the acid-activated slag compared with the raw slag may be due to an increase in the number of active adsorption sites by acid treatment.

The value of the separation factor, R_L , indicates the adsorption nature of the adsorbate with the adsorbent. The adsorption process is unfavorable if $R_L > 1$, favorable if $0 < R_L < 1$, linear if $R_L = 1$, and irreversible if $R_L = 0$ [26]. In the present study, the values of R_L fall in between 0 and 1, and have confirmed that all slag samples are favorable for Ni^{2+} and MB adsorption under the experimental conditions.

Evaluation of adsorption thermodynamics

To evaluate the thermodynamic parameters of Ni^{2+} and MB adsorption

Table 2. Isotherm parameters for the adsorption of Ni^{2+} and MB onto slag.

Adsorbate-Adsorbent	Q_m (mg/l)	K (l/mg)	R^2	R_L
Ni^{2+} - slag	36.49	0.62	0.9987	0.0040
MB - slag	0.68	24.90	0.9998	0.0020
MB - act. slag	1.98	19.73	0.9996	0.0013

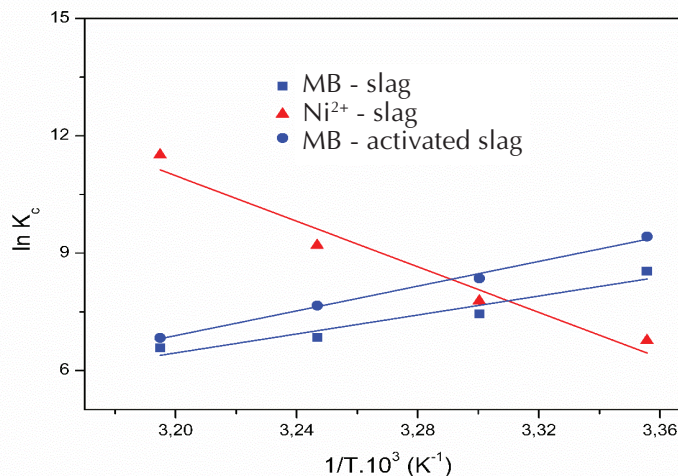


Fig. 8. Plot of $\ln K_c$ as a function of reciprocal of temperature ($1/T$) for the adsorption of Ni^{2+} and MB by slag.

Table 3. Calculated thermodynamic parameters for Ni^{2+} and MB adsorption on slag.

Samples	ΔG° (kJ/mol)				ΔH° (kJ/mol)	ΔS° (J/mol/K)	R^2
	298K	303K	308K	313K			
Ni^{2+} - slag	-16.76	-19.59	-23.56	-29.96	242.10	866.12	0.940
MB - slag	-21.16	-18.76	-17.52	-17.12	-100.87	-269.12	0.897
MB - act. slag	-23.35	-21.05	-19.61	-17.78	-131.36	-362.99	0.991

on the slag samples, the adsorption experiments were performed at different temperatures from 298 to 313 K. The equilibrium adsorption coefficient (K_c) for the adsorption process was calculated with the Equation 5 [27]:

$$K_c = \frac{C_{ad}}{C_e} \quad (5)$$

where: C_{ad} and C_e are equilibrium concentrations (mg/l) of Ni^{2+} and MB on the slag samples and in the solution, respectively. The thermodynamic parameters such as change in Gibbs free energy (ΔG°), enthalpy (ΔH°), and entropy (ΔS°) are calculated using the following equations [28]:

$$\Delta G^\circ = -RT \ln K_c \quad (6)$$

$$\ln K_c = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{RT} \quad (7)$$

where: R is the universal gas constant (8.314 J/mol/K), T is the absolute temperature (K). ΔH° and ΔS° were calculated from the slope $-\Delta H^\circ/R$ and intercept $\Delta S^\circ/R$ of the linear variation of $\ln K_c$ with the reciprocal of the temperature ($1/T$), as shown in Fig. 8. The obtained values of the thermodynamic parameters are presented in Table 3.

As can be observed from Table 3, the negative values of G° for nickel ions and MB adsorption at all temperatures indicate that the adsorption mechanism is a general spontaneous process and thermodynamically favorable [29]. The calculated thermodynamic parameters for MB adsorption on all the slag samples were negative. The negative values of ΔH° provide the exothermic

nature of the adsorption process. The negative ΔS° indicates the decrease of the degree of freedom at the solid-liquid interface during the adsorption of MB on slag. For nickel adsorption, the standard enthalpy and the entropy values were obtained as 242.10 kJ/mol and 866.12 J/mol/K, respectively. The positive values of ΔH° and ΔS° reflect that the adsorption of Ni^{2+} by the slag is an endothermic process, and the randomness at the solid-liquid interface during the adsorption increases. This type of absorption can be explained in terms of the magnitude of ΔH° . Physisorption generally has low enthalpy values of 20-40 kJ/mol, while the enthalpy of chemisorption lies in a range of 200-400 kJ/mol [30]. Therefore, nickel adsorption onto steel slag surface in the main can be attributed to a chemical adsorption process.

Conclusions

The steel slag was found to be a cheap material for the removal of Ni^{2+} and MB from aqueous solutions. The removal efficiency of Ni^{2+} and MB by the slag strongly depended on their initial concentration, contact time, initial pH, and adsorbent dose. The removal percentage of Ni^{2+} and MB dye was found to increase with an increase in the contact time, and the adsorbent dose was found to decrease with an increase in the initial adsorbate concentration. The optimal pH for Ni^{2+} removal was 5.0, which was lower than that for MB (pH 6.0). The adsorption of Ni^{2+} and MB dye on the steel slag saturated within 30 min and the adsorption processes could be well described by the Langmuir isotherm model, with maximum adsorption capacity of 36.49 mg/g for Ni^{2+} and 1.98 mg/g for MB. The activated slag had an adsorption capacity of about 2 times higher than the non-activated slag. The thermodynamic parameters indicate that the adsorption process is spontaneous in nature, thermodynamically favorable, endothermic (for Ni^{2+}), and exothermic (for MB). The results of this study show that the steel slag, a residue from steel plants that is readily available and easy to obtain at low cost, can be used as an effective adsorbent for the removal of Ni^{2+} ions and MB dye from wastewater.

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Using NMR, X-ray, and CD analysis in the study on natural products obtained from Vietnamese plant and fungi in terms of pharmaceutical product development

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Abstract:

NMR, X-ray analysis, and CD methods are powerful techniques for the study of absolute configuration of bioactive compounds from natural resources. This study presents the results of a joint-study between Vietnam and Taiwan on the bioactive compounds obtained from Vietnamese plants and fungi. Among the tested compounds, hexatenuin A displayed the most significant inhibition of superoxide anion generation and elastase release. These triterpenoids may be used as potential anti-inflammatory agents.

Keywords: absolute configuration, circular dichroism, NMR, X-ray analysis.

Classification number: 2.2

Introduction

Natural products are an important source for drug discovery. The determination of absolute configuration is one of the most challenging tasks in the structure elucidation of chiral natural products, especially those with complex structures. The available methods include NMR spectroscopy/chiral derivatization, analytical chemistry, X-ray crystallography for crystalline compounds, chemical synthesis, and chiroptical approaches [1]. Among these, X-ray crystallography probably remains the most powerful and effective approach. However, the complete structure elucidation of new compound may require considerable effort and involve many different spectroscopic and, sometimes, computational techniques.

The purpose of this review is to use several examples, representing different classes of natural products, to illustrate

the applicability of these approaches in determining the absolute configuration of natural products obtained from Vietnamese plants and fungi. Moreover, the purified constituents were examined for their anti-inflammatory activity. Among the tested compounds, hexatenuin A displayed the most significant inhibition of superoxide anion generation and elastase release. These triterpenoids may have potential to be used as anti-inflammatory agents.

Experimental

General experimental procedures

The optical rotations were measured with a JASCO P-2000 digital polarimeter in a 0.5 dm cell. The UV spectra were obtained with a Hitachi UV-3210 spectrophotometer while the IR spectra were measured with a Shimadzu FTIR Prestige-21 spectrometer. The ECD spectra were recorded on a JASCO J-720 spectrometer. The ¹H- and ¹³C-NMR

spectra were measured using Bruker AMX-400 and AV500 spectrometers with TMS as the internal reference, while the chemical shifts were expressed in δ (ppm). The ESIMS and HRESIMS were collected on a Bruker Daltonics APEX II 30e spectrometer. HPLC was performed on a Shimadzu LC-10ATVP (Japan) system, equipped with a Shimadzu SPD-M20A diode array detector at 250 nm, a Purospher STAR RP-8e c (5 μ m, 250 $\times$ 4.6 mm), Cosmosil 5C18 ARII (250 $\times$ 4.6 mm *i.d.* Nacalai Tesque Inc.), and Astec Cellulose DMP (150 $\times$ 4.6 mm *i.d.* 5 μ m) columns. The X-ray diffraction experiments were performed on a Bruker D8 Venture with a Photon 100 CMOS detector system equipped with a Cu Incoatec I μ S microfocus source (λ = 1.54178 Å).

Preparation of human neutrophils

Neutrophils were isolated by a standard method of dextran sedimentation, prior to their centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes. Blood was drawn from healthy human donors (20-30 years old) by venipuncture into heparin-coated Vacutainer tubes, using a protocol approved by the institutional review board at Chang Gung Memorial Hospital [2]. The blood samples were mixed gently with an equal volume of 3% dextran solution. After the sedimentation of the red cells for 30 min at room temperature, the leukocyte-rich plasma was collected. The leukocyte-

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rich plasma was transferred on top of a 20 ml Ficoll solution (1.077 g/ml) and spun down at 400 g for 40 min at 20°C. The granulocyte/erythrocyte pellets were resuspended in ice-cold 0.2% NaCl to lyse the erythrocytes. After 30 s, the same volume of 1.6% NaCl solution was added to reconstitute the isotonic condition. Purified neutrophils were pelleted and then resuspended in a calcium (Ca^{2+})-free Hank's balanced salt solution (HBSS) buffer at pH 7.4 and maintained at 4°C before use [2].

Measurement of superoxide anion generation

The assay of the superoxide anion generation was based on the SOD-inhibitable reduction of ferricytochrome c [2]. Briefly, after supplementation with 0.5 mg/ml ferricytochrome c and 1 mM Ca^{2+} , the neutrophils (6×10^5 cells/ml) were equilibrated at 37°C for 2 min and incubated with drugs or an equal volume of vehicle (0.1% DMSO, negative control) for 5 min. The cells were activated with 100 nM FMLP during the preincubation of 1 $\mu\text{g}/\text{ml}$ cytochalasin B (FMLP/CB) for 3 min. Changes in the absorbance, with a reduction in ferricytochrome c at 550 nm, were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Then calculations were based on the differences in the reactions with and without SOD (100 U/ml), divided by the extinction coefficient for the reduction of ferricytochrome c ($\epsilon = 21.1/\text{mM}/10 \text{ mm}$) [2].

Measurement of elastase release

The degranulation of azurophilic granules was determined by the elastase release, as described previously [2]. Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100 μM), the neutrophils (6×10^5 cells/ml) were equilibrated at 37°C for 2 min and incubated with drugs or an equal volume of vehicle (0.1% DMSO, negative control) for 5 min. The cells were

activated by 100 nM FMLP and 0.5 $\mu\text{g}/\text{ml}$ cytochalasin B while the changes in absorbance at 405 nm were continuously monitored to assay the elastase release. The results were expressed as the percentage of elastase release in the FMLP/CB-activated, drug-free control system [2].

Hexagonin A (16): white powder (CHCl_3); mp 184–185°C; $[\alpha]_D^{25} +57$ (c 0.6, MeOH); UV (MeOH) λ_{max} (log ϵ) 262 (2.65) nm; IR (neat) ν_{max} 2946, 1759, 1693, 1455, 1376, 1256, 1219, 1156 cm^{-1} ; $^1\text{H-NMR}$ (500 MHz, CDCl_3) (δ ppm): 4.71 (1H, br s, H-3), 4.32 (1H, ddd, $J = 11.5, 11.5, 5.0$ Hz, H-16), 3.72 (3H, s, CH_3 -4'), 3.40 (2H, s, CH_3 -2'), 2.27 (1H, dd, $J = 14.0, 11.5$ Hz, H-15), 2.18 (1H, m, H-20), 2.05 (2H, m, H-6, -11), 1.89 (1H, m, H-2), 1.84 (1H, m, H-12), 1.71 (1H, m, H-2), 1.60 (3H, m, H-7, -12, -22), 1.49 (3H, m, H-1, -7, -22), 1.41 (3H, m, H-1, -5, -17), 1.20 (1H, dd, $J = 14.0, 5.0$ Hz, H-15), 1.94 (3H, d, $J = 0.5$ Hz, CH_3 -31), 1.81 (3H, d, $J = 0.5$ Hz, CH_3 -27), 1.08 (3H, s, CH_3 -30), 1.00 (3H, s, CH_3 -19), 0.93 (3H, s, CH_3 -29), 0.95 (3H, d, $J = 6.5$ Hz, CH_3 -21), 0.88 (3H, s, CH_3 -28), 0.68 (3H, s, CH_3 -18); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) (δ ppm): 172.2 (C-26), 165.9 (C-1'), 167.2 (C-3'), 157.4 (C-24), 135.1 (C-9), 133.8 (C-8), 125.2 (C-25), 108.2 (C-23), 79.8 (C-16), 79.6 (C-3), 54.6 (C-17), 52.3 (C-4'), 48.6

(C-14), 45.3 (C-5), 43.5 (C-13), 41.8 (C-2'), 41.1 (C-22), 37.1 (C-10), 36.8 (C-4), 35.4 (C-15), 30.7 (C-20), 30.5 (C-1), 30.1 (C-12), 28.0 (C-30), 27.6 (C-28), 26.5 (C-6), 23.1 (C-2), 21.7 (C-29), 20.2 (C-11), 19.4 (C-21), 18.8 (C-19), 17.9 (C-7), 16.5 (C-18), 10.8 (C-31), 8.5 (C-27); ESIMS m/z 621 ($[\text{M}+\text{K}]^+$, 60), 605 ($[\text{M}+\text{Na}]^+$, 26), 521 (33), 505 (100), 483 (48); HRESIMS m/z 605.3451 ($[\text{M} + \text{Na}]^+$, calcd for $\text{C}_{35}\text{H}_{50}\text{O}_7\text{Na}$, 605.3454).

Results and discussions

A joint-study between Vietnam and Taiwan on bioactive compounds from the Vietnamese plant, *Clausena lansium* Skeels (Rutaceae), was conducted.

The methanol extract from the dried leaves of *C. lansium* was partitioned between H_2O and CHCl_3 . The purification of the CHCl_3 fraction by a combination of column chromatographic methods afforded eight new lactams, including γ -lactams (1-3), δ -lactams (4-7), and amide (8), along with seven known lactams (9-15), which were characterized from the leaves of *C. lansium* (Fig. 1). Their structures were elucidated using spectroscopic methods [3] and the absolute configurations were determined using electronic circular dichroism (ECD) and single-crystal X-ray diffraction analyses with Cu $K\alpha$ radiation.

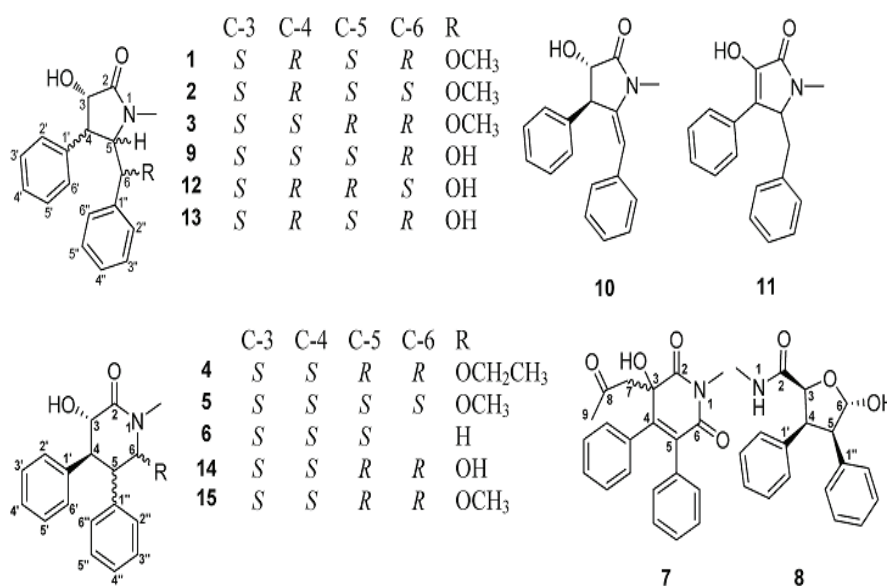


Fig. 1. The lactam compounds 1-15.

The ECD sign and red shift of the Cotton effect were shown to experimentally determine the C-3 configuration as well as the sign and the magnitude of the $n \rightarrow \pi^*$ Cotton effect, which are sensitive to the nature of the C-3 substituent [4]. Therefore, the C-3 configuration of compound **1** with a hydroxyl functionality was determined as S, because it displayed a positive Cotton effect near 230 nm. The absolute configuration of compound **1** was unambiguously defined, by a single-crystal X-ray diffraction analysis with Cu K α radiation, as 3S, 4R, 5S, and 6R (Fig. 2). Consequently, the structure of the 6-O-methylnecloclausenamide (**1**) was characterized, as shown in Fig. 1. The 2D structure of compound **2** was similar to compound **1**, while the relative configuration of the lactam ring was assigned as being similar to compound **1**, through the analysis of their NOESY spectra (Fig. 3). In addition, the absolute configurations at C-4, C-5, and C-6 were determined by the single-crystal X-ray diffraction pattern using the anomalous scattering of Cu K α radiation (Fig. 2). Therefore, the absolute configuration was determined as 3S, 4R, 5S, and 6S. In effect, the structure of 6-O-methyl-epi-necloclausenamide (**2**) was assigned as shown. The 2D structure of compound **3** was assigned to be identical to those of compounds **1** and **2** by a comparison of their UV, IR, MS, and NMR data [2]. The ECD spectrum of compound **3** showed a low-amplitude positive Cotton effect near 236 nm. The ECD spectrum of compound **12** showed a high-amplitude positive Cotton effect at 230 nm. Thus, the low-amplitude positive Cotton effect at 238 nm in the ECD spectrum of compound **3** (Fig. 4) suggested 3S and 4S absolute configurations [5]. By comparing the specific rotation and absolute configuration of compound **3** with the 16 stereoisomers of clausenamide, the 3S, 4S, 5R, 6S and 3S, 4S, 5R, 6R configurations could be further considered [3]. Therefore, the absolute configuration of 6-O-methyl-epi-

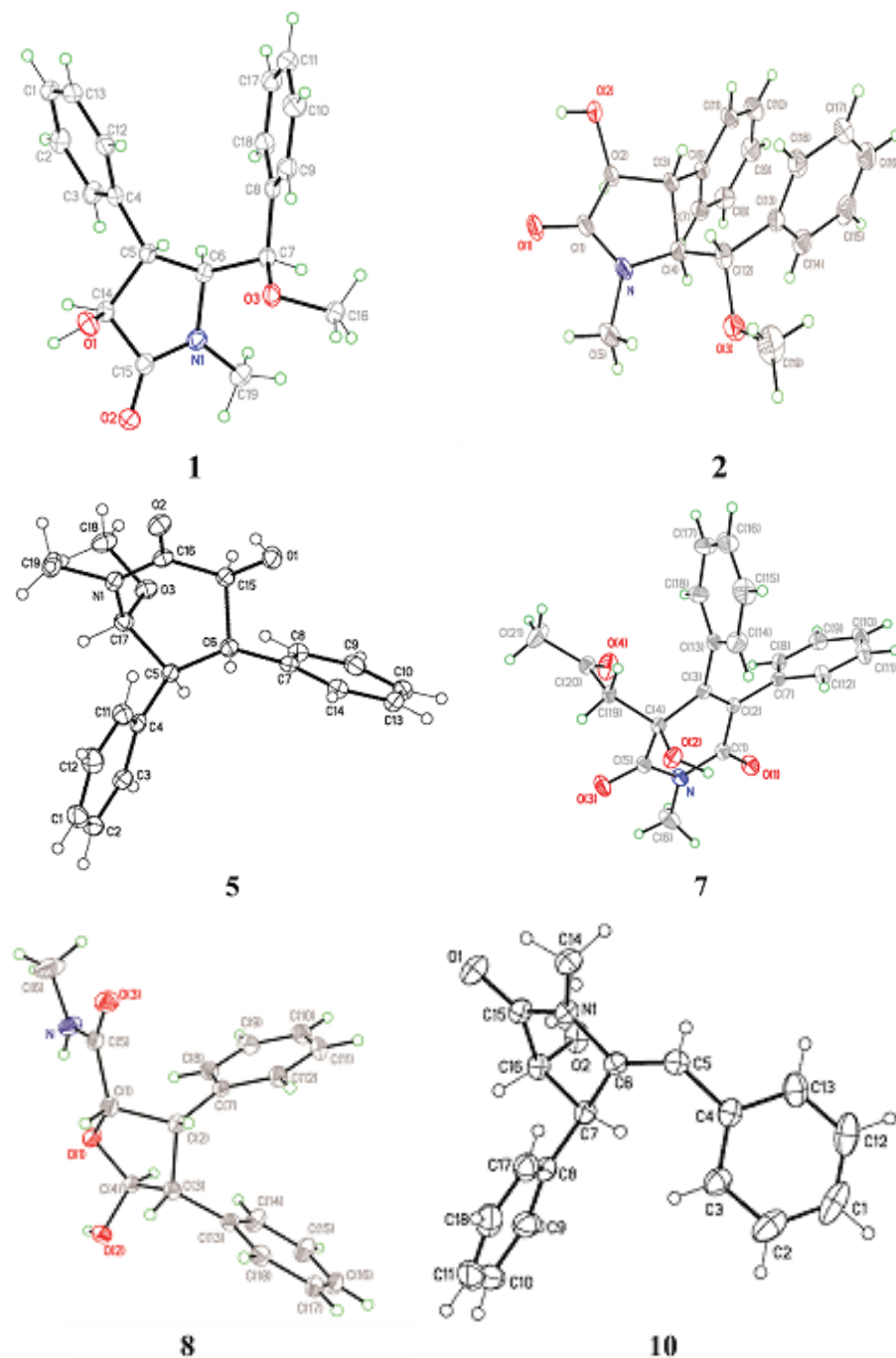


Fig. 2. ORTEP drawings of compounds **1**, **2**, **5**, **7**, **8**, and **10**.

cisnecloclausenamide (**3**) was established as 3S, 4S, 5R, and 6R. The absolute configuration of C-3 in compound **4** was deduced by the ECD spectrum. In this case, the ECD spectrum of compound **4** (Fig. 4) showed a positive Cotton effect at 231 nm, which evidenced a 3S absolute configuration. Consequently, the absolute configuration of compound

4 was deduced as 3S, 4S, 5R, and 6R, the structure of which was illustrated as shown. To determine the absolute configuration, compound **5** was subjected to a single-crystal X-ray diffraction analysis with Cu K α radiation (Fig. 2) which confirmed the structure unambiguously. Therefore, the absolute configuration was established as 3S, 4S,

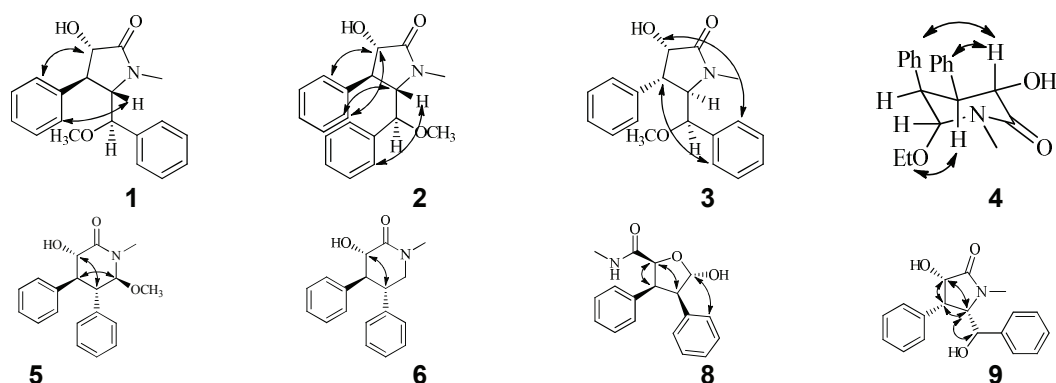


Fig. 3. Selected NOESY ($\leftrightarrow$) correlations for compounds 1-6, 8, and 9.

5S, and 6S (Fig. 2). Hence, compound **5** was characterized as lansamide-6. A positive Cotton effect at 223 nm in the ECD spectrum (Fig. 4) suggested a 3S absolute configuration. The absolute configuration was established as 3S, 4S, and 5S, while the structure of lansamide-7 (**6**) was characterized as shown. Based on these results and the single-crystal X-ray diffraction analyses using Cu K α radiation (Fig. 2), the structure of lansamide-8 (**7**) was identified as shown. The crystals of compound **7** were orthorhombic and belonged to the space group, *Pbca*. As shown in the ORTEP drawing (Fig. 2), the X-ray analysis revealed that compound **7** was a racemic mixture presumably originating from the reaction between pyridine-2,3,6-trione and acetone. From the spectroscopic analysis and the single-crystal X-ray diffraction data (Fig. 2), the absolute configuration was confirmed by the Flack parameter 0.0(2) and defined as 3S, 4S, 5R, and 6S. The structures of compounds **9** and **10** were confirmed by the HRESIMS data and single-crystal X-ray diffraction analysis (Fig. 2). These structures have been reported as synthetic products, but they were isolated from their natural sources for the first time. Compounds **12** and **13** were identified as (-)-clausenamide and (-)-neoclausenamide through the ^1H and ^{13}C NMR [1], the positive Cotton effect in the ECD spectrum [at 230 and 229 nm] (Fig. 4), single-crystal X-ray diffraction analysis (Fig. 2), and its negative specific rotation [-148.5

(c 0.8, MeOH) and -71.8 (c 1.8, MeOH)]. Compounds **14** and **15** were reported as racemates in a previous study [5], but the negative specific rotation [-107.8 (c 1.4, MeOH) and -117.1 (c 0.7, MeOH)] and a high-amplitude Cotton effect (Fig. 4) confirmed that they were pure enantiomers. Their structures were confirmed by the positive Cotton effects in their ECD spectra [at 230 and 231 nm] (Fig. 4) and single-crystal, X-ray diffraction analyses (Fig. 2).

Some relationships between the ECD spectra and the absolute configurations could be found from the above results. In the ECD spectra, δ -lactams **4**, **14**, and **15**, with 3S, 4S, and 5R absolute configurations, exhibited negative and positive Cotton effects near 210 and 230 nm, respectively. Compound **5** and **6**, possessing 3S, 4S, and 5S absolute configurations, displayed ECD spectra with a positive Cotton effect at 220 nm. For the γ -lactam group, compounds **1**, **12**, and **13**, with 3S and 4R stereochemistry, exhibited similar ECD spectra. However, the absolute configurations of compound **12** at C-5 and C-6 were different from those of compounds **1** and **13**. This implied that the absolute configuration of C-5 and C-6 had little contribution to the ECD spectra. In contrast, compounds **3** and **9** possessed 3S and 4S absolute configurations and showed different ECD spectra, as compared to those of compounds **1**, **12**, and **13**. This indicated that the C-4 phenyl group may have a significant influence on the Cotton effect near 230 nm. Furthermore, a comparison

of the ECD spectra of compounds **3** and **9** showed that the absolute configuration at C-5 may influence the wavelength of the Cotton effect.

In the other joint-study, air-dried and powdered fruiting bodies of *H. apiaria* were extracted with methanol and the combined extracts were concentrated under reduced pressure to produce a deep brown syrup. The crude extract was suspended in water and partitioned with ethyl acetate to afford ethyl acetate and water-soluble fractions. Purification of the ethyl acetate fraction by a conventional combination of column chromatographies yielded four new triterpenoids (**16-19**) and hexatenuin A [6].

Compound **16** was obtained as an optically active white powder, with $[\alpha]_D^{25} +57$ (c 0.6, MeOH). The positive-mode HRESIMS of compound **16** showed a pseudo-molecular ion peak at m/z 605.3451 ($[\text{M}+\text{Na}]^+$, calcd for $\text{C}_{35}\text{H}_{50}\text{O}_7\text{Na}$, 605.3454), corresponding to the molecular formula of $\text{C}_{35}\text{H}_{50}\text{O}_7$ with 11 indices of hydrogen deficiency (IHD). The UV spectrum of compound **16** exhibited an absorption maxima at 262 nm, compatible with an α,β -unsaturated carbonyl chromophore [7]. The IR absorption bands at 2946, 1759, and 1693 cm^{-1} suggested the presence of aliphatic C-H, lactonic carbonyl, and carbon-carbon double bond functionalities. The ^1H NMR spectrum of compound **16** displayed five methyl singlets at δ 0.68 (3H, CH_3 -18), 0.88

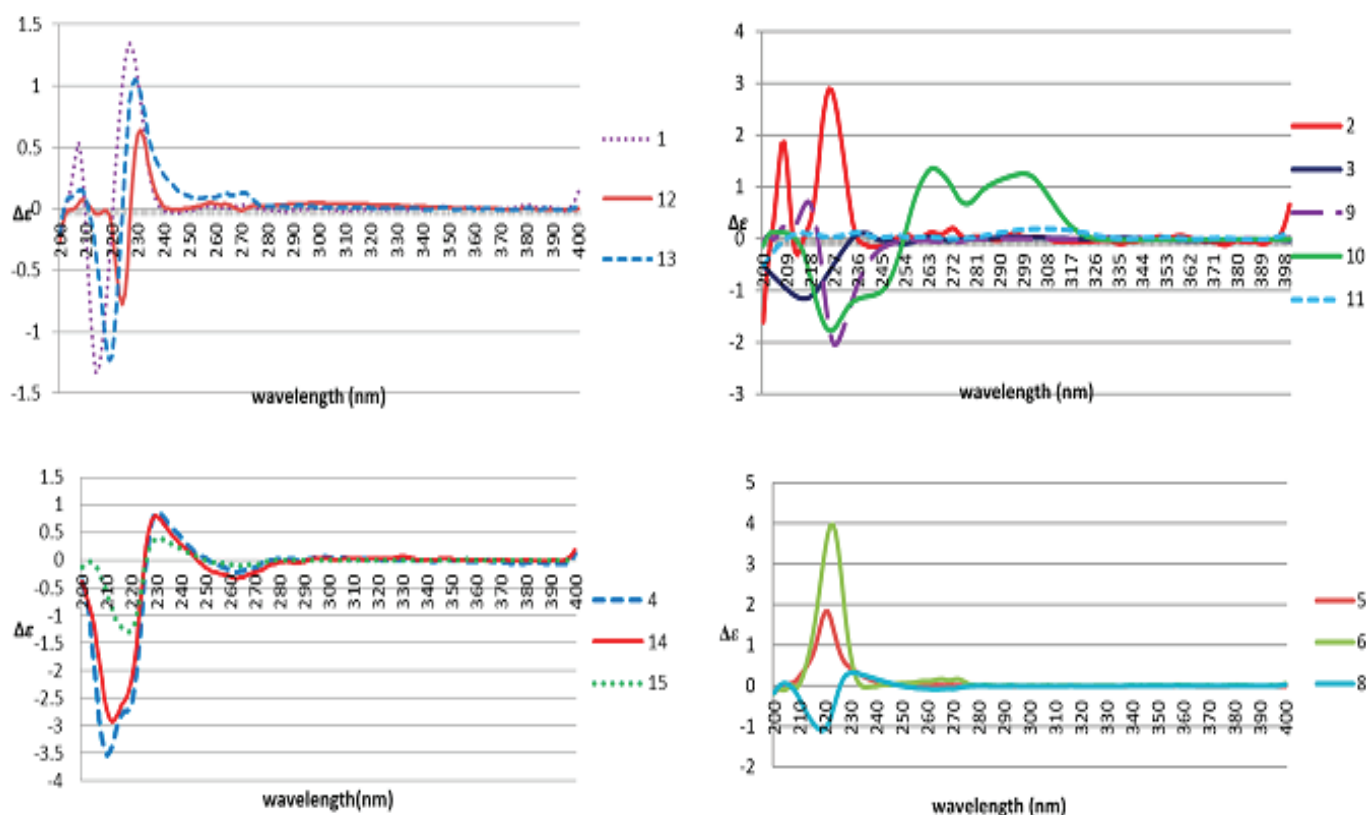


Fig. 4. ECD spectra of compounds 1-6 and 8-15.

(3H, CH₃-28), 0.93 (3H, CH₃-29), 1.00 (3H, CH₃-19), and 1.08 (3H, CH₃-30), respectively. In addition, one doublet methyl group at δ 0.95 (3H, J = 6.5 Hz, CH₃-21) suggested the presence of the lanostane skeleton. Two vinyl methyl signals at δ 1.81 (3H, d , J = 0.5 Hz, CH₃-27) and 1.94 (3H, d , J = 0.5 Hz, CH₃-31), along with the ¹³C NMR signals at δ 8.5 (C-27), 10.8 (C-31), 108.2 (C-23), 125.2 (C-25), 157.4 (C-24), and 172.2 (C-26), indicated a γ -lactone ring cyclized between C-23 and C-26. This was verified by the HMBC correlations from CH₃-31 to C-23, -24, and -25 as well as from CH₃-27 to C-24, -25, and -26, respectively. In the downfield region of the ¹³C NMR spectrum, there were two oxygenated methines at δ 79.6 (C-3) and 79.8 (C-16), one set of tetra-substituted double bonds at δ 133.8 (C-8) and 135.1 (C-9), and two ester carbonyl carbons at δ 165.9 (C-1') and 167.2 (C-3'). The location of the tetra-substituted double bond at C-8/C-9 was determined by the ³ J -HMBC correlations between

CH₃-19 and C-9 and between CH₃-30 and C-8. The HMBC cross-peaks from H-16 (δ 4.32, 1H, ddd , J = 11.5, 11.5, 5.0 Hz) to C-20 (δ 30.7), from H-3 (δ 4.71, 1H, $br\ s$) to C-29 (δ 21.7), C-1 (δ 30.5), C-5 (δ 45.3), C-1'; from CH₂-2' (δ 3.40, 2H, s) to C-1' and C-3'; and from CH₃-4' (δ 3.72, 3H, s) to C-3' evidenced that the C-16 had been oxygenated while the C-3 had been acetylated by the carbomethoxyacetoxy group. The elucidations provided above constructed the chemical skeleton of **1** with 10 IHDs. The last IHD was afforded by the cyclization between C-16 and C-23 through the ether linkage with a spiro structure. These spectra evidenced that compound **16** was very similar to the reported compound hexatenuin A [8], with the only difference being that compound **16** was the methyl derivative of hexatenuin A. The coupling constants of H-3 ($br\ s$) and H-16 (11.5, 11.5, 5.0 Hz) indicated their orientations to be equatorial and axial. The stereochemical configurations of H-3 and H-16 were

further established as β and β , according to the NOESY analysis and comparison of the spectral data of compound **16** and hexatenuin A [8]. The successive two-dimensional spectral experiments, including COSY, NOESY, HMQC, and HMBC accomplished the assignments of all the proton and carbon signals of compound **16**, and therefore its chemical structure was established as shown in Fig. 5 and named trivially as hexagonin A.

Compounds **17-19** were all obtained as optically active white powder, displaying similar UV spectra and IR absorption bands as those of compound **16**. Moreover, the proton resonances for the eight methyl groups, characteristic of the triterpenoid basic skeleton, were all observed in their ¹H NMR spectra. These data indicated that compounds **16-19** were structurally similar compounds (Fig. 6).

The purified triterpenoids, which were isolated in sufficient quantity,

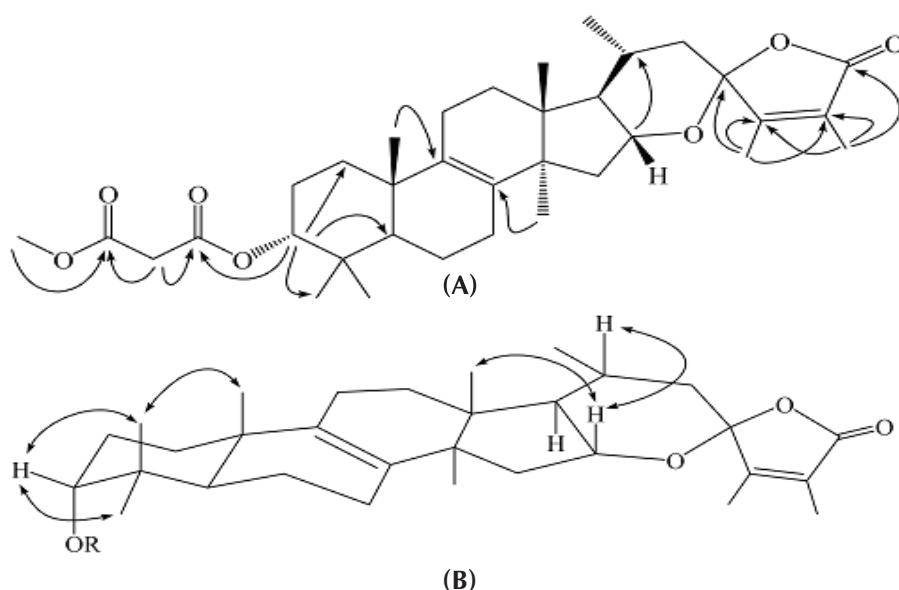


Fig. 5. Significant HMBC (A) and NOESY (B) correlations of compound 16.

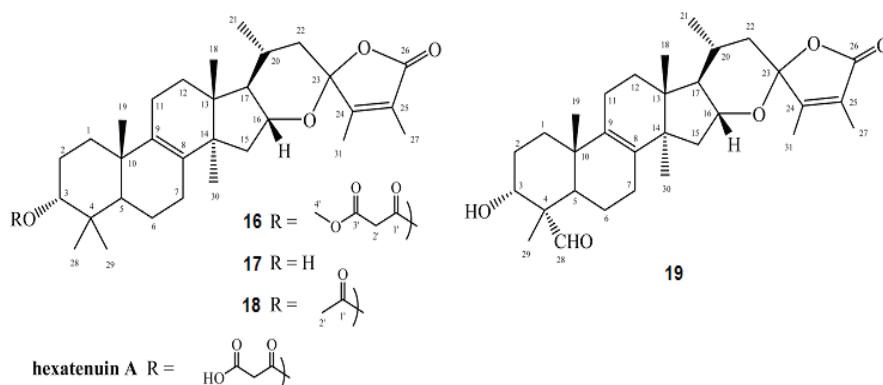


Fig. 6. Chemical structures of all the purified compounds.

Table 1. Inhibitory effects of purified samples from *H. apiaria* on superoxide anion generation and elastase release by human neutrophils, in response to N-Formyl-Lmethionyl-phenylalanine/Cytochalasin B (FMLP/CB).

Compound	IC ₅₀ (μM) ^a	
	Superoxide anion generation	Elastase release
16	>10	- ^b
17	>10	- ^b
18	>10	- ^b
19	6.0±1.0***	>10
hexatenuin A	1.9±0.2***	4.3±1.4***
LY294002 ^c	0.4±0.02***	1.5±0.3***

^aConcentration necessary for 50% inhibition. Results are presented as mean ± SD (n = 3-4). ***p < 0.001 compared with the control value. ^bIncreasing effects were observed. ^cA phosphatidylinositol-3-kinase inhibitor was used as a positive control for superoxide anion generation and elastase release.

were examined for their inhibition of superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB (Table 1). Among the examined constituents, hexatenuin A displayed the most significant inhibition of superoxide anion generation and elastase release, with IC₅₀ values of 1.9±0.2 and 4.3±1.4 μM, as compared to the reference compound LY294002,12 with IC₅₀ values of 0.4±0.02 and 1.5±0.3 μM for superoxide anion generation and elastase release, respectively. In addition, the following structure-activity relationships could be deduced from the bioactivity data. Hexagonins B (17) and D (19), which possess the basic triterpenoid skeleton without the malonyl substitution at C-3, did not show any anti-inflammatory bioactivity. Comparatively, hexagonin A (16), with its triterpenoid skeleton and malonyl and methyl ester functions, also failed to exhibit significant activity. Hexatenuin A, which had the triterpenoid skeleton as well as a free malonic acid group, displayed the most significant inhibitory effects in the bioactivity examination. Consequently, the free malonic acid function was important for anti-inflammatory activity. From the above data, it was concluded that the purified triterpenoids of *H. apiaria* are new potential leads for anti-inflammatory drug development and the starting fungus can be used as a health food with a possible and known mechanism of action.

Therefore, it is not surprising that intrinsic anti-inflammatory properties demonstrated in vitro with *H. apiaria* can be transferred in vivo after mushroom consumption as food or nutraceutical food. This study has identified the ability for food processing to anti-inflammatory. The process extraction for *H. apiaria* identified a five-step process that would address certain critical aspects in the design and development of functional food (Fig. 7).

Conclusions

A total of 15 lactams were isolated

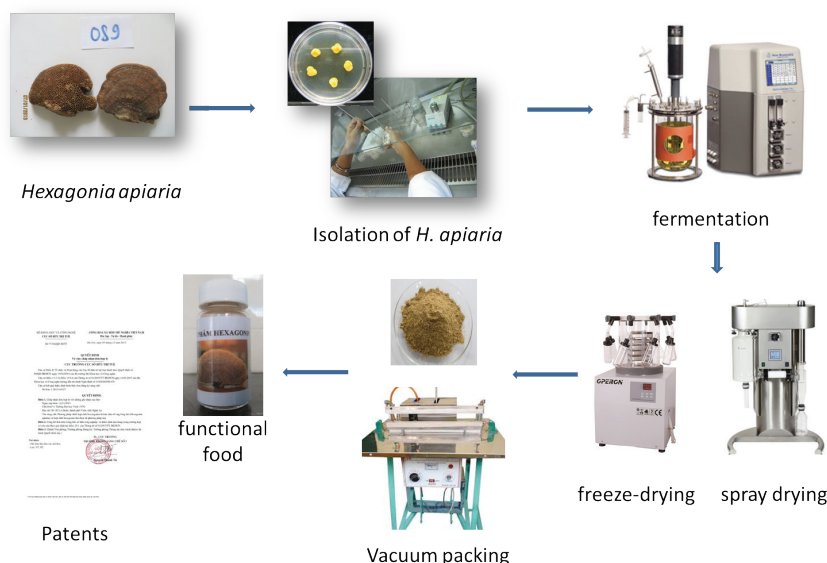


Fig. 7. The process of extraction for *H. apiaria*.

from the methanolic extract of *C. lansium*. This research work enabled the determination of the absolute configuration of these classes of compounds using MS, NMR, electronic circular dichroism (ECD), and single-crystal X-ray diffraction analyses with Cu K α radiation. In the other study, a chemical investigation of the fruiting bodies of *H. apiaria* resulted in the identification of five compounds, hexagonins A-D (16-19) and hexatenuin A. The purified constituents were examined for their anti-inflammatory activity. Among the tested compounds, hexatenuin A displayed the most

significant inhibition of superoxide anion generation and elastase release. These triterpenoids may have the potential to be used as anti-inflammatory agents. This study has identified abilities from food processing to anti-inflammatory. The process extraction for *H. apiaria* identified a five-step process that would address certain critical aspects in the design and development of functional food.

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Research and development of polyurea pavement marking paint

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Abstract:

Pavement markings make an important contribution to the safety of people and vehicles on roads. Many types of pavement marking materials have been used, and most recently, polyurea materials have been used as a binder in pavement marking paint. This type of paint has high retro-reflection and durability, so it has been widely applied for use at airports and on highways around the world. This article introduces the results of our research and the making of polyurea pavement marking paint using the formula: binder/harder ratio (1.3/1 by weight), 42% of pigment and filler, 10% of reflective balls, rheological additive, and dispersive additive to create outstanding features (retro-reflection > 260 mcd/m²/lux).

Keywords: pavement marking material, polyurea, retrorreflection.

Classification number: 2.3

Introduction

Outlined reflective traffic guides serve an important role in controlling the flow of traffic. The painted lines on roads include centerline stripes, lane dividing lines, and marked areas due to obstacles, parking areas, road signs, and walkways. The standards of these marking materials consider their durability, dry time, and their impact on the environment [1, 2].

Currently in Vietnam, solvent reflective marking paints based on alkyd resins or styrene acrylic are usually used in the transport sector. It has been found that signal reflective paints based on alkyd resins and solvents provide better visibility for pedestrians, both during the day and at night, compared to signal reflective paints that are based on thermosetting plastics. Under oxidation,

alkyd paints become brittle and lead to decreased adhesion on road surfaces, as well as developing glass particles in the paint. Besides that, solvent signal reflective paint also contains volatile organic compounds (VOC) that are not suitable for traditional usage, transport, and removal methods.

Reflective marking paints based on thermoplastics are also being widely used today on express ways, highways, and airplane runways. For highways, roads, and airplane runways, the abrasion resistance and reflectiveness of these coatings are still having many shortcomings, resulting in increasing maintenance and repair costs, being inefficient, and not ensuring traffic safety [1, 2].

Polyurea elastomer-based marking

paints contain two components that are new materials recently applied to the transport sector. Polyurea paints have several features, like two-component-modified polyurethane paints; polyurea coating is a unique coating technology, which is unlike polyurethane coating [3].

Two-component polyurea coatings are applied using specialized spray equipment that provides high-pressure application, fast drying time, and an achievement of optimal adhesion. The fast drying feature is contained within a wide temperature range and is unaffected by moisture. A fast drying time of paint leads to time to put the product into use more quickly. Polyurea coatings have color stability, abrasion resistance, and good adhesion to all surfaces. In particular, polyurea reflective marking paints have high reflectiveness and are sustainable over time. Polyurea materials claim to have a lifespan of up to five years [4, 5].

Despite their appearance in many countries, polyurea coatings have yet to be applied broadly in Vietnam, particularly in the field of transportation. In this study, with an aim of mastering the technology and initial identification of polyurea coating applications in the conditions of Vietnam, marking materials based on polyurea elastomer have been studied on a selection of basic components including: curing agent

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ratio, filler content, dispersing additives, rheology additives, and loading of glass bead.

Materials and experiment

Materials

- The binder is Bayer NDT1-Desmophen® (amine number 190 mg KOH/g, viscosity at 25°C: 1,000 mPa.s, the density at 20°C: 1.06 g/ml);

- The curing agent is polyisocyanate Desmodur VP LS 2371 (Bayer - Germany);

- Titanium oxide pigment, yellow organic pigment, calcium carbonate, and talc fillers (China);

- Dispersing additives including dispersants, DP-1 of BYK (solution of copolymers with acid groups, acid number: 53 mg KOH/g, specific weight: 1.03 g/ml, concentration of volatile substance: 52%);

- Dispersing additives including dispersants, DP-2 of Cognis (neutralization of acid salts polycarbonic with polyamines, the density: 0.88 to 0.90 g/cm³, acid number: 50 mg KOH/g);

- Rheological additives including rheological additives FS 1 (compound of hydrophilic silica surface of the carrier Aerosil, specific surface: 200 m²/g, average particle size: 12 nm, SiO₂ content: 99%);

- Rheological additives, FS 2 Elements (organic derivatives of hectorite clay, fine powder, specific weight: 1.8 g/cm³);

- The glass bead, DPI Class I, according to AASHTO M247.

Experiment

The determination of paint properties is according to the following tests: KU viscosity, smoothness, luminosity, impact resistance, oil resistance, salt resistance, alkali resistance, retro-reflectivity, and abrasion. The testing methods are specified in ISO standard 8787: 2011.

Results and discussions

Resin/curing agent

Based on a published document and standards prescribed about polyurea marking materials, the specifications for polyurea marking paints are chosen as follows [3-5]:

- Paints having 100% solid content;
- A mixing ratio between A and B

components is varied from 2:1 to 3:1 by volume;

- Loading of pigments, filler powder is greater than 15% by weight;

- Using amino resin NDT1 and isocyanate Desmodur VP LS 2371 to making polyurea coatings. The components are blended according to the formula in Table 1.

Table 1. Polyurea marking paint components.

	% by weight		
	Sample 01	Sample 02	Sample 03
<i>A component</i>			
NDT1	30	37	45
TiO ₂	15	15	12
Filler	27	20	17
Rheology agent FS-1	1	1	1
Dispersing agent DP-1	1	1	1
<i>B component</i>			
Desmodur VP LS 2371	28	28	28

Table 2. Drying time, adhesion, and abrasion of coating films.

Properties	Drying time (min)	Adhesion (MPa)	Abrasion (g)
<i>Sample 01</i>	5	3.5	0.08
<i>Sample 02</i>	8	3.0	0.07
<i>Sample 03</i>	12	4.0	0.06

Table 3. Paint with differences in titanium oxide content.

	% by weight		
	Sample 01	Sample 02	Sample 03
<i>A component</i>			
NDT1	37	37	37
TiO ₂	7	13	18
Filler	28	22	17
Rheology agent FS-1	1	1	1
Dispersing agent DP-1	1	1	1
<i>B component</i>			
Desmodur VP LS 2371	28	28	28

Then, prototyping and testing the properties of coating film including the drying time (minutes), the abrasion (g), and the adhesion (MPa). The results are shown in the Table 2.

From the obtained results, the ratio 1.3/1 of A/B is chosen to carry out the next survey.

Effect of pigments on properties of paint

The brightness of road markings has major influence on the ability to recognize road markings of driving. Typically, the rutile titanium oxide powder was used with the aim to increase the brightness of the paint. With amine compound NDT1 and isocyanate curing agent, the paints were conducted with titanium oxide content changing 7, 13, and 18% by weight (Table 3).

After fabricating the prototypes, the brightness of the paint was measured (Fig. 1). The results showed that the brightness of the coating increases when the content of titanium oxide is increased. Specifically observed was the brightness of the paint at 60% with 7% by weight of titanium oxide, further increased to 13 and 18% by weight at the brightness of 76 and 80%, respectively. The chosen content of titanium oxide in white paint polyurea was at least 13% by weight. Polyurea yellow paint is made with the following formula (Table 4):

The measured brightness of marking paint is 55% in accordance with standard yellow marking paint.

Effect of additives on properties of paint

The effect of dispersing additives signed DP-1 and DP-2 on the properties of paints having the same content of 1.0% by weight is shown in Table 5.

The results from Table 5 show that using dispersing agent DP-1 makes better dispersion of pigment powder and filler than that of DP-2.

The effect of rheology agent FS-1 and FS-2 on the properties of marking paints having the same content of 1.0% by weight is shown in Table 6.

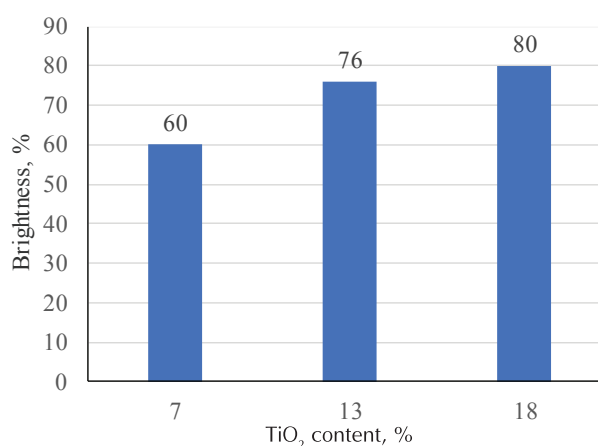


Fig. 1. Effect of TiO₂ content on the paint brightness.

Table 4. Formula of polyurea yellow paint.

% by weight	
<i>A component</i>	
NDT1	37
TiO ₂	8
Yellow pigment	5
Filler	22
Rheology agent FS-1	1
Dispersing agent DP-1	1
<i>B component</i>	
Desmodur VP LS 2371	28

Table 5. Effects of additives on properties of paint.

Dispersing agent	Appearance	Fineness (μm)
DP-1	Homogeneous. Normal surface	25
DP-2	A few small cracks appeared	30

Table 6. Properties of marking paint having rheology agent.

Anti-deposition additives	Flow ability	State
FS-1	Non flow	Non deposition
FS-2	Flow	Slight deposition
Not rheological additives	Flow	Deposition

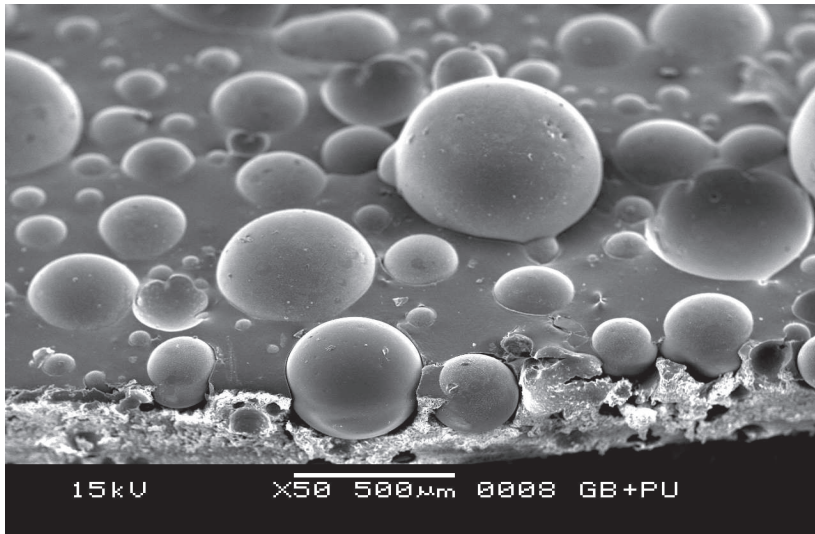


Fig. 2. SEM image of polyurea reflective paint.

Table 7. Effects of glass bead contents on the retro-reflectivity of polyurea marking materials.

Glass bead content (% by weight)	Retro-reflectivity (mcd/m ² /lux)
5	175
10	260
15	300

Table 8. The formula of polyurea marking paint (% by weight).

	White paint	Yellow paint
A component		
Amine NDT1 resin	37	37
TiO ₂	13	8
Yellow pigment	-	5
Filler	22	22
Rheology additive FS-1	1	1
Dispersing additive DP-1	1	1
B component		
Desmodur VP LS 2371	28	28

The results show that when rheological additives FS-1 are used, the viscosity of paint increases and the paint layers do not flow. Therefore, the rheological additive FS-1 was selected for making paint in the next experiments.

Effects of glass beads on retro-

reflectivity of paint

In order to investigate the influence of glass beads on the retro-reflectivity of paint film, the paint has been made according to the results shown above. Then the film was made and sprayed with type I glass beads of different

contents at 5, 10, and 15% by volume of paint. Fig. 2 shows an image of the paint surface under a scanning electron microscope (SEM). The results show that the contact area between the glass beads and the matrix depends greatly on the diameter of the glass beads. With glass beads of a diameter of less than 250 microns, the glass beads sunk to 3/4 diameter balls. Meanwhile, with glass beads of a diameter greater than 500 microns in diameter, only 1/3 of balls sunk into the paint matrix. Requirements for glass beads creating for the best retro-reflectivity paint and still ensuring good bonding strength with the matrix is that the sunken part of the ball in the paint matrix should be 2/3 of the ball. Glass beads having a diameter of about 250-500 microns are appropriate these requirements.

The results of retro-reflectivity of completely-dried paint films having glass beads are shown in Table 7.

The results show that the retro-reflectivity of polyurea marking paints increased with increasing content of glass beads and they were greater than 100 mcd/m²/lux. However, in order to maintain the retro-reflectivity of marking line with time it is better to choose the content of glass beads of 10% by weight.

Thus, it is pleased to offer the optimum formula for making elastomer marking paint as shown in Table 8.

Quality assessment of elastomer marking paint

The marking paint with optimal formula was made and then testing the following properties: viscosity, fineness, drying time, adhesion... The results were shown in Table 9.

Besides, the subject has also been constructed according to tests of the marking paint used at the scene. The marking paint gained the retro-reflectivity of over 260 mcd/m²/lux, adhesion on a cement, concrete road reached 3 MPa. The results showed

Table 9. Quality assessment of elastomer marking paint.

	Unit	Requirement	Results
Color	-	$\geq Y 35$	Y 35
Stability		≥ 8	8
Viscosity	KU	60÷80	80
Drying time	min	≤ 15	≤ 8
Luminosity:			
- White paint	%	≥ 75	≥ 76
- Yellow paint	%	≥ 50	≥ 55
Flexibility	mm	≤ 12	4
Adhesion:			
- Concrete	%	≥ 90	95
- Asphalt	%	≥ 80	92
Anti-shaded color	%	≤ 3	≤ 3
Impact resistance	-	The substrate is not exposed through the paint film	The substrate is not exposed through the paint film
Oil resistance			
Salt resistance			
Water resistance		The coating is not peeling or blistering.	The coating is not peeling or blistering.
Alkali resistance			

that the quality standards of painting materials was achieved, and even exceeded the requirements prescribed under TCVN 8787: 2011.

Conclusions

- The composition suitable for road marking paints made on the basis of polyurea elastomer including amino

resin NDT1 at 37%, titanium oxide powder at 13% (white paint) and at 8% (yellow paint), organic yellow powder at 5%, fillers at 22%, dispersing additives DP-1 at 1%, rheology additives FS-1 at 1%, isocyanat plastic at 28%, and glass beads at 10% by weight of the paint.

- Polyurea reflective road marking

paint has fast drying time (less than 8 minutes); greater adhesion to concrete bases of 3 MPa; and retro-reflectivity of more than 260 mcd/m²/lux in standard conditions. This is truly the appropriate use for the airport runway and the highway in Vietnam.

- Reflective marking materials based on polyurea are made to both meet the specifications defined in TCVN 8787: 2011 and to have many advantages, especially adhesion to concrete pavement (3 MPa, greater than regulation of 1.2 MPa), and high flexibility, remaining highly durable with retro-reflectivity. With their advantages, reflective marking materials based on polyurea elastomer will improve expected durability and high reflective ability; contribute in the work of ensuring traffic safety.

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QTL analysis on rice genotypes adapted to acid sulfate soils in the Mekong river delta, Vietnam

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Abstract:

Three target points in acid sulfate soils have been identified as: 1) Aluminum (Al) toxicity; 2) Iron (Fe) toxicity; 3) Phosphorous (P) deficiency; and 4) Droughts at the seedling stage. The exploitation of gene pools from wild rice species fruitfully obtained a true introgression of desirable traits into high yielding varieties (HYVs), such as AS996 (IR64/*Oryza rufipogon*), which are tolerant to Al-toxicity, have short durations, high yields, and adaptability to acid sulfate soils. Major QTLs on chromosome 3 were detected to control Al-toxicity as identified through an analysis of the RIL population of IR64/*O. rufipogon* on control relative root length (RRL). RM232 was considered as a good marker linked to the target quantitative trait locus (QTL) on chromosome 3, then SR28 and OSR29 on chromosome 9 were also used.

QTL mapping by 126 SSRs through 225 individuals of the F₆ RILs population of AS996/OM2395 was carried out to find the *P-uptake* gene on chromosome 12. The promising genotype of OM4498 from the BC population of IR64/OMCS2000 was selected through MAS with RM235 and RM247 on chromosome 12 linked to QTL, which controls the P-deficiency tolerance.

Based on the leaf bronzing index (LBI), SSR markers were used to select promising genotypes tolerant to iron-toxicity, such as RM315 and RM212 on chromosome 1, and RM252 and RM211 on chromosome 2. The intervals among RM315-RM212 on chromosome 1, RM6-RM240 on chromosome 2, and RM252-RM451 on chromosome 4, were continually studied through further fine mapping.

A backcrossing mapping population that included 217 individuals of BC₂F₂, was set up from OM1490/WAB880-1-38-18-20-P1-HB to detect the QTLs relating to drought tolerance (DT). The QTL was located in the intervals between RM201-RM511 on chromosome 9. BAC clones 13A₉ and 7O₃ were used as pinpoints on the high resolution map for new markers designed from their sequences. The markers became useful to help rice breeders possibly select the improved genotypes adapting to drought stress in the seedling stage.

Keywords: aluminum tolerance, drought tolerance, iron-tolerance, P-deficiency tolerance.

Classification number: 3.1

Introduction

Acid sulfate soils (*Sulfaquefs* and *Sulfaquents*) account for 30.1 and 48.5% in the Mekong River Delta and Red River Delta, respectively [1]. Thus, acid sulfate soils have become the main constraint for rice production in the Mekong delta.

Four target points in acid sulfate soils have been identified as aluminum (Al) toxicity, iron (Fe) toxicity, phosphorous (P) deficiency, and drought stress at the seedling stage. The problems and constraints vary across ecosystems; therefore, the solutions to the problems will vary accordingly. The research thrushes each ecosystem to address these particular problems. Currently, water management and agronomic practices have been recommended. Rice varietal improvement is also considered as a key approach. QTL analysis was performed using the software package QGEN from Cornell University and MapL from Japan University. MapMarker/QTL (IRRI) was also used to find the location of major and minor genes. The threshold for declaring a QTL for P deficiency tolerance was at LOD > 3. All markers were tested for the expected 1:1 ratio.

Tolerance to Al-toxicity

Since the aluminum (Al) forms of soils and their solubility have a high pH of 5 or less, Al-toxicity is one of the major growth limiting factors of acidic soils [2]. Roots injured by high Al-concentrations are usually stubby, thick, dark-colored, brittle, poorly branched, and have reduced root length and volume.

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Al-toxicity may inhibit shoot growth by limiting the supply of nutrients and water due to poor subsoil penetration or lower root hydraulic conductivity. Y. Tang, et al. (2000) [3] mapped a gene for Al-tolerance on the long arm of chromosome 4H of barley, 2.1-cM proximal to the marker Xbcd117, and 2.1-cM distal to the markers Xwg464 and Xcdo1395. P. Wu, et al. (2000) [4] identified several QTLs conferring Al-tolerance in a random inbred mapping-population derived from Azucena and IR1552. V.T. Nguyen, et al. (2001) [5] also detected five QTLs for Al-tolerance scattered across five chromosomes with a major QTL located on chromosome 1. V. Nguyen, et al. (2002) [6] found ten QTLs located on nine chromosomes for Al-tolerance using a doubled-haploid population derived from the cross of CT9993 x IR62266. Mapping using Indica x japonica populations identified QTLs associated with a transgressive variation where alleles from a susceptible *aus* or *Indica* parent enhanced Al-tolerance in a tolerant *Japonica* background [7].

Three populations of *O. rufipogon* were collected by Duncan Vaughan and Bui Chi Buu in 1989 at Tram Chim - bird sanctuary (Dong Thap Muoi), which area has strong acid sulfate soils, and its pH varies from 2.8 to 3.2 [8].

A total of 274 RFLPs from Cornell University and RGP digested by *EcoRI*, *EcoRV*, *DraI*, *HindIII*, and *XbaI* exhibited 14.0, 12.5, 19.8, 27.7, and 19.5% degrees of polymorphism, respectively. A population of 171F₆ recombinant inbred lines were derived from the cross of IR64 x *O. rufipogon* (acc. 106412). A genetic map, consisting of 151 molecular markers covering 1,755 cM with an average distance of 11.6 cM between loci, was constructed (Table 1). The seedling stage, a major QTL for RRL, explained 24.9% of the phenotypic variations, and was found on chromosome 3 of the rice varieties (Fig. 1 and 2). These results indicated the possibilities to use MAS and pyramiding QTLs for enhancing Al-tolerance in

Table 1. QTL mapping by 126 SSRs through 225 individuals of the F₆ RIL population of AS996/OM2395 [10, 11].

Chromosome	cM	Number of SSRs	Mean of genetic distance between two markers
1	507.5	18	28.19
2	206.7	14	14.76
3	795.9	12	66.33
4	216.7	13	16.70
5	196.6	11	17.87
6	101.4	6	16.90
7	319.2	13	24.55
8	115.7	7	16.52
9	99.9	8	12.48
10	79.9	5	15.98
11	115.9	7	16.55
12	150.1	12	12.50
Total		126	23.05

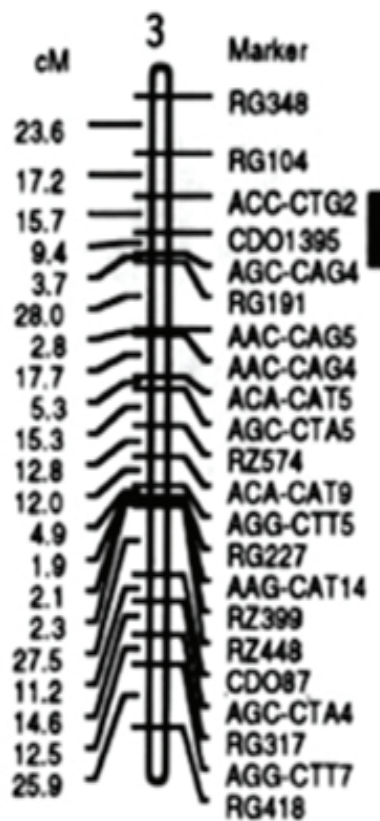


Fig. 1. QTLs controlling Al-tolerance related to RRL on chromosome 3.

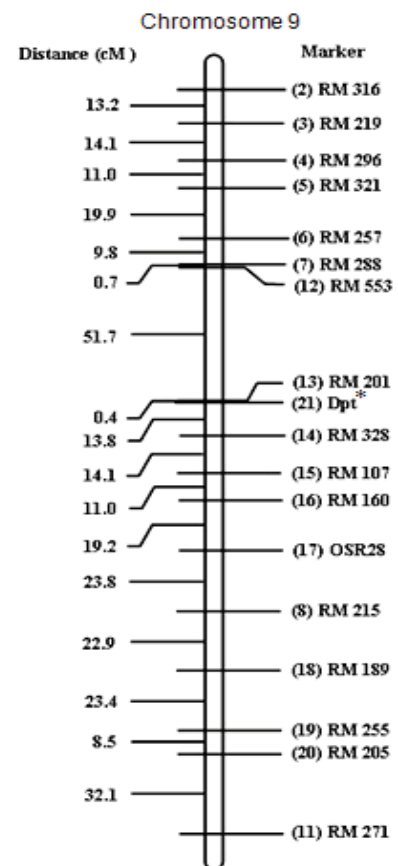


Fig. 2. Fine mapping on chromosome 9 from BC₂F₂ of OM1490/WAB880-1-38-18-20-P1-HB [12, 13].

Table 2. Putative QTLs detected for RRL by interval mapping analysis [9].

Interval	Chromosome	Length (cM)	Additive effect (DPE)	LOD	R ²
RG406-RZ252	1	6.5	0.100 (O)	2.4	9.0
CDO1395-RG391	3	0.6	0.167 (O)	8.3	24.9
RZ629-RG650	7	29.8	0.126 (O)	5.4	22.5
RG28-RM223	8	31.0	0.104 (O)	2.5	20.8
RM201-WALI7	9	10.0	0.109 (O)	2.6	9.9

DPE (direction of phenotypic effect): The allelic genetic effect and the O and I observed shows that the favorable alleles were derived from *O. rufipogon* and IR64, respectively; LOD: The maximum-likelihood of LOD score for the individual QTL; R²: Phenotypic variation explained by the individual QTL.

Table 3. Nature of gene variation for important characters under P-stress [20].

Trait	(H ₁ /D) ^{1/2}	2σ ² gca/(2σ ² gca + σ ² sca)	H ² _{ns} (%) (Narrow sense heritability)
Tilling capacity	1.94	0.16	19.70
Growth duration	0.98	0.56	33.90
Filled grains/pan.	5.80	0.01	3.10
Root dry weight	0.81	0.03	20.90

rice varieties [9]. AS997 was officially released and has become a leading variety adapted to acid sulfate soil areas in the Mekong river delta so far. The exploitation of the gene pool from wild rice species fruitfully displayed a true introgression of desirable traits into high-yielding varieties (HYVs), such as AS996 (IR64/*O. rufipogon*), which is tolerant to Al-toxicity and has short duration, high yield, and adaptability to acid sulfate soils.

Major QTLs on chromosome 3 were detected to control Al-toxicity, and this was observed through the analysis of the RIL population of IR64/*O. rufipogon* on RRL (Table 2) [9].

Tolerance to P-deficiency

P-deficiency in soils is a major yield-limiting factor for rice production. Increasing the P-deficiency tolerance of rice cultivars may represent a more cost effective solution than relying on fertilizer application [14]. The QTL linked to marker C443 on chromosome 12 displayed a major effect. Two of the three QTLs were detected for internal

P-use efficiency, which included a major one on chromosome 12, that coincided with QTLs for P-uptake; however, whereas Indica alleles increased P-uptake they reduced P-use efficiency [14]. Three QTLs that were identified for dry weight and four QTLs for P-uptake together explained 45.4 and 54.5% of the variation for the respective traits. M. Wissuwa, et al. (2002) [15] finally identified the gene *Pup1*, which controls P-deficiency tolerance on chromosome 12, in acidic soils. Y.J. Zhang, et al. (2010) [16] identified the interval of R3375-R367 on chromosome 12, which controls P-deficiency tolerance. Common quantitative trait loci (QTLs) for P-deficiency tolerance have been mapped on chromosomes 6 and 12 [14, 15, 17]. P-deficiency has been identified as the main factor in preventing the realization of high-yielding potentials of modern varieties in lowland rice production as well [18]. This problem is aggravated by the high P-fixing financial capacity of many soils commonly found in rice growing regions [19].

The allelism test and QTL map

analysis were conducted among progenies of mapping populations of Kasalath 47/OM4495 (BC₂F₃) and AS996/OM2395 (BC₂F₃).

The genetic nature of some characters related to P-deficiency tolerance was studied using diallele analysis. Suitable materials were chosen as OM723-11, OM850, IR64, IR50404, OM997, and IR59606. The tillering ability was considered as a good selection criteria. Maximum tiller numbers were scored at 45 days after transplanting the hybrids and their parents, constituting a 6 x 6 diallel set. However, shoot dry weight is the most sensitive plant parameter to P-deficiency, followed by root dry weight and the number of tillers. The proportion of dominant and recessive genes in the parent (K_D/K_R = 1.6) was more than one unit, which means that the dominant gene actions were more important under P-stress. The tendency of + ve alleles was clear (H₂/4H₁ = 0.37) showing the higher the root dry weight, the better tolerance to P-deficiency.

The variance ratio 2σ²gca/(2σ²gca + σ²sca) was computed from expected components of the mean square assuming a fixed model to access the relative importance of additive and non-additive gene effects in predicting progeny performance (Table 3).

The tolerance variety of AS996 to P-deficiency is one derivative of *O. rufipogon*, whereas high-yielding varieties of OM2395 are sensitive. The SSR linkage map consisted of 116 polymorphic SSR markers which showed the location of QTLs associated with relative shoot length, RRL, relative shoot dry weight, relative root dry weight under the Yoshida solution treatments of P-deficiency (0.5 mg P/liter), and P-adequate (10.0 mg P/liter). The map length was 2,905.5 cM with an average interval size of 23.05 cM. Based on the constructed map, a major QTL for P-deficiency tolerance was located on chromosome 12. Several minor QTLs were mapped on chromosomes 1, 2, 5, and 9. The study indicated that the candidate genes linked to RM235 and RM247 on chromosome 12, had an interval distance of 0.2 cM (Fig. 3 and Table 4) [10, 11].

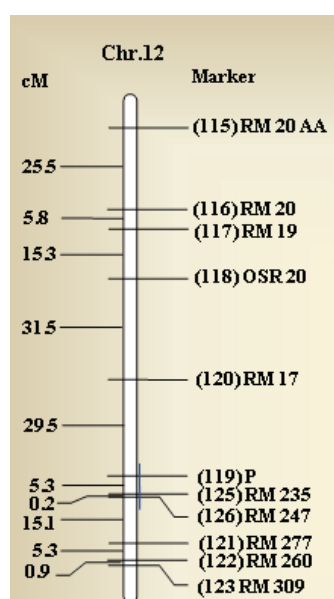


Fig. 3. QTL controlling P-uptake under acidic soils on chromosome 12.

Phosphorous-uptake 1 (*Pup-1*) controlling P-deficiency tolerance was considered as one of the most promising QTLs to develop rice genotypes (*Oryza sativa* L.) that are tolerant to abiotic stress. Gene-based molecular markers which were distributed among QTLs were fine-mapped as a 278-kb region [21] to be useful for rice breeders.

DT at the seedling stage

Acid-sulfate toxicity normally combines with drought stress at the seedling stage in dry seasons (April-May) to be harmful to rice crop in the Mekong River Delta. Crop tolerance connected to drought is genetically and physiologically complicated. Many morpho-physiological traits putatively contribute to DT, and multiple genes or quantitative trait loci (QTLs) typically control each of these traits. It is influenced by the environment to a great extent. Developing DT rice varieties has not been very successful despite the efforts made by breeders because they are done through practical breeding programs. Populations are typically segregating for maturity, making it difficult to accurately, repeatedly, and uniformly time and manage relevant water stress levels for selections. In most rice growing areas,

Table 4. Interval mapping analysis of the target characters.

Index	Interval marker	Chromosome	P-value	Centi-Morgan
8-9 (RSL)	RM307-RM237	1	0.001	10.8
63-64 (RSDW)	RM291-RM261	5	0.000	12.0
125-126 (RSDW and RSL)	RM235-RM247	12	0.001	0.2

RSL: Relative shoot length; RSDW: Relative shoot dry weight.

Table 5. QTL mapping by 232 SSRs through 225 individuals of a BC₂F₂ population of OM1490/WAB880-1-38-18-20-P1 [12, 13].

Chromosome	cM	Number of SSRs	Mean of genetic distance between two markers
1	355.5	24	14.81
2	337.0	25	13.08
3	221.8	19	11.67
4	187.9	18	10.43
5	183.2	17	10.77
6	120.9	20	6.04
7	189.0	18	10.50
8	180.9	17	10.64
9	290.4	20	16.13
10	133.3	15	8.88
11	177.2	18	9.84
12	186.6	21	8.88
Total	2,553.7	232	10.97

yield reductions due to drought have been observed. To overcome this problem, it was proposed to improve DT by marker-assisted selection (MAS) for DT. A marker-assisted back-crossing (MABC) breeding program was conducted to improve the root morphological traits. This variety, the recurrent parent in the MABC, was not previously used for QTL mapping. The donor parents as WAB880-1-38-18-20-P1, IR65195-3B-2-2-2-2, and WAB881 SG9 from IRRI, and were crossed with OM1490 and OM4495 (Indica genotypes). Using 20 marker assays in a total of 229 lines of BC₂F₂ were evaluated for root length (RL), spikelet fertility (SF), DRR (drought recovery score), and yield (Y). The target segment on chromosome 9

(RM201) was significantly related to root length and DT under drought stress treatments, confirming that this root length QTL from OM1490/WAB880-1-38-18-20-P1, OM1490/WAB881 SG9, and OM4495/IR65195-3B-2-2-2-2 (Table 5). The data suggested that DT for yield components is largely associated with genetic and physiological factors independent from those determining the traits *per se*. The implications of these results for developing an efficient strategy of marker-assisted selection for DT are discussed.

BAC clones 13A₉ and 7O₃ were used as pinpoints on the high resolution map for new markers designed from their sequences. The markers became useful to help rice breeders possibly select

improved genotypes that are adapting to drought stress in the seedling stage (Fig. 2 and 5) [22]. The rice variety OM6162 was well-adapted to drought prone areas, and has been released by MARD through the marker-assisted backcrossing (MAB) approach from C50/Jasmine 85/C50 [22]. Molecular breeding approaches, such as marker-assisted backcrossing, marker-assisted recurrent selection, and genome-wide selection, have been suggested to be integrated into crop improvement strategies to develop drought-tolerant cultivars that will enhance food security in a changing and more variable climate [23].

Iron-toxicity tolerance

‘Bronzing’, the symptom of iron-toxicity in rice, is caused by high ferrous (Fe^{2+}) concentrations found in flooded soils in many of the lowlands and swamps in India, West Africa, and other regions. Molecular markers linked to genes for tolerance to ferrous (Fe^{2+}) toxicity in rice seedlings were identified by using 175 DNA markers mapped on all of the chromosomes of a double haploid population derived from a cross between an upland variety, Azucena, and the Indica variety, IR64 [24]. In preliminary screening using toxic and non-toxic solution cultures, no leaf bronzing was observed in Azucena under Fe^{2+} stress with 250 mg Fe^{2+} /L at pH 4.5 for four weeks, but clear symptoms appeared in the IR64 variety [25].

Based on the leaf bronzing index, SSR markers were used to select promising genotypes tolerant to iron-toxicity, such as RM315 and RM212 on chromosome 1, and RM252 and RM211 on chromosome 1. The intervals between RM315-RM211 on chromosome 1 (Fig. 4), RM6-RM240 on chromosome 2, and RM252-RM451 on chromosome 4 (Table 6) were continued studied through further fine mapping (Fig. 5). Marker RM252 was finally recommended (Table 7).

J.L. Wan, et al. (2005) [27] conducted the study on F_2 and equivalent F_3 populations derived from Japonica/Indica crosses of rice and Longza 8503/

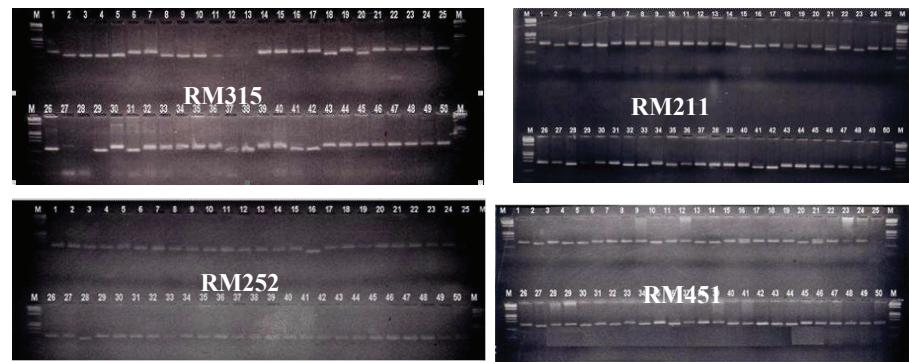


Fig. 4. PCR products at the loci RM315 (left) and RM211 (right) on chromosome 1; loci RM252 (left) and RM451 (right) on chromosome 4.

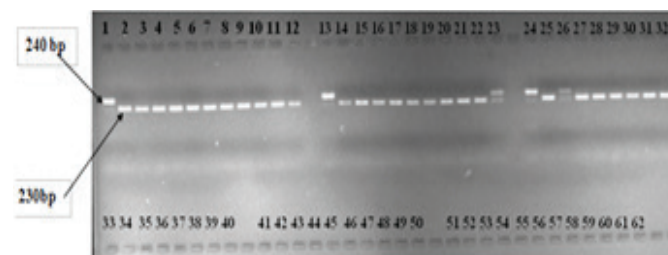


Fig. 5. PCR products at the locus RM23805 on chromosome 9 from BC_2F_2 of OM1490/WAB880-1-38-18-20-P1-HB [12, 13, 22].

Table 6. SSRs linked to the putative QTLs concerning to iron-toxicity tolerance under the iron concentration of 100 ppm in Yoshida nutrition solution [26].

Chrm.	Marker	F-primer	R-primer	Motif
1	RM315	GAGGTACTTCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG	(AT) ₄ (GT) ₁₀
2	RM6	GTCCCTCCACCAATTC	TCGTCTACTGTTGGCTGCAC	(AG) ₁₆
4	RM252	TTCGCTGACGTGATAGGTTG	ATGACTTGATCCCAGAACG	(CT) ₁₉
9	RM201	CTCGTTTATTACCTACAGTACC	TACCTCCTTTCTAGACCGATA	(CT) ₁₇

Table 7. Phenotypic and genotypic assessment to estimate the accuracy of the SSR markers related to iron-tolerance.

Marker	Number of individuals	Homozygous R	Homozygous S	Heterozygous	Predictability (%)
RM6	24	22	2	0	91.67
RM240	24	16	8	0	66.67
RM252	24	20	2	2	83.33
RM451	24	12	12	0	50.00

IR64, and they were raised under iron-enriched solution cultures, and are used to map QTLs that control ferrous iron-toxicity tolerance. Leaf bronzing index, plant height (PH), and maximum root length (MRL) were evaluated. QTLs

controlling LBI were located at the region of RM315-RM212 on chromosome 1, RM6-RM240 on chromosome 2, and RM252-RM451 on chromosome 4.

Ethylene production of rice roots significantly increased when grown under

Fe-depleted conditions. Fe-limiting conditions increased ethylene production and signaling in rice varieties [28]. Molecular properties of GR (glutathione reductase) (gene *OsGR*) from rice (*Oryza sativa* L.) was considered as reducing the deleterious effects of unfavorable abiotic conditions such as iron-toxicity [29].

Rice breeding for acid sulfate soils will be considered as a key activity in the coming years when considering how to narrow yield gap in less favorable areas. Priorities will be considered as marker-assisted selection combined to the advantages of conventional breeding methods. Vietnam needs to increase capacity building biotechnology to rice improvement and to receive assistance in preparing pre-breeding materials especially by IRRI. The integration of biotechnology tools with conventional breeding methods offers new opportunities to increase rice productivity and sustainability, achieve better progenies tolerant to acid sulfate toxicity.

The potential of genetic diversity has not been adequately utilized. We need the collaboration to make better use of this potential latest biotechnological methods employed in conjunction with conventional rice breeding program.

Conclusions

QTL mapping is an important activity connecting genome research to varietal improvements, which is a key application to be applied to breeding for acid sulfate soil adaptations.

PCR-based markers in MAS are to be identified to have high levels of accuracy and efficiency with the emphasis on chromosomes 3 and 9 for Al-toxicity tolerance, then chr. 9 for drought tolerance, chr.12 for P-deficiency tolerance, chr.1 and 4 for iron-toxicity tolerance.

One of the important applications on molecular linkage map is to allow “molecular dissection” of complex traits through design and analysis of QTL mapping experiments. Drought and iron-stresses have been considered as the

most difficult traits to be phenotyped.

Potential GxE interactions and epistasis associated with QTLs make it more difficult to apply QTL-MAS to genetic improvement of the complex trait.

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Evaluation on the yield of some rice varieties with tolerance to salt stress, a case study

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Abstract:

In recent years, sea water has intruded into paddy fields along the coastal areas of the Mekong River Delta. This phenomenon, e.g abiotic stress, has caused rice yields to decrease significantly. A set of six new varieties of rice, including a variety control, IR28, were tested in Long An Province, a region subjected to abiotic stress, from August 2013 to December 2013. The experiments were arranged according to randomized complete block design, with three replications. Results showed that one new mutant cultivar of rice named CTUSM1 was tolerant to soil and water conditions (ECe, EC 6 dSm⁻¹) at the seedling stage; with a yield of 4.43 ton/ha, amylose content of 16.56%, protein content of 6.78%, and a significant resistance to pests and diseases.

Keywords: ECe (electrical conductivity extract), high yield, saline-tolerant rice, seedling stage.

Classification number: 3.1

Introduction

Rice is very sensitive to salinity stress and is currently listed as the most salt sensitive cereal crop, with a tolerance threshold of 3 dSm⁻¹ for most cultivated varieties [1], whereas, generally, a soil is only considered saline (salt-affected) if it has an ECe (electrical conductivity of its saturation extract) above 4 dSm⁻¹ [2]. Even with an ECe as low as 3.5 dSm⁻¹, rice loses about 10% of its yield, with 50% yield loss having been recorded for rice at ECe 7.2 dSm⁻¹ [3].

Rice plant response to salinity varies according to the growth stage. In the vast majority of rice cultivars, plants at the early seedling phase are most sensitive to salinity [4-5]. According to [6], salinity stress during seedling phase can reduce plant dry weight by two-

fold compared to when stress occurs in the ripening phase. When employing irrigated rice planting in coastal areas, salinity may occur at any stage of plant growth. Therefore, it is important to determine the response to salinity in rice plants throughout the growth stage.

Salt infected soils are around 700.000 ha during the dry season from December to May annually [7]; Can Giuoc and Can Duoc districts are located in the southeast of Long An province, which is affected by the Vam Co and Soai Rap rivers, where rice-shrimp farming is popular. During this time, farmers take advantage of salt water by using it for their shrimp cultures. In the following season, farmers use natural rain water (from June to December) to remove salt from the paddy fields, and then begin planting rice. Although the salinity of

soil is decreased, it is still high due to the previous shrimp culture season. Therefore, the aim of this research is to identify rice varieties (1) with high salinity tolerance at the seedling stage, and (2) that can escape salt water which intrudes into canals from December on via a shorter maturity term - around 120 days.

Material and method

Material and location

Experiments were carried out in the autumn-winter crop of 2012 and of 2013 in the two districts Can Giuoc and Can Duoc of Long An province. Four varieties (CTUSM1, CTUSM2, BN2, and OM5629 x TP6) were developed and selected up to F7 by crossing (OM5629 x TP6) and mutation method of temperature shocked (CTUSM1 and CTUSM2) [8].

Method

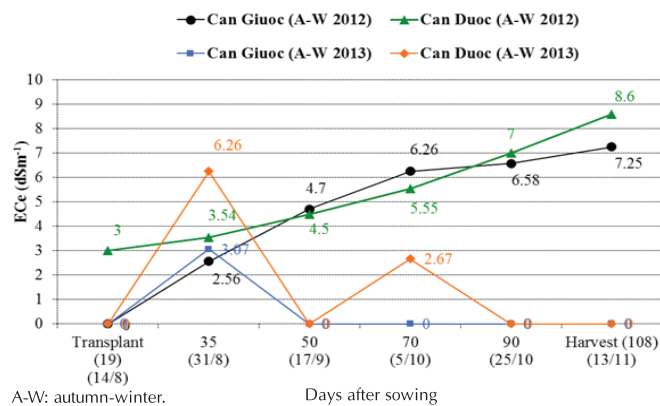
Experiments were designed by a randomized complete block with 3 replications, 6 treatments (CTUSM1, CTUSM2, OM4900, BN2, OM5629 x TP6, IR28 (variety control).

VCU testing (Procedure to conduct tests for Value of cultivation and use of rice varieties, 2011) applied the fertilizer formula: 100N-60P₂O₅-50K₂O, phosphate fertilizer was used in all trials as basal dressing, nitrogen and potassium fertilizers were applied according to the time (Table 1).

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Table 1. Ratio of Nitrogen and Potassium fertilization according to time (% by weight).

Time application	N	K ₂ O
Before transplanting	50	30
The first when recovering and tillering (10 days after transplanting)	30	40
The second at panicle initiation	20	30

**Fig. 1. ECe of water in the autumn-winter 2012 and autumn-winter 2013 crops at Can Giuoc and Can Duoc districts, Long An province.****Table 2. ECe and pH of soil through the growth stage of the rice plant.**

Location	Stages	Transplanting		Flowering		Harvest	
		ECe (dSm ⁻¹)	pH	ECe (dSm ⁻¹)	pH	ECe (dSm ⁻¹)	pH
Can Giuoc (A-W 2012)		4.56	7.30	5.68	6.78	9.12	6.34
Can Giuoc (A-W 2013)		2.97	6.68	4.37	6.59	3.27	6.26
Can Duoc (A-W 2012)		5.68	6.94	8.75	5.95	10.96	6.45
Can Duoc (A-W 2013)		4.80	6.99	5.92	6.35	5.78	6.91

The following methods were followed for analysis of amylose content [9]; protein content [10]; Gelatinization temperature [11]; Gel consistency [12]; Grain length and shape [13].

Water EC and salinity were measured by EC meter (Hanna instrument HI 2550). Five points in the experimental paddy fields were recorded randomly, then the means were calculated;

Soil samplings followed [14]: pH, ECe were sampled at four growing stages of rice: transplanting, panicle initiation, ripening, and harvesting.

Method to analyze data: Excel and

SPSS programs were used to carry out data analysis. An F-test was used to determine the differences among treatments. A Duncan test was applied to compare means among treatments.

Results and discussions

Salinity dynamics

EC of water: The EC at the seedling stage (from transplant to 35 days after sowing) varied from 2.5 to 6.2 dSm⁻¹ in the autumn-winter 2012 and autumn-winter 2013 crops in the Can Giuoc and Can Duoc districts (Fig. 1). Most of plants in the IR28 (control) died, while other varieties developed successfully.

At the panicle initiation stage (50 days after sowing, DAS), salinity decreased to fresh water conditions (0 dSm⁻¹) in the autumn-winter 2013 term while the other season ranged from 4.5 to 4.7 dSm⁻¹. During the boosting stage (70 DAS), salinity increased from 2.7 to 6.3 dSm⁻¹. In 2012, at the harvesting stage (90-108 DAS), salinity increased from 7.3 to 8.6 dSm⁻¹ while in 2013 salinity decreased to fresh water conditions (0 dSm⁻¹) due to high rain fall. According to [15, 16], rice is very sensitive to salinity at the seedling stage; its height, root length, emergence of new roots, and dry matter should be expected to decrease significantly at EC 5-6 dSm⁻¹. Thus, our new five varieties can be considered tolerant at the seedling stage.

ECe of soil: The ECe of soil at the seedling stage in the A-W 2012 and A-W 2013 crops ranged from 2.97 to 5.68 dSm⁻¹ in these two districts. And the pH in these two districts was around neutral: 6.68-7.30 (Table 2). At the stage from flowering to harvest, the ECe of soil increased from 5.68 to 9.12 dSm⁻¹ in the season of A-W 2012 in Can Giuoc district while at the Can Duoc site it increased from 8.75 to 10.96 dSm⁻¹ in the same season (A-W 2012).

Agronomical characteristics, yield components and yield

The term from germination to maturity of rice varieties spanned from 97 to 110 days over the autumn-winter 2012 and autumn-winter 2013 periods in the Can Giuoc and Can Duoc districts of Long An province (Table 3). According to research of [17], maturity reached in from 90-105 days is suitable for shrimp-rice farming models. Most of these rice varieties with a suitable maturity term [18, 19] have shown that an ideal phenotype of rice should be high yield, with the plant height being semi-dwarf (about 90-130 cm). According to [20], the Mot Bui Do variety of traditional rice has been chosen as a main cultivated variety for rice-shrimp models in some Mekong River Delta provinces such as

Table 3. Maturity and height of rice varieties at Can Giuoc, Can Duoc districts in A-W 2012.

Variety/line	Maturity (days)				Height (cm)			
	A-W 2012		A-W 2013		A-W 2012		A-W 2013	
	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc
CTUSM1	100	100	105	105	93.5 ^{ab}	107 ^c	93.5 ^{ab}	108 ^{bc}
CTUSM2	100	100	104	103	84.4 ^b	97 ^d	84.4 ^b	105 ^{bc}
OM4900	105	105	104	105	99.2 ^a	112 ^b	99.2 ^a	122 ^a
BN2	101	101	96	96	83.2 ^b	110 ^{bc}	83.2 ^b	109 ^b
OM5629 x TP6	105	105	104	105	98.6 ^a	116 ^a	98.6 ^a	126 ^a
IR28	97	97	96	96	82.8 ^b	102 ^c	82.8 ^b	102 ^c
<i>F</i>					*	*	*	*
<i>CV (%)</i>					6.29	1.90	6.29	2.86

Table 4. Yield component of rice varieties.

Variety/line	Panicle/m ²				Spikelets/panicle				Weight 1000 grains (g)			
	A-W 2012		A-W 2013		A-W 2012		A-W 2013		A-W 2012		A-W 2013	
	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc
CTUSM1	235 ^c	243 ^b	251.3 ^b	286 ^a	97 ^b	83 ^b	105.7 ^a	81 ^b	23.2 ^b	23.7 ^b	23.2 ^b	22.8 ^{bc}
CTUSM2	261 ^b	244 ^b	291.3 ^a	297 ^a	83 ^c	80 ^b	79.0 ^b	87 ^b	22.6 ^b	22.3 ^c	22.2 ^{bc}	22.2 ^{bc}
OM4900	192 ^d	195 ^c	196.0 ^c	205 ^b	101 ^{ab}	79 ^b	114.0 ^a	90 ^b	24.9 ^a	25.6 ^a	25.2 ^a	25.6 ^a
BN2	260 ^b	230 ^b	249.3 ^b	290 ^a	81 ^c	76 ^b	79.5 ^b	79 ^c	22.7 ^b	21.6 ^d	23.4 ^b	21.9 ^c
OM5629 x TP6	195 ^d	165 ^d	203.7 ^c	176 ^b	107 ^a	116 ^a	114.7 ^a	116 ^a	24.8 ^a	25.4 ^a	24.9 ^a	25.2 ^a
IR28	283 ^a	322 ^a	201.3 ^c	201 ^b	50 ^d	27 ^c	50.4 ^c	51 ^d	21.9 ^c	22.6 ^c	21.6 ^c	23.3 ^b
<i>F</i>	*	*	*	*	*	*	*	*	*	*	*	*
<i>CV (%)</i>	4.52	3.39	3.60	8.45	4.10	6.03	8.12	6.39	1.60	1.59	3.24	3.00

Bac Lieu, Ca Mau, Kien Giang, yielding a plant height from 100-120 cm, this height being considered as sufficient for shrimp-rice models, so the plant

height of all six tested varieties/lines of this experiment appear to be suitable phenotypes for high yield shrimp-rice models.

There is a wealth of research on yield components and yields. In [21-25], ideal phenotypes of high performance rice were examined, with following components analyzed: panicles per hill (8-10 panicle for transplanting) or 3-4 panicles/hill (sowing), number of panicle/m² was 270-300 panicle, with 150 spikelets/panicle and a spikelet rate reaching over 80% (Table 4).

As for the ECe, it changed a lot from the seedling stage to the harvest stage. Final yields of varieties/lines in this experiment ranged from that of control IR28 (which exhibited the lowest yield (0.57-1.40 ton/ha)) to that of the mutant line CTUSM1 (3.52-4.43 ton/ha) which exhibited the highest yield (Table 5). IR28 died after one to two weeks DAS, while CTUSM1 tolerated these conditions. The yield of CTUSM1 was also higher than that of MTL119, which exhibited a tolerance at the seedling stage of 6‰ tested by Yoshida solution (4.4 ton/ha in fresh water soil; 2.0- 3.0 ton/ha in saline soil, ECe 1.4 dSm⁻¹, 0 ton/ha in ECe 2.11 dSm⁻¹). [26, 27] reported that OM9605 and OM5953 could tolerate salt concentration at 6-8 dSm⁻¹, giving yields from 5 to 7 ton/ha under fresh water conditions.

Evaluation of pest resistance in the experimental field

Rice leaf blast caused by fungus *Pyricularia oryzae* appeared on CTUSM2 and OM4900 varieties were scored at level 3 while CTUSM1 was at level 0. Moreover, OM4900 was also affected by neck blast. Other varieties/lines were not sensitive to this fungus. Leaves of six varieties/lines were damaged by rice leaf folder *Cnaphalocrosis* at level 3, damage was about 11-20% from tillering to maturity (Table 6).

Table 5. Yield of rice varieties.

Variety/line	Actual yield (ton/ha)				Theoretical yield (ton/ha)			
	A-W 2012		A-W 2013		A-W 2012		A-W 2013	
	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc	Can Giuoc	Can Duoc
CTUSM1	3.87 ^a	3.52 ^a	4.43 ^a	3.78 ^a	5.28 ^a	4.82 ^{ab}	6.14 ^a	5.28 ^a
CTUSM2	3.31 ^b	3.11 ^b	3.58 ^c	4.36 ^a	4.91 ^a	4.37 ^c	5.14 ^{bc}	5.73 ^a
OM4900	3.19 ^b	2.69 ^c	3.26 ^c	2.86 ^b	4.83 ^a	3.98 ^{cd}	5.63 ^{ab}	4.72 ^b
BN2	3.21 ^b	2.46 ^c	3.68 ^{bc}	3.37 ^{ab}	4.75 ^a	3.81 ^d	4.63 ^c	5.02 ^{ab}
OM5629 x TP6	3.75 ^a	3.22 ^{ab}	4.17 ^{ab}	3.66 ^{ab}	5.20 ^a	4.86 ^a	5.82 ^{ab}	5.14 ^{ab}
IR28	1.26 ^c	0.71 ^d	0.57 ^d	0.79 ^b	1.09 ^b	1.97 ^c	1.19 ^d	1.40 ^c
<i>F</i>	*	*	*	*	*	*	*	*
<i>CV (%)</i>	5.20	6.50	29.23	14.72	5.93	6.17	10.23	9.54

Table 6. Pest resistance of six rice varieties in the paddy field experiment.

(Damage level)

Variety/line	Pyricularia oryzae	Xanthomonas oryzae pv. oryzae	Bipolaris oryzae	Agrotis ipsilon	Cnaphalocrosis	BPH
CTUSM1	0	0	0	1	3	3
CTUSM2	3	0	0	1	3	3
OM4900	3	0	0	1	3	0
BN2	1	0	0	1	3	0
OM5629xTP6	0	0	0	1	3	3
IR28	0	0	0	1	3	3

BHP: Brown plant hopper.

Table 7. Some rice quality traits of varieties/lines in the basic experiment

Variety/line	AC (%)	P (%)	GC (level)	Length of grain (mm)	Ratio of L/W	GT (level)
CTUSM1	16.56 ^{bc}	6.78	3 ^b	7.1 ^{abc}	3.2 ^c	3
CTUSM2	17.77 ^b	5.48	3 ^b	6.7 ^d	3.0 ^d	2
OM4900	16.01 ^{bc}	5.90	1 ^c	7.0 ^{bc}	3.2 ^{bc}	2
BN2	23.52 ^a	6.52	5 ^a	7.3 ^a	3.5 ^a	2
OM5629xTP6	14.88 ^c	6.31	3 ^b	7.2 ^{ab}	3.4 ^{ab}	2
IR28	24.90 ^a	6.39	5 ^b	6.9 ^c	3.5 ^a	7
<i>F</i>	*	ns	*	*	*	
<i>CV (%)</i>	7.60	11.64	13.70	1.91	2.72	

AC: amylose content; P: protein content; GC: Gel consistency; GT: Gelatinization temperature; L/W: Length/Wide.

Quality traits of 6 varieties/lines

Among all varieties in this experiment, amylose content of BN2 and IR28 was the highest (20-25%) while the other CTUSM1, CTUSM2, OM4900, OM5629 x TP6 had low amylose content ranging from 14.88 to 16.56% (Table 7).

Conclusions

The basic experiment of VCU (Value of Cultivation and Use) in the paddy field results was conducted over two years: 2012 and 2013 in Can Giuoc and Can Duoc districts, Long An province, an elite line CTUSM1 appeared excellent - it could tolerate salt at the seedling stage - under soil and water conditions of E_Ce and EC, respectively < 6 dSm⁻¹, and a high yield of 4.43 ton/ha was observed. High quality features were observed: amylose content 16.56%, protein content 6.78%, length of grain 7.1 mm, resistance to pests and diseases in the paddy field. CTUSM1 should be checked for the adaptable ability in various ecological areas.

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Research results on robusta coffee breeding in Vietnam

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Abstract:

In the research and development of coffee trees in Vietnam, particularly breeding is one of the most important activities and always prioritized by the Government. Since 1976, the Western Highlands Agriculture and Forestry Science Institute (WASI, former Coffee Research Institute), has invested a lot of efforts in selecting and creating new coffee varieties with good characteristics, namely high yield and big-sized beans, that have contributed to the enormous coffee development, especially to Robusta coffee. During 40 years, WASI has created and made them recognized by the Ministry of Agriculture and Rural Development with total 11 Robusta varieties. Of which, TR4, TR9, and TR11 are largely used in the cultivation in the Central Highlands, TR14 and TR15 are very promising to adapt to new challenge of climate change, and TRS1 is a good solution for quick seedling supplies for the replanting program. These new varieties are highly recommended for the coffee development program till 2020 and in the coming years.

Keywords: breeding, coffee, high yield, new variety, Robusta, Vietnam.

Classification number: 3.1

Background

During the last 40 years, a number of research projects have been done by the Western Highlands Agriculture and Forestry Science Institute, and it has selected 11 new Robusta clones for coffee replanting in the Central Highlands's provinces of Vietnam. These new varieties, especially the three normal ripening clones TR4, TR9, and TR11 and the two late ripening ones TR14 and TR15 have much higher yield and bean quality potentials compared to those of old cultivars [1]. The late ripening clones not only offer good

characteristics such as high yield and good bean quality but also allow a delay of the first irrigation timing in the middle of dry season, thus we can save one irrigation application compared to other normal ripening clones.

Besides, with the high demand for coffee plantlets to serve replanting, the production of grafted plantlets is not sufficient due to the present capacity in Vietnam. Therefore, the research of good hybrid seeds taken from elite clones is necessary and by that way, TRS1 variety is selected. In fact, TRS1 is hybrid seeds collected from the seed producing garden growing selected clones: TR4,

TR9, TR11, and TR12 [2]. Seedlings are better than clone production because of the production cost and propagation capacity. All Robusta varieties released by WASI are largely planted in the Central Highlands. They have shown a very good adaptation to the ecological condition of the region as well as the ability to give high yield and good quality beans [3].

Methodology

Materials

Robusta clones TR4, TR9, and TR11 were selected in Dak Lak's coffee growing areas. The late ripening clones TR14 and TR15 were selected from Indonesia's imported materials in 1996.

The synthesis variety TRS1 (seed) was created from open-pollinated one in seed producing garden growing TR4, TR9, TR11, and TR12 clones.

The TR4 clone was officially recognized as a national variety in 2006 by the Ministry of Agriculture and Rural Development (Decision No 1086 QĐ/BNN-KHCN on April 14, 2006); TR9 and TR11 in 2011 (Decision No 175/QĐ-TT-CCN on May 4, 2011); TRS1 in 2015 (Decision No 324/QĐ-TT-CCN, on November 5, 2015).

Research methodology

Clones or hybrids, after passing comparison trials and having initial evaluations, are reselected to go through

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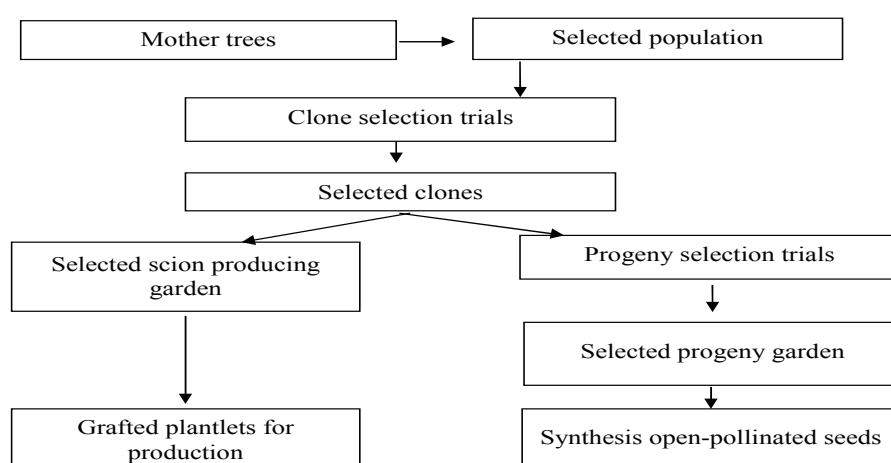


Fig. 1. Robusta varieties selection diagram

Table 1. Trial results of 3 robusta clones planted in Buon Ma Thuot - Dak Lak.

Clone	3 harvests' average yield (tons /ha)	Berry/green bean ratio	100 bean's weight (gr)	Bean's proportion remained on screen 16 (%)	Leaf rust index (%)
TR4	7.3	4.0	18.4	80.0	0.0
TR9	6.7	4.3	22.5	94.3	0.0
TR11	6.6	4.2	18.3	88.2	0.0



Fig. 2. TR4 trial in Dak Lak.

other trials in the Central Highlands provinces: Dak Lak, Gia Lai, Dak Nong, and Lam Dong (Fig. 1).

Clone comparison trials are RCBD with three blocks, 10-30 trees per block, total experimental area: 0.5-1.0 ha per

province. The hybrid variety TRS1 is designed to compare with original varieties; experimental garden is the control one that is an average yield garden with the same care conditions and is grown at the same place and period.

Trial periods: TR4, TR11, and TR12 clone trials were implemented from 1999 to 2010; the late ripening clones TR14 and TR15 were from 2006 to 2015; the hybrid coffee TRS1 was from 2010 to 2015.

Monitoring and evaluation indicators

Growth: Stem diameter, tree height, number of primary branches, branch length, nodes per branch, and fruit bearing nodes, fruits per node.

Yield: An average yield of 3 harvests or more in the trial areas was compared with the average yield of mass planting with the same care conditions.

Green bean indicators: Samples are taken from each region with the number of 5-10 samples and for average two harvests. Each sample weighs 2.0 kg of berries. Analyzing and evaluating indicators: Berry/green bean ratio, Weight of 100 beans (g), proportion of beans remained on screen No. 16 (6.3 mm).

Leaf rust resistance ability on the field.

Data analysis and processing methods

Data is processed by biological statistics methods using EXCEL, SAS 9.2 software with F and LSD comparison test.

Results and discussions

Evaluation results on selecting 3 Robusta clones TR4, TR9, and TR11

As shown in Table 1, the three harvests' average yields of 3 clones are rather high in the condition of Dak Lak. Among them, TR4 clone shows dominant yield potential at 7.3 tons per ha (Fig. 2). TR9 and TR11 clones can also give high yields (more than 6.0 tons per ha). These three clones are the highest and most stable clones in term of yield potential compared to other six clones recognized by MARD at the same period.

Bean size and 100 beans' weight of these three clones are very impressive: 100 beans' weight of TR4 and TR11 clones is more than 18 grams. The TR9 clone especially has very big fruit size with 100 beans' weight which reaches 22.5 grams. In terms of bean proportion remained on the screen No.16, in average, these three clones get more than 80%, in which, TR9 gets 94.3%.

In Gia Lai province, these three clones are grown since 2005. The results on yields, bean qualities and leaf rust indexes are shown in Table 2.

Results in Table 3 show that the three harvests' average yields of TR4 and TR9 clones are almost the same (4.1-4.2 tons per ha), and the TR11 clone has lower yield. In general, Gia Lai's clones give quite high yields but a little lower than those in the condition of Dak Lak province. The bean size of these clones in Gia Lai is also big and they show high resistance to leaf rust disease.

When being planted in Lam Dong province, the three harvests' average yields of TR4 and TR11 clones are almost the same (4.8-5.2 tons per ha), while the TR9 clone has lower yield.

Especially in the conditions of Lam Dong province, these clones' bean size is very large with more than 85% of the beans at grade I (on screen No 16). The TR9 clone has 99.2% of grade I beans, and its 100 beans' weight reaches 29.6 grams.

Trials were implemented in the main coffee growing regions. Yield monitoring results show that in trial regions, these clones give yields varying from 3.3 to 4.0 tons per ha. These clones show good adaptation to the conditions in the Central Highlands (Table 4).

Evaluation results on selecting Robusta late ripening clones TR14 and TR15

Table 5 shows that in the three trial places, the Robusta late ripening clones give the four harvests' average yield higher than the control one. These clones can reach from 5.13 to 5.24 tons per ha

Table 2. Trial results of 3 robusta clones planted in Iagrai - Gia Lai.

Clone	3 harvests' average yield (tons/ha)	Berry/green bean ratio	100 beans' weight (gr)	Bean proportion remained on screen 16 (%)	Leaf rust index (%)
TR4	4.2	4.2	15.3	63.2	0.0
TR9	4.1	4.5	17.0	75.0	0.0
TR11	3.7	4.4	15.7	65.0	0.0

Table 3. Trial results of 3 robusta clones planted in Bao Loc - Lam Dong.

Clone	3 harvests' average yield (tons/ha)	Berry/green bean ratio	100 beans' weight (gr)	Bean proportion remained on screen 16 (%)	Leaf rust index (%)
TR4	5.57	4.0	18.4	76.7	0.0
TR9	5.20	4.3	23.0	89.5	0.0
TR11	5.10	4.2	18.9	83.0	0.0

Table 4. Characteristics of 3 clones planted in trial regions.

Clone	3 harvests' average yield (tons/ha)	Berry/green bean ratio	100 beans' weight (gr)	Bean proportion remained on screen 16 (%)	Leaf rust index (%)
TR4	5.2	3.9	21.6	86.9	0.0
TR9	4.8	4.0	29.6	99.2	0.0
TR11	5.0	4.0	22.8	95.8	0.0

Table 5. Yields of Robusta late ripening clones (4 harvests' average yield).

Clone	Yield (tons/ha)			Average yield	Time from flowering to harvesting (months)
	Dak Lak	Gia Lai	Lam Dong		
TR14	5.63 a	5.15 cd	4.93 d	5.24 a	12
TR15	5.30 bc	5.19 bc	4.90 d	5.13 a	12
TR16	5.42 ab	5.15 cd	5.06 cd	5.21 a	10
TR6 (control)	4.67 e	4.46 e	4.46 e	4.58 b	11
Average	5.25 a	5.02 b	4.84 c	CV(%) = 3.24	

while the TR6 only reaches 4.58 tons per ha. This difference is statistically significant.

Due to the interactions between regional condition and these late ripening clones, four harvests' average

yield varies largely from 4.46 to 5.63 tons of green bean per ha, more than one ton per ha of difference. Especially in Dak Lak province, the TR14 clone gives the highest average yield (5.63 tons per ha) significantly compared with those in



Fig. 3. The TR14 trial in Dak Lak.

Table 6. Bean quality and leaf rust resistance index of Robusta late ripening clones in trial places.

Place	Clone	100 beans' weight (gr)	Bean proportion remained on screen 16 (%)	Berry/green bean ratio	Leaf rust index (%)
Dak Lak	TR14	20.9	97.8	4.3	0
	TR15	24.9	98.1	4.2	0.1
	TR16	20.0	94.3	4.3	0
	TR6 (ck)	19.4	92.7	4.3	0
Gia Lai	TR14	20.5	95.4	4.2	0
	TR15	22.0	97.9	4.2	0
	TR16	19.1	93.2	4.3	0
	TR6 (ck)	18.4	91.5	4.4	0
Lam Dong	TR14	22.4	95.7	4.3	0
	TR15	23.5	96.3	4.3	0
	TR16	20.2	91.2	4.3	0
	TR6 (ck)	19.2	90.0	4.5	0

Table 7. Results on coffee cupping of Robusta late ripening clones in Buon Ma Thuot - Dak Lak.

Clone	Aroma	Flavor	Acidity	Body	Overall evaluation
TR14	Typical	Fair	Average	Good	Good
TR15	Typical	Fair	Average	Good	Good
TR16	Typical	Fair	Average	Good	Good
TR6 (ck)	Typical	Fair	Average	Good	Good

(Evaluated by: CAFECONTROL - Central Highlands Branch).

Gia Lai and Lam Dong provinces and with other clones in the three trial places (Fig. 3).

In all trial places, TR14, TR16, and TR6 (control) clones are not affected by leaf rust disease. The TR15 clone is affected slightly (0.1%) and only in Dak Lak but not in Gia Lai and Lam Dong.

Bean quality of these late ripening clones is rather good. The weight of 100 beans in all three regions reaches from 19.1 to 24.9 gram; much higher than the control - TR6 clone whose weight is from 18.4 to 19.4 gram. In trial regions, this weight reduces with this following order: Lam Dong > Dak Lak > Gia Lai. However, for the TR15, its weight in Dak Lak is higher than in Lam Dong. This can be explained by a better ability of the TR15 clone's dry matters in severe conditions compared to other clones (Table 6).

In the three trial regions, most of the Robusta late ripening clones have the proportion of beans on the screen No. 16 more than 90% and the low ratio of cherry/bean in the range of 4.2 to 4.5 (Table 6).

Besides the evaluation of green bean quality, results on coffee cupping in Table 7 show that: flavor and body of the samples are both good with typical aroma. The acidity is average, and overall, the cupping is evaluated at good quality. This evaluation shows that the dominant characteristics of these Robusta late ripening clones are bean quality and also the cup, one factor that is focused much nowadays.

According to the trial evaluation results on the adaptation of three Robusta late ripening clones in the main coffee growing areas of some Central Highlands provinces like Dak Lak, Dak Nong, and Lam Dong, it is clear that: These clones give very high yield (more than 4.5 tons per ha), especially in the conditions of Dak Lak (four harvests' average yield reaches 5.25 tons per ha) and especially with the TR14 clone (5.63 tons per ha) compared with other clones.

Table 8. Growth situation of the TRS1 variety in trial regions (18 months old).

Place	Diameter (mm)	Height (cm)	Number of primary branches	Branch length (cm)	Nodes/branch	Fruit bearing nodes	Fruits/node
Dak Lak	5.3	129.2 b	17.9 c	115.8	37.9 a	26.5 a	24.9
Gia Lai	4.9	142.0 a	22.7 ab	110.9	23.9 b	14.9 c	19.9
Dak Nong	5.8	144.0 a	19.8 bc	117.3	38.5 a	9.1 d	21.5
Lam Dong	4.9	145.7 a	24.5 a	110.3	24.5 b	22.2 b	21.7
CV (%)	9.9	4.2	7.6	5.1	9.9	9.6	8.5
LSD ($\alpha = 0.05$)	ns	11.7	3.2	ns	6.1	3.5	ns

ns: non-significant.

Table 9. Main characteristics of the TRS1 variety in trial regions.

Trial place	2 harvests' average yield (tons/ha)	100 beans' weight (gr)	Bean proportion remained on screen 16 (%)	Berry/ green bean ratio	Leaf rust index (%)
Dak Lak	4.05	20.8	90.9	4.6	5.4
Gia Lai	4.57	19.3	84.1	4.7	2.4
Dak Nong	4.43	18.2	85.7	4.5	12.4
Lam Dong	3.59	17.9	80.6	4.7	8.8
Average	4.31	19.1	85.3	4.6	7.2
Increase compared to the control	50.2	25.4	27.7	5.4	

In short, these clones are resistant to leaf rust disease and can give rather higher yield (more than 5 tons per ha) than the control TR6. The green bean quality is good with very big bean size, and they ripen from 15 to 25 days later than control clone.

Comparison and selection results of the Robusta hybrid variety TRS1

In Table 8, after 18 months, the

research on the TRS1 variety's growth situation in the trial regions shows that: This variety can be adapted very well in all regions with an average stem diameter from 4.9 to 5.8 mm, the number of primary branches reaches from 17.9 to 24.5 pairs. The variation of all growth factors in trial zones is very low with CV under 10%.

The research results on trial models;

the TRS1 variety in all regions shows that: the 2 harvests' average yield of TRS1 in the Central Highlands reaches 4.31 tons of green bean per ha; the average 100 beans' weight is approximately 19.1 grams; the proportion of grade I beans accounts for about 85.3% and the leaf rust disease index is about 7.2% (Table 9). In general, TRS1 shows a good adaptation with better yield and other factors than those which were commonly planted a long time ago in the Central Highlands.

Conclusions and recommendations

- New selected clones and varieties of Robusta coffee (TR9, TR11, TR14, TR15, and TRS1) show better yield, quality, etc. than all previous ones planted in the Central Highlands

- Using these new clones and varieties for replanting programs in Vietnam. Especially, TRS1 can be supplied in massive quantity for big programs and in large areas. TR14 and TR15 should be considered to be grown in regions with less irrigation water.

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Single port laparoscopic colectomy for colonic cancer

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Abstract:

Objectives: The evaluation of feasibility, safety, and outcomes of single port laparoscopic colectomy (SPLC) for colonic cancer. Prospective study of 114 patients with colonic cancer (84 right side, 17 left side, and 13 sigmoid colon) who underwent SPLC between October 2010 and October 2016 in Hue Central Hospital. **Results:** Mean age of patients was 56.1 ± 15.2 (25-87 years), male/female ratio was 70/44. Mean BMI was 23.5 ± 2.2 kg/m². Procedures included 84 right hemi-colectomies (73.7%), 17 left hemi-colectomies (14.9%), and 13 sigmoidectomies (11.4%). Six cases required additional ports, and 19 were converted to open surgery. Mean tumor size was 3.7 ± 2.7 cm. Stage I: 6.1%; stage IIa: 43.9%; stage IIIa: 36.8%; and stage IIIc: 13.2%. Mean operation time was 160.5 ± 75.5 min. There had been no deaths/intraoperative complications. Mean incisional length (including extraction) was 5.5 ± 2.2 cm. Mean lymph nodes was 16.2 ± 4.5 . Mean hospital stay was 7.5 ± 6.1 days. The surgical site infection rate was 3.5%, and 1 patient required reoperation (anastomotic leakage: 0.9%). After 32.2 ± 7.5 (3-65 months) follow-up, there were no late surgical complication, incisional hernia, or incision metastasis. There were five local recurrences, and three patients developed liver metastasis in the open conversion group. The overall survival time after 2 years was 87.5%, and after 5 years, it was 59.4% (Kaplan-Meier). **Conclusion:** SPLC is feasible and safe procedure for the treatment of colon cancer. The cosmetic value is better (short incision confounded by umbilicus). Other outcomes were equivalent to conventional laparoscopy.

Keywords: colon cancer, single port laparoscopic colectomy.

Classification number: 3.2

Introduction

Colonic cancer is among the most commonly occurring cancers (ranking 2nd in women and 3rd in men) in the world and the 4th cause of death after lung, stomach, and liver cancer [1]. In Vietnam, colorectal cancer is the 4th leading cause of death after gastric cancer, lung cancer, and liver cancer [2].

Since Jacobs successfully performed the first laparoscopic right hemi-colectomy for a cecal tumor in 1991, laparoscopy for colon cancer has been widely used, and it is as effective as open surgery in oncological terms [3].

In 2008, P. Bucher, et al. reported the first case right hemicolectomy by single port access laparoscopy and in 2009, single-port access laparoscopic radical left colectomy was successfully performed and reported [4].

SPLC allows intra-abdominal operation through a small umbilical incision [5]. With the development of techniques and instruments (Harmonic-Scapel, Ligasure, etc.), SPLC is being widely and successfully applied around the world [5, 6].

This study aimed to evaluate the feasibility, safety and early results of this

technique.

Materials and methods

Materials: 114 colon cancer cases confirmed by preoperative colonoscopic biopsy. All tumors were ≤ 8 cm, stage III or under, non-invasive to adjacent organs, with no distant metastasis on CT-scan. Patients were ASA 1-3.

Methods: Prospective study: general characteristics of patients, surgical procedures, conversion rate, complications during and after surgery, the results of surgery, and hospital stays were collected for analysis.

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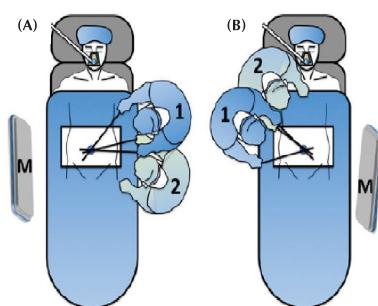


Fig. 1. Position of patient, surgeon, and assistant. (A) Right colon, (B) Left colon and sigmoid colon.

Equipment: Karl-Storz endoscopes 30° (5 mm diameter, 60 cm length), SP from COVIDIEN, long and conventional laparoscopic instruments, staplers, etc.

Technique: The patient was underwent general anesthesia, supine for right colon cancer and in lithotomy position for left colon and sigmoid colonic cancer (Fig. 1).

Insufflation; longitudinal incision through the umbilicus (2-2.5 cm);

placement of SP device; insufflation of CO₂ into the peritoneal cavity (12 mmHg).

Ligation of blood vessels close to their origins; mobilization of the colon; removal of the SP device; and expanding the incision, if necessary.

Placement of the incision protective device; removal of the colon and tumor; and anastomosis performed by hand or stapler (Figs. 2-5).



Fig. 2. SP right hemi-colectomy.



Fig. 3. Specimen with ascending colonic cancer.

MACROSCOPIC HISTOLOGIC FEATURES

Colectomy specimen contain a bowel segmental length 20cm, diameter 4cm. Distance 6cm from tumor to distal margin resection. Many mesocolonic lymph nodes 0.2-1.0cm

(1) Proximal margin resection, (2) Distal margin resection

MICROSCOPIC HISTOLOGIC FEATURES

Colonic adenocarcinoma with large nucleus, eccentrically placed nuclei, abundant cytoplasm, a tubular microscopic pattern, invaded into the submucosa of the colon.

19 evaluated lymph nodes: Negative

Both margin resections: Negative

CONCLUSION

Well differentiated colonic adenocarcinoma

(pT2N0M0, Stage I)

Fig. 4. Post-op pathology with 19 lymph nodes examined.



Fig. 5. Scar after 2 years.

Results

General characteristics of patients

	n=114	%
Male/Female	70/44	1.6/1
Mean age	56.1±15.2	
Min	25	
Max	87	
BMI (kg/m ²)	23.5±2.2	
Adenocarcinoma (colonoscopy + biopsy)	114	100
Tumor size/CT scan (cm)	3.7±2.7	
Min	3	
Max	8	
CEA (ng/ml)		
< 5	52	45.6
≥ 5	62	54.4

Surgical techniques

Procedures performed	N	%
Right hemi-colectomy	84	73.7
Left hemi-colectomy	17	14.9
Sigmoidectomy	13	11.4

Conversion

Open conversion	n=19	16.7%
Right colon	17	14.9
Left colon	2	1.8
Additional trocars	n=6	5.3%
Right colon	4	3.5
Left colon	2	1.8

Causes for open conversion

Causes	n=19	16.7%
Peritumoral inflammation	7	6.1
Tumoral invasion	12	10.5

Operative results

	Min	Max	Mean
Operating time (minutes)	140	300	160.5±75.5
Lymph nodes examined	12	25	16.2±4.5
Incisional length (cm)	4	10	5.5±2.2
Drain	n=5		4.4%
Postoperative pain (days)	2	6	4.1±2.5
Bowel movement return (days)	2	5	3.5±1.2
Hospital stay (days)	6	16	7.5±6.1
Incisional infection	n=4		3.5%
Anastomotic leakage	n=1		0.9%

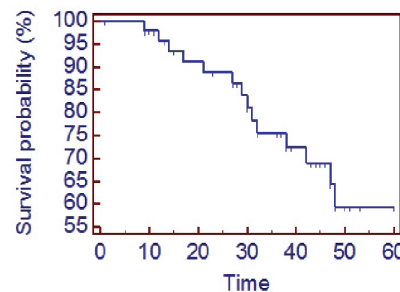
Staging

Stage	n	%
Stage I	7	6.1
Stage IIa	50	43.9
Stage IIIa	42	36.8
Stage IIIc	15	13.2
Stage IV	0	0

Postoperative follow-up

Mean time follow-up 32.2±7.5 (3-65) months	N	%
Postoperative CEA ≥ 5 ng/ml	25	21.9
Liver metastasis	3	2.6
Local recurrence	5	4.4
Lung metastasis	0	0
Incisional hernia	0	0
Incisional metastasis	0	0

Overall survival time (Kaplan-Meier)



After 2 years: 87.5% and after 5 years: 59.4%.

Discussions

Based on our experience, in 114 patients with colonic cancer undergoing SPLC, there were 19 conversions, six needed additional ports; there were no deaths, no intra-operative complications, and only one case of anastomotic leakage. Four patients had surgical site infections. This study showed that SPLC treatment of colon cancer is feasible and safe.

Although the patients had been selected before the surgery, the general characteristics of the patients in our study were similar to other studies. The mean age of the study: 56.1±15.2 years.

This result was similar to those of other domestic or international studies [7, 8].

Regarding the sex, there were 70 males and 44 females in our study. This factor was different from the results of several studies, including those of William Tzu-Liang Chen's study [9] and B. Peter's study [1]. However, this difference did not affect the outcomes of SPLC.

The overweight patients are more difficult since mesenteric blood vessel cannot be seen easily [10]. It is well recognized that this is one of the factors that leads to the conversion from SP laparoscopy to conventional laparoscopy or laparotomy [9]. This influence is especially evident in SP laparoscopy. Indeed, I. Diego, et al. found the conversion rate was higher in the group with BMI over 25 kg/m² compared to the group with BMI less than 25 kg/m² [11]. The average BMI in our study was 23.5±2.2 kg/m². The conversion rates in other studies of SP laparoscopy had relatively low BMI, such as the meta-analysis study of T. Makino, et al. had BMI of 25.5 kg/m² [3], the meta-analysis study of A.K. Fung, et al. had 25.8 kg/m² [7]. Even in the randomized comparative study of William Tzu-Liang Chen, et al., BMI was 23.3 kg/m² [9]. Thus, we could consider the BMI in our study was similar to other studies.

Our selection of patients with tumor size on CT scan averaged at 3.7±2.7 (2-8) cm. Randomized comparative study of H.T. Papaconstantinou, et al., in the SP laparoscopic group, the average tumor size was 4±2 (0.9-9.5 cm) [12]. Thus, the criteria for patient selection related to the size of our tumor was similar to other authors.

Another factor related to SPLC in colon cancer is surgical procedure. Most published studies have evaluated the feasibility and safety of right SPLC [1, 6, 9, 11, 13]. Only a few studies

studied left SPLC [14, 15]. Most authors agree that SP laparoscopy of left colectomy or sigmoidectomy, which requires dissection in the pelvis and freeing the splenic flexure, is more difficult. According to F. Leblanc, et al. both medial to lateral and lateral to medial approaches were used to ligate the vessels for 11 right and 6 left colectomies [16]. The small rate of left hemicolectomy or sigmoidectomy in our study was not due to that reason. In our study, there were only 17 left colon cancers. Most of the patients with left colon cancers hospitalized in our department were in intestinal sub-occlusion or occlusion situation; thus, we couldn't select them for our study.

Operating time for SP laparoscopy was longer than conventional laparoscopy in the randomized comparative study of William Tzu-Liang Chen, et al. [9] with an average operating time of 175 minutes [9]. In the Papaconstantinou, et al. the study showed that operating time was equal between the two groups, where the average operating time was 144 minutes [12]. In our study, the average operating time was 160.5 ± 75.5 (140-300) minutes, which was longer than Papaconstantinou, et al. since there were 17 left hemi-colectomies (14.9%), 13 sigmoidectomies (11.4%), and 19 converted cases in our study, where the operating time was long. In contrast, there was no left hemi-colectomy in the study of Papaconstantinou, et al. [12]. The average operating time in SP laparoscopic group of C.G. Huscher [8] was 147 ± 61 minutes, which may be related to the high rate of cancer stage 1 and 2 (12/16), accounting for the shorter operating time compared to our study. According to T. William, et al. [9], SP laparoscopy should be performed by experienced surgeons in laparoscopic surgery, and when this technique becomes routine, the time factor is no longer different between the two techniques. We also found that our

operating time was shorter in the latter half of our series.

There were 19 conversions (16.7%) to open surgery in our study. The conversion rate in our study was higher than several other studies. The comparative study of William Tzu-Liang Chen, et al. [9] had three cases of conversion (3/18), where one case was of open surgery, and 2 cases were of traditional laparoscopy. In the study by Papaconstantinou, et al., 26 patients in SP laparoscopic group had 3 conversions, where two cases were converted to traditional laparoscopy and one to open surgery [12]. This even suggests that the selection of patients with low BMI and small tumor size is very important for SP laparoscopy. The reasons for conversion in our study were tumor invasion to adjacent organs (12/19 cases) and peritumor inflammation (7/19). In cases of peri-tumor inflammation, we added first additional port in RLQ or LLQ. If no progress was apparent, we converted to open surgery. We have been successful in 5 cases. In tumor invasion situations, we decided to convert to open surgery immediately without adding more ports.

In the studies of A. Keshava, et al. [6] and William Tzu-Liang Chen, et al. [9], the average incision length was of 4 cm (3-6). In our study, the average incision length was 5.5 ± 2.2 cm (4-10). We discerned, our incision was longer because several tumors in our study were of a bigger size, and so, we had to extend the incision to extract these tumors. In the studies of William Tzu-Liang Chen, et al. [9], the average tumor size was 2 cm (0.5-6), but for our study, it was 3.7 ± 2.7 cm (3-8).

Only 2 cases in SP laparoscopic group of William Tzu-Liang Chen, et al.'s study [9] had complications related to surgery, and no cases needed re-intervention. Research by B. Peter, et al. on 50 cases of SP laparoscopy had one case of anastomotic leakage and 4

cases of abdominal incision infection [1]. A study of 22 cases of right SPLC by A. Keshava, et al. (BMI 27) had 2 cases of re-intervention [6]. Our study had five cases of complications, including 4 cases of wound infection and one case of anastomotic leakage that required re-intervention. Thus, our rate of complications was similar to studies by other authors and not a risk factor for SP laparoscopy.

In our study, other results such as postoperative pain, bowel movement return, length of hospital stay were similar to the results of conventional laparoscopy [2].

Until recently, all four studies comparing SP laparoscopy with conventional laparoscopy showed that SP laparoscopy was feasible and safe, in both technical terms as well as oncological terms. However, these studies are yet to see any other outstanding advantages of SP laparoscopy as compared to conventional laparoscopy, except aesthetically [8, 9, 12, 17]. Only a comparative study by J.T. Poon, et al. on 50 patients recorded that SP laparoscopy had lesser postoperative pain and shorter hospital stay; however, the authors did not affirm the conclusions since the number of patients was small [17].

The results of postoperative follow-up had an average of 32.2 ± 7.5 (3-65) months recorded. There was no incision hernia or metastasis. CEA ratio above 5 ng/ml was 21.9%, which is similar to the recognition of H.T. Papaconstantinou, et al. [12]. In our study, 5 patients had local recurrences and 3 liver metastases. All patients in the group of open conversion were in stage IIIC. Thus, we concluded that the recurrence rate in our study was not related directly to SP laparoscopy.

Conclusions

SPLC in the treatment of colon cancer is feasible, and safe. The cosmetic value is better (shorter incision, hidden

by umbilicus). Other results are similar to conventional laparoscopy.

However, a large number of patients are required accurate assessment of the long-term results, particularly in terms of oncology.

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Minimally invasive direct coronary artery bypass: preliminary results at University Medical Center of Ho Chi Minh city

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Abstract:

Background - Objectives: In the recent years, minimally invasive direct coronary artery bypass (MIDCAB) is under rapid development worldwide. The number of MIDCAB is growing dramatically in developed countries. This study aimed for the assessment of indications, techniques, and short-term result of MIDCAB and the experience in building a new technique in our center.

Method: We reported 4 patients who underwent minimally invasive coronary artery bypass at the University Medical Center of Ho Chi Minh city.

Results: 4 patients were operated with MIDCAB procedure. Mean ICU time was 1.4, mean mechanical ventilation time was 5.7 hours, and in-hospital time was 8.4 days. In postoperative time, patients recovered quickly; they experienced less pain than normal and returned to normal activities in a short time.

Conclusions: In our very first experiences with MIDCAB procedure, the early outcomes are satisfactory with low morbidity and no mortality. MIDCAB is safe and feasible, provided that patient selection is good and safety protocols are followed.

Keywords: coronary artery bypass, left minithoracotomy, minimally invasive cardiac surgery.

Classification number: 3.2

Background

First described in 1910 by Alexis Carrels, coronary artery bypass grafting is one of the three major areas of adult heart surgery. The first selected graft is the internal thoracic artery. In 1955, vein grafts were put into use. In 1958, off-pump coronary surgery was first performed by Longmire [1].

Coronary artery disease has so far

been widespread, with a long-term survival of around 77% after 10 years (Domburg, et al.) [2].

Currently, in Vietnam, coronary artery bypass surgery has been widely performed in heart centers. At University Medical Center of Ho Chi Minh city, coronary artery bypass surgery has become a routine surgery.

Technically, this is one of the most

meticulous surgery, requiring the surgeon to not only have good strategies and be knowledgeable, but also have good skills.

The classic opening is the median sternotomy. Internal thoracic artery, saphenous vein, gastroepiploic artery have been used as graft materials. Surgery is performed with cardiopulmonary bypass (cardiac arrest) or off-pump technique (using a specially designed holder to fix the heart), or under the support of cardiopulmonary bypass without cardiac arrest.

The advantage of median sternotomy is a large surgical field, where the surgeon can operate easily and conveniently. However, this approach also has its own disadvantages.

One of the disadvantages of median sternotomy is the risk of postoperative deep sternal wound infection. In coronary artery bypass surgery via median sternotomy, there is an increase in the risk of sternal dehiscence. In addition, there are general drawbacks such as postoperative pain, slower recovery than less invasive surgery, increased hospital stay, and increased costs [3]. Since the internal thoracic artery is the first choice graft, when this artery is harvested, blood supply to the sternum decreases, leading to increased risk of infection and reduced bone healing. From 2005

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to 2010 in India, Okonta, et al. found that the mean length of stay for sternal wound infection was 23.5 ± 8.9 days, which was much longer than the length of hospital stay after surgery without these complications [4].

However, until now, total arterial coronary artery bypass grafting is still the gold standard for cases of triple vessels disease, having the best long-term graft patency, long-term mortality, as well as lower incidence of cardiovascular events than other methods [5].

Currently, triple vessels disease with stentable lesions in the left circumflex and right coronary systems in high-risk patients (elderly, obesity, diabetes mellitus), median sternotomy and bilateral internal thoracic arteries harvesting may increase the postoperative mortality and complications, particularly deep sternal wound infection and long recovery time of the patient. Therefore, the trend of minimally invasive surgery has opened a new direction for these patients: coronary intervention in combination with surgery.

Method

A total of 4 patients underwent MIDCAB surgery via left anterior thoracotomy at the Department of Cardiovascular surgery of the University Medical Center of Ho Chi Minh city from January 2017 to October 2017 (Table 1).

In four patients, there were three cases of chronic total occlusion of the Left anterior descending artery (LAD), one early stent stenosis of LAD in relatively young patients (54 years), the patient and his family chose less invasive coronary artery bypass surgery. All patients underwent MIDCAB via left anterior thoracotomy.

Techniques

Patients were placed in supine position with a cushion under the left scapula to facilitate exposure; two first patients were anesthetized with double lumen endobronchial tube selective left lung isolation, single lumen endotracheal tube was used for the following two patients. An incision of 7 cm was made, intercostal space was selected depending on the lesions on the LAD. If lesions of

the LAD are at the first or the middle part, we choose the IV intercostal space (ICS), if LAD lesions are at the third part, we choose the fifth ICS (Fig. 1).

A special thoracic retractor (Geister's Thoragate) is specially designed to harvest the left internal mammary artery (LIMA). The goal is to harvest to the origin of the artery in order to avoid stealing blood from the collateral branches of the internal thoracic artery to the chest wall. The median duration of chest harvesting in four patients was 46.5 minutes. The two early patients were longer than the two following ones.

After being harvested from the chest wall, the LIMA was cut down to check the blood flow and ensure no dissection or damage that affect the flow. The pericardium was opened at the level of the LAD. Traction sutures were placed to give better exposure to this artery. In the first two cases, we used supported cardiopulmonary bypass from the femoral vessels; in the latter two cases, we performed the complete off-pump LIMA-LAD anastomosis (Figs. 2, 3).

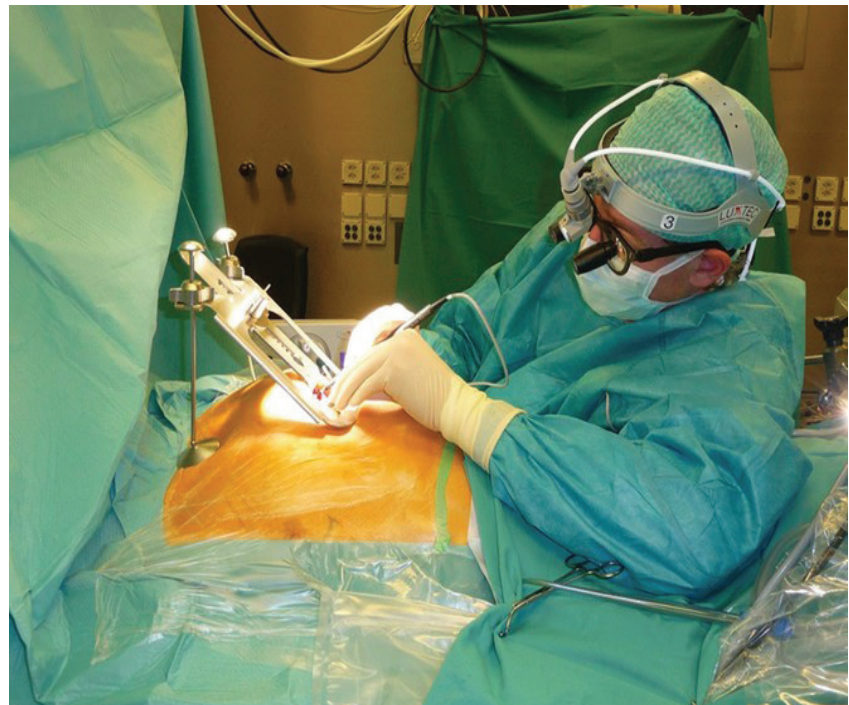
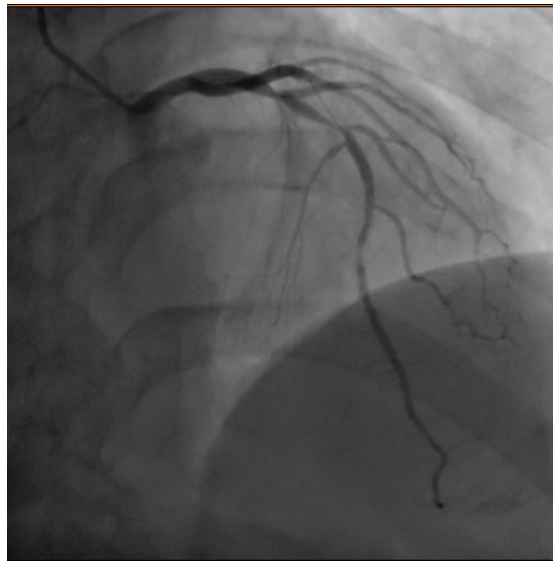
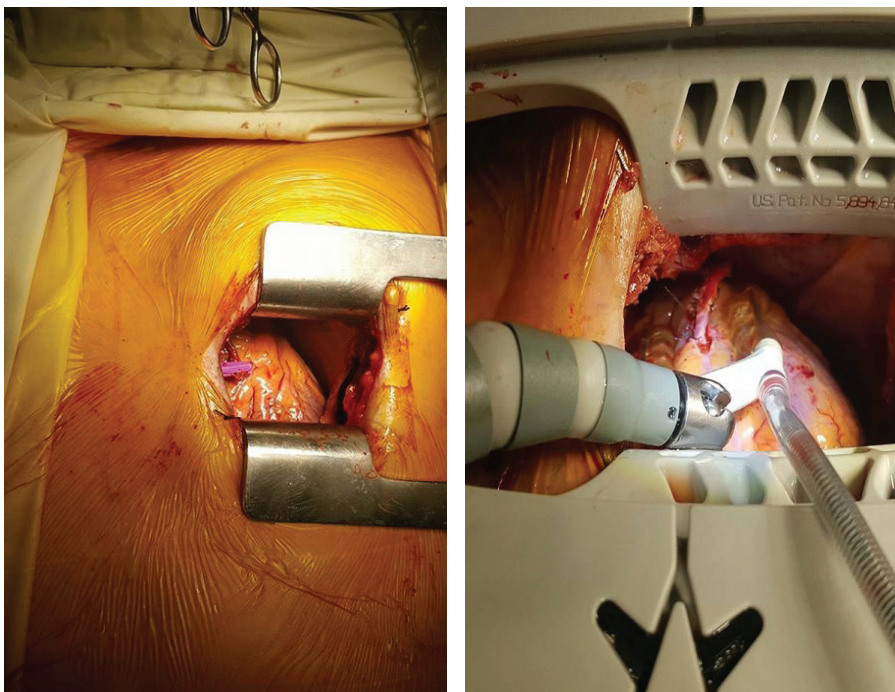


Fig. 1. Thoragate retractor (left) and surgeon's position (right).

Table 1. Description of the variables.

Variables	Results
Number of patients	4
Mean ventilation time (hour)	5.7
Mean ICU time (days)	1.4
Mean postoperative time (days)	8.4

**Fig. 2. LAD stent restenosis.****Fig. 3. LIMA harvested (left) and LIMA - LAD anastomosis (right).**

After performing the anastomosis, hemostasis was checked, a chest tube and a pericardial drainage were placed and the thoracotomy was closed.

Results and discussions

By the 1980s, coronary artery bypass surgery had been established as a widespread and safe surgery. Since the 1990s, less invasive cardiac surgery has been widely accepted to meet the needs of patients (less traumatic, cosmetic) and the requirements of economic benefits (rapid recovery, reduction in hospitalization time).

As a result, new surgical instruments and peripheral cardiopulmonary bypass techniques have been developed (outside the thoracic aorta and the vena cava) to help create a limited access to the surgical field while maintaining the quality of the operation.

Minimally invasive cardiac surgery uses a variety of approaches such as ministernotomy, minithoracotomy and small trocar holes (total endoscopic and robotic surgery). This type of cardiac surgery reduces bleeding, pain, and the incidence of surgical site infections. Additionally, it helps patients recover quickly, reduces hospital stay, and reduces medical costs. Many studies have shown that all of the techniques performed in cardiac surgery with classic sternotomy are applicable in less invasive cardiac surgery without altering the prognosis of the patient, even when performed for patients with high surgical risk.

A meta-analysis of P. Modi, et al. from 43 studies published between 1998 and 2005 (two RCTs, 17 case-control studies, 24 cohort studies), found that compared to conventional full sternotomy, minimally invasive cardiac surgery did not increase mortality, and postoperative cerebrovascular accident. Reoperation due to bleeding was significantly higher but tended to decrease with time. Moreover, infection was significantly lower (1.8% vs. 7.7%, $p = 0.03$). The level of postoperative pain was reduced, and the recovery time to normal activities was faster (4 weeks vs. 9 weeks, $p = 0.01$) [6].

In 1998, Duhaylongsod, et al. described LIMA harvesting through a small thoracotomy with thoracic

endoscopy, which contributed to put the first steps in minimally invasive coronary artery bypass surgery [1].

The LIMA-LAD anastomosis has been shown to have a very good durability in treating modalities for this very important coronary artery. If the artery is severely stenosis and the lesion is complicated, and unstentable, harvesting the LIMA via a small thoracotomy with the usage of a special retractor helps avoid the median sternotomy. This is especially beneficial for patients with type 2 diabetes, obesity as it decreases chest instability and the risk of mediastinal infections.

According to Y. Ling, et al.'s report on minimally invasive coronary artery bypass surgery, the median duration of LIMA harvesting was 43 minutes, mean mechanical ventilation time was 9 ± 7 hours, mean ICU time was 24 ± 18 hours, the mean units of red blood cell transfused was 0.79 ± 1.58 , and 30 day mortality was 0.5% [7] (Table 2).

R. Birla, et al. conducted a research to compare the minimally invasive coronary artery bypass surgery and the conventional

off-pump coronary artery bypass (OPCAB) grafts on single vessel disease, which demonstrated no difference in mortality, recurrent myocardial infarction, postoperative cerebrovascular accident, atrial fibrillation, and reoperation [8] (Tables 3, 4).

At the moment, minimally invasive coronary artery bypass surgery is indicated for the following cases:

- Single vessel disease of LAD and/or diagonal branches with complex, unstentable lesions.
- Stent restenosis of LAD, unstentable lesions.
- Three-vessel disease in high-risk patients, unstentable, revascularisation of the most important cardiac muscle part perfused by LAD is indicated.
- Three-vessel disease in high-risk patients and it is feasible to stent the LCx and RCA.
- Patients with coronary artery disease who wish to undergo minimally invasive surgery on the LAD in combination with stent placement in the other branches (right coronary arteries and arteries) [7].

Table 2. In-hospital clinical outcomes and 30-day mortality (N = 200).

	MIDCAB (n=138)	Hybrid (n=62)	Total (N=200)
30-day mortality, N (%)	1 (0.7 %)	0 (0.0 %)	1 (0.5 %)
Perioperative MI, N (%)	1 (0.7 %)	0 (0.0 %)	1 (0.5 %)
Duration of mechanical ventilation, hour	9.93 ± 8.65	7.79 ± 4.43	9.27 ± 7.65
LOS in ICU, hour	24.17 ± 17.83	24.48 ± 18.03	24.27 ± 17.85
PRBC, units	0.86 ± 1.63	0.61 ± 1.47	0.79 ± 1.58
PAF, N (%)	10 (7.2 %)	4 (6.5 %)	14 (7.0 %)
Stroke, N (%)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)
Renal failure, N (%)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)
Incision complications, N (%)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)

ICU: intensive care unit; LOS: length of stay; MI: myocardial infarction; PAF: postoperative atrial fibrillation; PRBC: packed red blood cell.

Table 3. Intensive care unit length of stay for MIDCAB and OPCAB groups.

	MIDCAB (n=74)	OPCAB (n=78)	p-value
Length of stay	38.36 hours	47.87 hours	>0.5
Ventilation duration	5.04 hours	5.35 hours	>0.5

Table 4. Comparison of early postoperative outcomes between MIDCAB and OPCAB groups.

	MIDCAB (n=74)	OPCAB (n=78)	p-value
Mortality	0 (0%)	0 (0%)	-
Reoperation for bleeding	0 (0%)	2 (2.7%)	0.2
Atrial fibrillation	17 (22.9%)	12 (15.4%)	0.3
Wound infection	4 (5.4%)	2 (2.7%)	0.4
Cerebrovascular accident	2 (2.7%)	0 (0%)	0.1

Collaboration between Cardiac surgeons and Interventional Cardiologists:

Cardiologists play an important role in the selection of patients with minimally invasive coronary artery bypass surgery based on the indications. The collaboration between cardiac surgeons and interventional cardiologists to select the patient ensures patient safety, and provides a new option for patients, especially those at high risk for surgery.

Conclusions

In our very first experiences with MIDCAB procedure, the early outcomes are satisfactory with low morbidity and no mortality. MIDCAB is safe and feasible, provided that patient selection is good and safety protocols are followed.

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Establishment of an in-house one-step real-time RT-PCR assay for detection of Zaire ebolavirus

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Abstract:

Ebola virus is a deadly causative agent with a high mortality rate of up to 90%, therefore it has been classified by the Center for Disease Control and Prevention (CDC) as a category A biological agent. The World Health Organization (WHO) recommended using RT-PCR based assays to rapidly detect the virus. In the present study, we established an in-house assay for detection of Zaire ebolavirus via real-time RT-PCR. The nucleotide sequence of the Zaire ebolavirus nucleoprotein (NP) gene was retrieved from the Genbank for designing primer pairs and probes using Primer Express 3.0 software. The RNA positive control was generated by in vitro RNA transcript synthesis. The optimal components in the 20 µl final volume of the real-time RT-PCR assay were 10 µl 2X QuantiTect Probe RT-PCR master mix, 0,6 µM of each primer, 0,1 µM of the probe, 0,2 µl RT mix and 5 µl of RNA template. The thermal cycle conditions were as follows: 50°C for 30 min, 95°C for 15 min, then 45 cycles of 15 s at 94°C, 60s at 60°C. The limit of detection of the assay was 100 copies/reaction and 1414 FFU/ml with the positive RNA panel and sample panel of RNA extracted from cell culture supernatants of cells infected with Zaire ebolavirus 2014/Gueckedou-C05, respectively. The specificity of this assay was 100% when tested with the positive RNA panel of Ebola virus and other haemorrhagic fever viruses. In conclusion, we successfully established an in-house real-time RT-PCR assay for detection of Zaire ebolavirus in Vietnam with a limit of detection of 1414 FFU/ml and specificity of 100%.

Keywords: ebola virus, real-time RT-PCR, Vietnam, Zaire ebolavirus.

Classification number: 3.2, 3.5

Introduction

Ebola virus (EBOV) is a fetal causative agent of severe hemorrhagic fever epidemic with a high mortality rate of up to 90%. The virus was firstly discovered in 1976 when it caused two simultaneous outbreaks in Sudan and Zaire (now Democracy Republic of Congo) [1]. The recent Ebola outbreak in Western Africa was the largest in history

with more than 28,602 suspected cases and 11,301 deaths. The cause of this outbreak was then identified as a Makona variant of Zaire ebolavirus [2]. The WHO declared the outbreak of EBOV disease in West Africa as a “Public health emergency of international concern” and called for a substantial global response in order to control this epidemic [3].

EBOV belongs to the *Filoviridae*

family consisting of the five species: Zaire ebolavirus (ZEBOV), Sudan ebolavirus (SEBOV), Reston ebolavirus (REBOV), Bundibugyo ebolavirus (BEBOV) and Tai Forest ebolavirus (TEBOV) [1]. EBOV is an enveloped, negative-sense, and single-strand RNA virus with its genome (19 kb in length) encoding for 7 proteins including nucleoprotein (NP), viral protein (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), matrix protein (VP24), and RNA dependent RNA polymerase (L). No available vaccines or antiviral drugs exist for prevention and treatment of the EBOV disease. Therefore, early detection of suspected cases is critical for the management, surveillance and control of this deadly epidemic. Real-time RT-PCR assays were used routinely in the laboratory of clinical virology due to high sensitivity, specificity and rapid results, therefore the WHO recommended the use of a real-time RT-PCR assay as the first choice for detection of EBOV in clinical virology laboratories [4]. However, commercial real-time RT-PCR kits approved by the FDA were not available before the arrival of the epidemic in late 2013. Other relevant assays including ELISA, require a Bio safety level 4 (BSL-4) facility for isolation and viral culture [5]. Therefore, a simple, sensitive, and accurate assay based on real-time PCR, which is affordable in countries of limited resources, is essential for

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early detection of EBOV in inactivated specimens [6]. This study aims to establish and evaluate a real-time RT-PCR assay for detection of ZEBOV.

Materials and methods

Preparation of ZEBOV RNA positive standard

The 1306 bp nucleotide sequence of a partial NP gene and 3' untranslated region (3'UTR) of recently epidemic ZEBOV strain (GenBank: KJ660348) was chemically synthesized and inserted into the pIDTBlue vector (4 µg) by IDT (USA). This plasmid was linearized by digestion with *PciI* restriction enzyme for *in vitro* RNA transcription with a Transcript Aid T7 High Yield Transcription Kit (Thermo Scientific), and the synthetic viral RNA transcripts were purified using a GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. The RNA level was measured by a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific) and then converted to the number of copies per µl. The RNA transcript was stored at -80°C for further use.

RNA extraction

RNA samples were extracted from 140 µl of clinical samples collected from patients in recently Ebola stricken Guinea and from cell culture supernatant of cells infected with ZEBOV2014/Gueckedou-C05 and other haemorrhagic virus species including SEBOV, REBOV, TEBOV and the Marburg virus [Leiden-BNI 2008], and plasma of patients infected with dengue virus, Zika virus and chikungunya virus for assay cross-reactivity and specificity evaluation using QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. All clinical samples were inactivated before doing extraction by using an AVL buffer and absolute ethanol; then samples were incubated at 60°C for 60 minutes under

BSL-4 conditions in the department of virology at Bernhard Nothch of Tropical Medicine (BNITM), Hamburg, Germany. Extracted RNA samples were prepared at a concentration of 10^6 copies/ml.

One-step real-time RT-PCR assay

A one-step real-time RT-PCR assay was optimized by using QuantiTect Probe RT-PCR Master mix (Qiagen) in a final volume of 20 µl including 5 µl of RNA. Real-time RT-PCR assays were performed using the Rotor-Gene Q Instrument (Qiagen) as well as LighCycler 2.0, LighCycler 480 II Instrument (Roche) with thermal cycle parameters as follows: 50°C for 30 min, 95°C for 15 min, then 45 cycles of 15 s at 94°C and 60 s at 60°C. Fluorescent signals were recorded during each annealing step of the amplification cycle and a threshold signal was chosen at 0.1 to determine the threshold cycle (Ct) value during the analysis process for the Rotor-Gene Q Instrument and automated mode for Roche Instrument. All experiments were tested in duplicate within or between runs.

A 10-fold serial dilution from 10^6 to 10^0 copies/µl of transcribed RNA and RNA extracted from cell culture supernatant of infected cells with ZEBOV 2014/Gueckedou-C05 (1.65×10^5 - 1.65×10^0 FFU) was used to determine the limit of detection (LoD). The LoD was defined as the lowest RNA concentration detected in all runs of the 20 replicates.

Statistical analysis

The regression and the coefficient of variation (CV) of the mean Ct value for each standard concentration within and between individual PCR runs were analyzed by using statistical excel.

Results

ZEBOV RNA positive standard

The transcribed ZEBOV RNA was yielded with a high concentration of

1,400.3 ng/µl (1.44×10^{12} copies/µl) and 2.01 A260/A280 ratio. Moreover, the RNA transcript was determined by specific size 1806 base in gel agarose electrophoresis (Data not shown). Additionally, the quality of RNA transcript was evaluated by using our previously developed one-step RT-PCR assay for EBOV detection. The RT-PCR product of the ZEBOV RNA in a 10^6 copies/µl concentration is a specific and thick band 830 bp in length (RT mix (+)), whereas there is no band for RT mix (-) RT-PCR (Lane 2 and 3, Fig. 1). Positive RT-PCR product was confirmed exactly by direct sequencing (Data not shown).

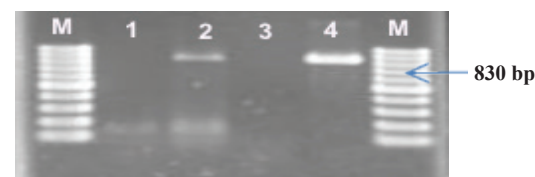


Fig. 1. Evaluation of ZEBOV RNA transcript by one-step RT-PCR assay. M. Marker 100 bp (Thermo Scientific), 1. Negative control; 2. RT-PCR with enzyme RT mix, 3. RT-PCR without enzyme RT mix, 4. Positive control plasmid.

Development and optimization of one-step real-time RT-PCR

Design primer and probe: A nucleotide sequences of the NP gene retrieved from the Genbank database was used for alignment with Clustal W to identify the conserved region for designing a primer and probe. We used Primer Express 3.0 software to design primers in highly conserved regions of the NP gene. The primer and probe sequences were as follows: EBOV-forward: 5'-GACAAATTGCTCGGAATCAC-3'; EBOV-reverse: 5'-ATCTTGTGGTAATCCATGTCAG-3' and probe: 5' FAM - CAGTGAGACTCGGCGTCATCCAGA - TAMRA 3' that amplified 103 bp in length real-time PCR product (Fig. 2). The primer-probe sequences were checked with a Blast primer tool.



Fig. 2. Nucleotide sequences and sites of primer pairs and probe for a ZEBOV real-time RT-PCR assay.

Table 1. Results of analytical sensitivity.

Concentration	1st Exp Ct	2nd Exp Ct	3rd Exp Ct	Mean Ct	SD	CV
E6	26.39	26.01	27.1	26.5	0.55	0.30
E5	30.16	29.75	32.26	30.7	1.34	1.81
E4	34.67	34.09	36.24	35.0	1.11	1.23
E3	38.84	38.23	40.54	39.2	1.19	1.43
E2	40.41	39.7	43.71	41.2	2.13	4.57
E1	-	-	-	-	-	-

SD: standard deviation, CV: coefficient of variation.

Optimization of the one-step real-time RT-PCR assay: Concentrations of primers and probes were optimized in a final volume of 20 μ l reaction mixture containing 5 μ l of RNA template to obtain

minimal Ct. Primer concentrations were tested from 0.1 to 0.6 μ M and probe concentrations were tested from 0.05 to 0.4 μ M. The optimal reaction was obtained at a primer concentration of

0.6 μ M (for both primers) and a probe concentration of 0.1 μ M.

Limit of detection and specificity of one-step real-time RT-PCR assay

The analytical sensitivity of the real-time RT-PCR assay was evaluated in triplicates on a sample panel ranging from 10^0 to 10^6 copies/ μ l which was created by serial dilutions of the synthetic viral stock RNAs. The threshold line was chosen at 0.1 during analysis and the data collected were analyzed by linear regression ($r^2 = 0.99$). The results showed that the one-step real-time RT-PCR assays could detect in samples at the concentration of 10^2 copies/reaction (Table 1).

Additionally, the diagnostic sensitivity of the assay was assessed by determination of the LoD, defined as the last dilution at which all replicates were positive. The results have shown the diagnostic sensitivity was 1.65×10^1 FFU/reaction, mean 1414 FFU/ml equivalent, indicating a good sensitivity (Fig. 3 and Table 2).

$$\text{Concentration (FFU/ml)} = \frac{1.65 \times 10^1 \text{ FFU/reaction}}{0.14 \text{ ml (V extract sample)} \times \frac{5 \mu\text{l (V RNA/reaction)}}{60 \mu\text{l (V elute RNA)}}} = 1414 \text{ FFU/ml}$$

The LoD of each test was determined to be the lowest concentration resulting in 95% positive detection of 20 replicates. Furthermore, we also evaluated the sensitivity of the assays on several clinical specimens with different viral loads measured with a Realstar Ebola PCR kit in BNITM. Therefore, the diagnostic sensitivity of the assay was confirmed at the 1.65×10^1 FFU/reaction, it was also set as the LoD for the assay. End-point real-time RT-PCR products also showed specific bands with a length of 103 bp on agarose gel (Fig. 4).

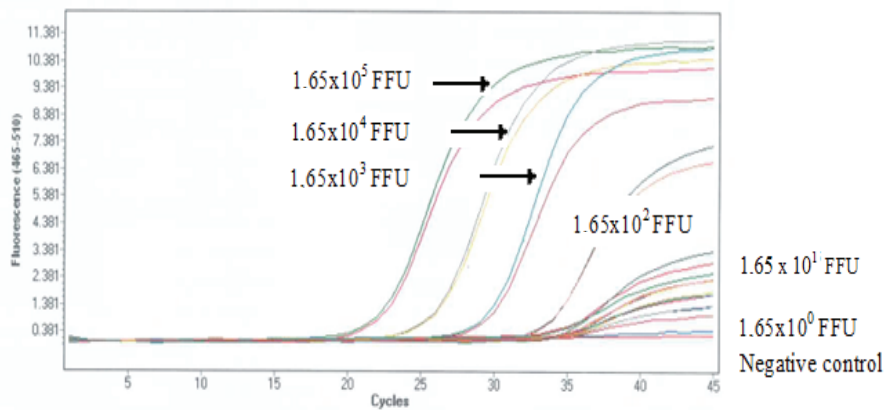


Fig. 3. Concentration dilutions from 1.65×10^5 to 1.65×10^0 FFU/reaction.

Table 2. The diagnostic sensitivity and specificity of real-time RT-PCR.

Sample panel ZEBOV RNA	Quantity (FFU/reaction)	Mean Ct value	Replicates	Assay results
Diluted E-1	1.65×10^5	22.3	6	100% Positive
Diluted E-2	1.65×10^4	26.31	6	100% Positive
Diluted E-3	1.65×10^3	29.68	6	100% Positive
Diluted E-4	1.65×10^2	33.62	9	100% Positive
Diluted E-5	1.65×10^1	34.72	20	100% Positive
Diluted E-6	1.65×10^0	-	6	100% Negative
Other viruses				
Sudan EBOV Gulu			3	100% Negative
Reston EBOV			3	100% Negative
Tai Forest EBOV			3	100% Negative
Marburgvirus Leiden			3	100% Negative
Marburgvirus Popp			3	100% Negative
Dengue virus			3	100% Negative
Chikungunya virus S27			3	100% Negative
Zika virus			3	100% Negative

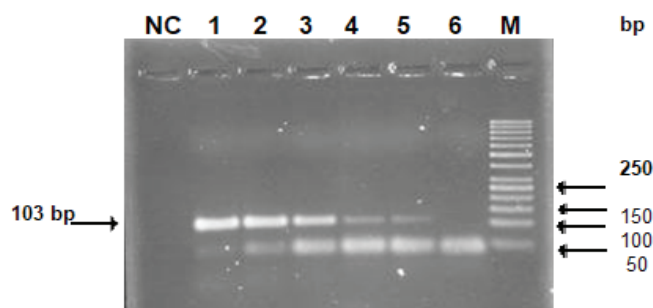


Fig. 4. Representative agarose gel 2% of end-point products of one-step real-time RT-PCR ZEBOV RNA from 1.65×10^5 to 1.65×10^0 FFU/reaction. M: marker 50 bp (thermo scientific), NC: negative control; 1-6: 1.65×10^5 - 1.65×10^0 FFU.

The cross-reactivity and specificity of the assay were tested with RNAs extracted from the supernatant of cell-cultures infected with other EBOV species: SEBOV, REBOV, TEBOV, and Marburg virus [Leiden-BNI 2008], dengue virus, Zika virus and chikungunya virus. There was no cross-reaction of the assay with any of the other EBOV species which were observed. The diagnostic specificity was 100% of all tested samples which were negative for ZEBOV and closely other hemorrhagic fever viruses.

Discussions

EBOV disease is a major public health issue in the world. Among five EBOV species, ZEBOV caused a majority of the outbreaks in Africa with the highest case-mortality rate of up to 90%. After the three week period of incubation, EBOV disease presents with unspecific symptoms and is usually difficult to differentiate from other tropical diseases [7]. Therefore, diagnostic laboratory assays play an important role in confirming or excluding suspected cases [5]. In recent years, several methods for detecting EBOV have been developed for use in clinical virology laboratories, including the use of several assays under Emergency Use Authorization, and others evaluated in a field setting. Due to the fact that EBOV is categorized as a high-hazard pathogen, diagnostic methods including viral culture and isolation require it to be handled in a BSL-4 facility. However, in resource-limited countries, the WHO and CDC have advised that EBOV can be tested in BSL-2 conditions by nucleic acid testing if specimens are inactivated by appropriate methods.

The first real-time PCR assay was developed by Gibb, et al. to detect and differentiate between ZEBOV and SEBOV in patient samples collected during the 2000 Gulu outbreak [8] sensitive, and specific laboratory diagnostic test is needed to confirm outbreaks of Ebola virus infection and to distinguish it from other diseases that can cause similar clinical symptoms. A one-

tube reverse transcription-PCR assay for the identification of Ebola virus subtype Zaire (Ebola Zaire). In addition, the real-time PCR assay measured the viral load in the patients' plasma, which has been shown to be associated with the outcome of the disease. Recent studies have shown that most patients in Western Africa with high viral load associated with a poor prognosis and higher mortality rate [9]. However, there was not a commercial real-time PCR assay approved by the FDA for use upon emergence of the EBOV outbreak in Western Africa, whereas, various laboratory-developed assays have demonstrated significant variability in regards to their sensitivity of detection as well as their reliability [4, 10, 11].

In this study, we established an in-house assay for detection of recent ZEBOV by one-step real-time RT-PCR. Ideally, optimization of assays needs to be performed on EBOV-RNA samples extracted from the stock viral strains, but it is very difficult to acquire this material in Vietnam because there have yet to be any reported cases of EBOV infection. Therefore we used RNA transcribed *in vitro* from a plasmid containing the NP gene of EBOV to generate both the acceptable standards for the optimization of components and appropriate reaction conditions, as well as for the evaluation of the analytical sensitivity of the assay. Furthermore, we validated the established assay with an RNA sample extracted from inactivated cell culture supernatant of infected cells with ZEBOV 2014/Gueckedou-C05 and several clinical samples to determine the LoD and diagnostic sensitivity at the BNITM in Hamburg, Germany. Results showed that the analytical sensitivity of the assay obtained was at a concentration of 10^2 copies/reaction, whereas, specificity was 100% as tested with RNA extracted from other EBOV species and close other hemorrhagic fever viruses. When tested on RNA extracted from the supernatant of infected cells with ZEBOV 2014/Gueckedou-C05 indicated

the LoD at a concentration of 1414 FFU/ml and 100% of positive clinical samples. Importantly, we also optimized one-step real-time RT-PCR using a total volume of 20 μ l per reaction, making this assay save more reagents. One notable point, the established assay performed on both the Rotor-Gene Q and LightCycler instrument showed a similar performance. Compared with previous studies, the established assay in this study had higher sensitivity and specificity. When comparing this assay to others it can be said to be affordable in cost and to provides accurate results in a short period of time. In addition, the volume of RNA template and related requirements should be considered when comparing this assay to others. Therefore, it is very important to standardize and optimize with more extensive reagents and then validate these assays further in regards to international WHO reference materials.

In conclusion, we developed a highly specific, sensitive assay for the detection of ZEBOV by one-step real-time RT-PCR with the LoD concentration of 1414 FFU/ml, and specificity of 100%. This assay could be used to detect ZEBOV in samples taken from subjects suspected of infection, after returning from travel in infected regions.

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Preparation and characterization of liposomes encapsulating *Calophyllum inophyllum* oil

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Abstract:

Calophyllum inophyllum oil, also known as Tamanu oil, is reported to treat a wide range of skin problems such as acne, eczema, psoriasis, herpes, hemorrhoids, and injuries caused due to wounds, among others. Liposomes, which are effective carriers for topical treatment of dermal diseases, could enhance the therapeutic efficiency of Tamanu oil. Therefore, the purpose of this study was to formulate and characterize liposomes loading Tamanu oil. Liposomes encapsulating Tamanu oil with different ratios of Phospholipon 90G and L- α -lecithin were prepared using the thin-film hydration technique. Liposomal formulations were characterized in terms of aspect, particle size, size distribution, zeta potential, and morphology by using light microscope and dynamic light scattering analysis (DLS). Furthermore, the best formulation was tested with the storage stability after 30 days, and Tamanu oil loaded in the liposomes was identified as dictated in Vietnamese pharmacopoeia. The data demonstrated that an average liposome diameter of 53 nm with a narrow polydispersity (0.289) was obtained at a Phospholipon 90G to L- α -lecithin molar ratio of 4:6, and a Tamanu oil to phospholipid mass ratio of 1:3 approximately. In addition, according to the DLS results, the particle size and the zeta potential were quite stable at 2-8°C during 30 days of storage. The study achieved the promising results for developing a novel formulation containing Tamanu oil, which may be valuable to treatment of skin diseases.

Keywords: liposome, Tamanu oil, thin-film hydration method.

Classification number: 3.3

Introduction

Calophyllum inophyllum L., Guttiferae, locally called Tamanu, is a tropical tree that is widely distributed throughout Africa, Asia, and Pacific countries. *Calophyllum inophyllum* oil extracted from the Tamanu nuts is composed of a mixture of lipids and other components including xanthone, flavon, and terpene derivatives. Traditionally, Tamanu oil has topically been used for skin care and to relieve skin problems for centuries. Ever since the first half of

the 20th century, a number of researches have reported the pharmacological properties of Tamanu oil. Those include anti-inflammatory, antimicrobial, wound-healing, tissue-regenerative, and skin-protective properties. However, the direct application of pure Tamanu oil on the skin presents some disadvantages: The permeable efficiency of Tamanu oil across the skin is low due to the hydrophobicity of its lipid compositions; Tamanu oil is slightly rubefacient, so, long-term dermal exposure to the pure

oil can lead to skin irritations; just like other oils, it can clog skin pores, often resulting in acnes and other skin infections [1, 2].

Tamanu oil came to be used in cosmetics about 40 years ago, and it was approved for clinical uses about 20 years ago [3]. Liposomes are used as a drug delivery system offering several benefits including biocompatibility, adjustable membrane to control their pharmacokinetic properties, increasing efficacy and therapeutic index of active agents [4, 5]. In terms of liposomal composition, liposomes are nanometric or sub-micrometric vesicles consisting of an internal aqueous core and one or more external phospholipid bilayer. This makes it possible for liposomes to load both hydrophobic and hydrophilic molecules. Hydrophobic compounds are inserted into a lipid layer, while hydrophilic compound can be entrapped in an aqueous center. This contributes to protecting these compounds from degradation and any other adverse environmental factors, thereby improving their stability. Moreover, owing to possessing a similar lipid bilayer with that of the skin, liposomes are easily attracted to dermal cells in different ways, for instance, adsorption, endocytosis, lipid exchange, or fusion. It is for this reason that the use of liposomes as a topical drug delivery system in the treatment of skin diseases facilitates the penetration of active ingredients into the deeper layer of the skin where their action should occur [6, 7]. Finally, the entrapment of drugs into liposomal vesicles overcomes some

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inconveniences that are present in free drugs, such as irritation, unpleasant odor, clogging pores. Due to these benefits, liposomes are likely to be a promising choice for loading Tamanu oil in order to enhance clinical efficacy of the oil.

In addition, when it comes to physicochemical properties of liposomes that influence skin permeation of active ingredient entrapped in liposomes, a few researches indicated that small-sized liposomal vesicles appear to bring out a higher degree of penetration, and some surfactants act as a skin penetration enhancer.

For these reasons, this study was aimed at the preparation and characterization of liposomes containing Tamanu oil. In this way, liposomal suspensions encapsulating Tamanu oil with various organic solvents dissolving the lipid phase, speed of rotor/stator homogenization, lipid compositions, and tween 80 concentration have been studied based on their particle size, size distribution, and stability in order to optimize the liposomal formulation loading Tamanu oil.

Then, the result would be useful in the creation of effective liposomal topical formulations loading Tamanu oil for cosmetic and dermatological applications.

Materials and methods

Materials

The *Calophyllum inophyllum* oil (CIO) was provided by the Traditional Medicine Institute in Ho Chi Minh city (Vietnam). Phospholipon 90G (PL) from soybean and L- α -Lecithin (LL) from egg yolk were purchased from Lipoid® and Calbiochem®, respectively. The solvents for phospholipids including chloroform, ethanol 90 percent, and petroleum ether (30-60) were purchased from Sigma Aldrich (Germany). Tween 80 which was used to enhance the solubility of phospholipids in ethanol was purchased from Sigma Aldrich (Germany).

Methods

Preparation of liposomes loading Tamanu oil:

Liposomes were prepared by the thin-film hydration technique. In brief, the lipid phase (consisting of accurately weighed quantities of CIO, and a PL-LL mixture in different molar ratios) was dissolved in organic solvent (ethanol, chloroform, or petroleum ether) in a round bottom flask; the organic solvent was then removed under reduced pressure by using a rotary evaporator (Buchi R-200/205) at 70°C, then a thin lipid layer appeared in the flask. Thus, the lipid film obtained was kept on evaporating for three hours to eliminate the trace of the organic solvent. Finally, the hydration of the film with 50 ml of distilled water was carried out on the rotary evaporator under fast spin, at 50°C, for one hour to favor the vesicle formation. Liposomes were stored at temperatures between 2-8°C.

Homogenization of prepared liposomes:

The prepared liposomal suspension was homogenized by using Rotor/Stator Homogenizer, heated at 50°C (homogenization takes place at higher temperatures than phase transition temperatures of lipids) for 15 minutes. The rotor speed was set up at 11,000 rpm, 15,000 rpm, and 19,000 rpm in turns.

Measurement of liposome size and zeta potential:

The mean particle size, size distribution, and zeta potential analysis of the liposomes were determined at 25°C by Dynamic Light Scattering using a Malvern Zetasizer (Malvern Instrument Limited, Malvern, UK). Experiments were run in triplicate.

Storage stability studies:

The liposomal suspension was stored in darkness at 8°C and 25°C for 30 days. The storage stability of the liposomes was based on the change of both their particle size and polydispersity index (PDI) measured by the Malvern

Zetasizer.

Light microscopy:

The liposomal vesicles were monitored for their morphological attributes with the help of a digital optical microscope at 100X Objective (Olympus, Moticam 1000, Japan).

Identification of Tamanu oil in liposomes:

Thin-layer chromatography identification test: 10 μ l of standard solution (0.1 g of CIO dissolved in 0.5 ml diethyl ether), test solution (0.1 g of liposomes sediment dissolved in 0.5 ml diethyl ether), and placebo solution (0.1 g of a mixture of PL, LL, and tween 80, dissolved in 0.5 ml diethyl ether) were applied on a parallel line of the thin-layer chromatography plate coated with silica gel-G. The plate was placed in a pre-saturated chromatographic chamber with a solvent system consisting of a mixture of benzene and ethyl acetate (8:2). The chromatogram was developed with the developing solvent system until the solvent front had moved about three-fourths of the length of the plate. Thus, the plate was removed from the chamber, the solvent front was marked and dried at a temperature from 100-105°C for 5 minutes. The location of the spots on the plate was observed under UV light (at the wavelengths of 254 nm and 365 nm). Later, the plate was sprayed with a vanillin-sulfuric acid reagent, and heated at 100-105°C for 5 minutes. This step was carried out to detect the presence of other compositions that weren't visible under UV light. The test solution chromatogram should be mainly similar to that of the standard solution and different from that of the placebo solution.

Results and discussions

Homogenization of liposomes loading Tamanu oil

The multi-lamellar vesicle (MLV) liposomes prepared by thin-layer hydration method are fairly large (several micrometers) and heterogeneous. In this way, it is compulsory to reduce and homogenize the liposomal particle size.

The particle size and size distribution of the liposomal vesicles obtained after homogenization using rotor/stator homogenizer at different rotor speeds are presented in Table 1.

The particle size of the non-homogenized liposomes was about four times larger than that of the homogenized liposomes. Furthermore, the rotor/stator homogenization decreased the size distribution of the liposomal vesicle, which related to the stability of the liposomes. These results showed that the homogenization played an important role in the preparation of liposomes.

The particle size and size distributions of liposomes obtained through homogenization at 15,000 rpm and at 19,000 rpm are mainly similar. Moreover, the homogenization at high speed may result in degradation of Tamanu oil and also lipid materials. Therefore, the speed of 15,000 rpm was chosen to homogenize liposomal vesicles.

Organic solvent used to dissolve the lipid phase

Solubility study proved that the lipid phase consisting of CIO, PL, and LL was dissolved well in chloroform or in a mixture of ethanol and petroleum ether (8:2). Yet it formed a suspension after being dispersed in ethanol.

Hypothesis: The majority of CIO compositions are lipophilic, which could dissolve the particles of phospholipid during the preparation of the thin-layer film. This might lead to a homogeneous layer film.

Therefore, ethanol, chloroform, and petroleum ether were chosen to dissolve the lipid phase. The particle size and size distribution of the liposomal vesicles were prepared by using these three different solvents as shown in Table 2. With regard to the particle size, there is no significant change between the formulations. Despite the increase in the polydispersity index, this value is a little small and is acceptable. Besides, the trace of the organic solvents such as chloroform, petroleum ether in a topical

Table 1. Particle size and polydispersity index of formulations homogenized at different speeds.

Formulation	Particle size (d.nm)	Polydispersity index
H0 (non-homogenized)	410.4	0.568
H11 (11,000 rpm)	119.26	0.292
H15 (15,000 rpm)	106.26	0.298
H19 (19,000 rpm)	106.06	0.279

Table 2. Particle size and polydispersity index of formulations prepared by using three different organic solvents dissolving the lipid phase.

Formulation	Particle size (d.nm)	Polydispersity index
A1 (ethanol)	110.70	0.309
A2 (chloroform)	114.04	0.270
A3 (ethanol:petroleum ether (8:2))	114.98	0.286

product can cause adverse side effects in cases of long-term application. As a result, ethanol was used as an organic solvent for dissolving the lipid phase.

Encapsulation of Tamanu oil

Almost all the compositions in CIO are lipophilic. So, the CIO was added to the lipid phase (passive encapsulation method) and would be integrated into the phospholipid bilayer during the formation of the thin-layer film. The amount of CIO into the lipid phase was established by evaluating the stability (storage at a temperature between 2-8°C for 30 days) of liposomal suspensions with a varying amount of CIO.

At concentrations of up to 24.1%, the liposomal suspensions obtained were stable during the storage phase (no surface phenomena were observed).

Optimization of lipid composition

The composition of the bilayer, in particular phospholipids, influences the fluidity as well as the stability of liposomes considerably. First of all, the stability of liposomal suspensions with different molar ratios was investigated on the basis of the centrifuge stability testing (centrifugation at 17,000 rpm

for 30 minutes). It was seen that the change of the lipid molar ratio could result in the change in the stability of liposomal vesicles. To be more precise, the formulations containing a mixture of PL and LL with molar ratios from 8:2 to 4:6 presented a higher stability than the others. Then, the particle size and the size distribution of these formulations were measured in order to find out the best formulation. The results are displayed in Table 3.

The potential zeta is a good tool to investigate the stability of liposomal products. In this study, the incorporation of two kinds of phospholipids contributes to the augmentation of potential zeta (from -20 mV to -42 mV). This suggests that the incorporation of PL and LL improved liposome stability. The formulation with a mixture of PL and LL at the ratio of 6:4 gave the smallest mean particle size. Therefore, this ratio was chosen for the liposomal formulation loading CIO.

Influence of tween 80 on physical properties of liposomes

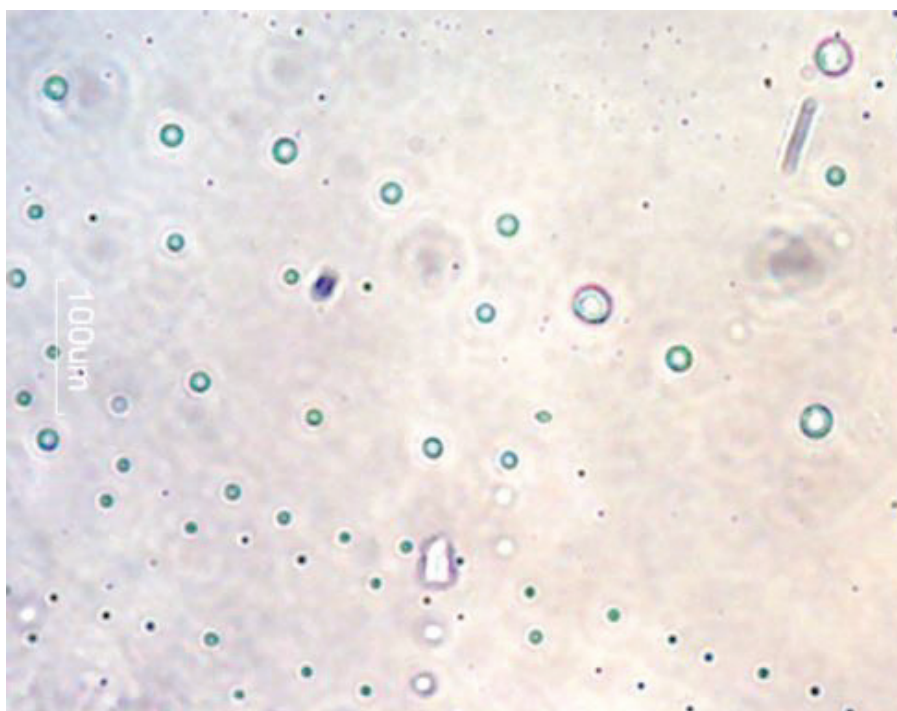
The application of liposomes as a tropical and transdermal drug delivery system necessitates some specific

Table 3. Particle size distribution and potential zeta of different formulations with various molar ratios of PL and LL.

Formulation	Particle size (d.nm)	Polydispersity index	Potential zeta (mV)
B0 (10 PL: 0 LL)	143.08	0.333	-20.3
B2 (8 PL: 2 LL)	118.18	0.302	-42.0
B3 (7 PL: 3 LL)	114.06	0.309	-48.7
B4 (6 PL: 4 LL)	107.88	0.301	-53.7
B5 (5 PL: 5 LL)	110.70	0.309	-56.7
B6 (4 PL: 6 LL)	110.22	0.298	-56.8

Table 4. Particle size and polydispersity index of formulations with and without different concentrations of tween 80.

Formulation	Particle size (d.nm)	Polydispersity index	Potential Zeta (mV)
C0 (0% w/w)	-	-	-
C1 (5% w/w)	109.8	0.301	-53.7
C2 (10% w/w)	102.74	0.276	-54.5
C3 (15% w/w)	97.38	0.418	-53.9
C4 (20% w/w)	106.32	0.455	-54.2

**Fig. 1. Optical microscope image at 100X objective of the liposomal formulation.**

properties such as elasticity of liposomes. It was reported that the surfactant acted as an “edge activator” which enhanced the flexibility of liposomes. This helps the encapsulated agent to penetrate to the deeper layer of the skin. In the present paper, the effect of tween 80 is evaluated as well as that of its concentration on the particle size, and the size distribution of liposomal vesicles is also evaluated, which is summarized in Table 4. The film of the tween 80-free formulation (C0) did not completely detach during the hydration because of the hydrophobicity of the film compositions. The use of the surfactant allowed the film to become more fluid and to form vesicles easily. At concentrations up to 10% (w/w), tween 80 was not only beneficial to the hydration, but it also contributed to the reduction in the particle size and the size distribution of liposomal vesicles. However, at the range from 15-20% (w/w) of tween 80 in the liposomal formulation, liposomal particles became more heterogeneous. Therefore, the content of 10% (w/w) of tween 80 was chosen to be added to the formulation for preparation of liposomes encapsulating the CIO.

Characterization of the final liposomal formulation

Physical appearance of liposomal suspension: The liposome suspension is homogeneous, and light green in color. The morphological attributes of liposomes before homogenization, as observed through an optical microscope, is shown in Fig. 1. Although the liposomes viewed by using the optical microscope were giant liposomes, the optical microscope image provided the morphology of liposomes loading CIO. The image showed that Tamanu oil (green color) was encapsulated into the liposomal bilayer.

Storage stability: The stability results which displayed minimal changes of particle size and size distribution of the final liposomal formulation are summarised in Table 5.

Table 5. Particle size and polydispersity index of the final liposomal formulation before and after storage for 30 days at the different temperatures.

Formulation	Particle size (d.nm)	Polydispersity index	Potential zeta (mV)
S0 (day 0)	106.26	0.289	-49.8
S01 (30 days stored at 2-8°C)	100.9	0.300	-43.0
S02 (30 days stored at 25-30°C)	108.2	0.301	-51.4

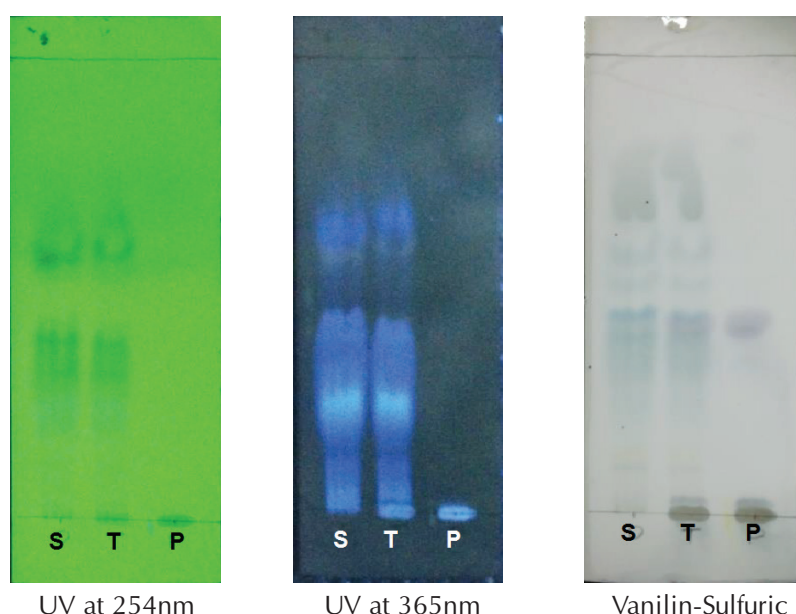


Fig. 2. Chromatograms of the standard solution (S), the test solution (T), and the placebo solution (P), stained under a UV light at 254 nm, 365 nm, and with Vanilin-Sulfuric respectively.

The particle size and the size distribution of liposomal suspension had slightly modified after 30 days of storage. The liposomal vesicles entrapping Tamanu oil were fairly stable during the storage even at temperatures between 25-30°C.

Identification of Tamanu oil into liposomes: The chromatograms (Fig. 2)

showed that each separated spot obtained from the test solution corresponds to that of the standard solution. As a result, all components of CIO were encapsulated into the liposomal vesicles.

Concluding remarks

In the present study, liposomal formulation trapping *Calophyllum*

inophyllum oil was optimized with the lipid phase comprising 65.9% (w/w) of the Phospholipon 90G: L- α -lecithin combination in the 6:4 molar ratio, 10% (w/w) of tween 80, 24.1% (w/w) of CIO. The average size of the prepared liposomes was small (mean diameter of 102.74 d.nm) and homogeneous (PDI of 0.276). The high negative charge of liposomal vesicles (-54.5 mV), and the minimal modification of particle size as well as the polydispersity index of liposomal vesicles after the storage stability studies indicated a good stability of suspension of liposomes encapsulating Tamanu oil. Interestingly, ethanol was used as a solvent dissolving the lipid phase in order to avoid the toxicity of the trace of organic solvent.

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Evaluating the systematic position of *Ehretia asperula* Zoll. & Moritz based on ITS1, *matK* and *trnL-trnF* DNA sequences

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Abstract:

Ehretia asperula, popular as a medicinal herb that has potential on cancer treatment, has a limited research about its phylogeny relationships. With many innovative advancements in molecular biology, it is more easy and reliable to identify the taxonomic position of a species by molecular markers- DNA barcode. In this study, we used three different markers, ITS1, *trnL-trnF*, and *matK*, to evaluate *E. asperula*'s systematic position. Based on ITS1 sequence, *E. asperula* belongs to clade *Ehretia* I and is a close relative of *E. resinosa*. Moreover, ITS1 was suggested to be use as a suitable DNA barcode in order to identify *E. asperula*.

Keywords: *Ehretia asperula*, ITS1, *matK*, phylogenetics, *trnL-trnF*.

Classification number: 3.5

Introduction

Ehretia asperula Zoll. & Moritz is an accepted name of a species from *Ehretia* genus (Ehretiaceae family). It was first described by Zollinger and Moritz in mid 1840s [1]. In Vietnam, it is present mainly on the mountainous area of the North [2]. Historically, *E. asperula* was used in ethnopharmacology and folklore. The ethnic minorities have been using *E. asperula* for treatments of various ailments, especially for liver diseases, such as hepatitis, liver cirrhosis and even liver cancer [2]. Besides, *E. asperula* appears to be effective in prevention of acne, jaundice, hypertension, and diabetes [2]. A research recently revealed the pharmacological potential of *E. asperula* in cancer treatment [3].

Although many patients who applied folklore procedure using *E. asperula* in combination with other medicinal herbs or with modern treatments recovered from cancer, and prolonged their life [3], no clinical trial has been published using *E. asperula* in cancer therapy. Likewise, the underlying mechanism responsible for its inhibitory effect on cancer is still unclear. Yet there is no study of *E. asperula* molecular and its phylogenetic relationship has not been reported.

The *Ehretia* genus has nearly 50 species distributed mainly in high altitude areas of Asia, Africa, and Australia [1]. In Vietnam, they represent seven species [3], in which, *E. asperula* shares many characteristics in common with other *Ehretia* species. *E. asperula* is

a climbing shrub, having bristle-covered reddish to greyish brown branches. Leaves are blade lanceolate in which the base is narrowly rounded and 5-7 mm in length, and have 7 nerves on each side. Inflorescences are terminal and lateral on short branches, and 5-10 cm in length, where individual flower's base is 2-4 mm long. Flowers have separated five calyx-lobes and white petals. Fruits are 1 cm-long globoses with 3 separate parts. Seeds have pink skin. *E. asperula* produces flowers from March to May and bears fruits from August to December [2].

The taxonomic relationship of *Ehretia* species was clarified by studies based on molecular data. On the basis of the ITS1 information, *Ehretia* was a sister group of *Bourreria* (Ehretiaceae family) and is composed of three major clades. Among them, *Ehretia* III has a closer relationship with *Ehretia* II compared to *Ehretia* I [4]. Additionally, the ITS1 secondary structure was applied to build phylogenetic trees at higher taxonomic levels, and seems to be good at giving a well-resolved tree. Recently, Gottschling, et al. [5] elucidated the phylogeny relationship of Boraginales by using concatenated ITS nuclear and plastid rps16 *trnL-trnF*, *trnS-trnG* sequences. An additional clade

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Ehretia IV, in which *E. microphylla* is a presentative, was inferred from extent analysis.

In this study, ITS1, *trnL-trnF*, and *matK* regions were used to investigate the systematic position of *E. asperula*. As inferred from the data, *E. asperula* belongs to *Ehretia* I and is a close relative of *E. resinosa*. Moreover, we contributed three sequences to DNA barcode database of *Ehretia* genus, and suggested ITS1 as an appropriate DNA barcode.

Materials and methods

Three leaf specimens of *E. asperula* were collected at three different locations in the Hoa Binh province by a colleague at Vietnam national museum of nature, and were preserved in silica gel. Genomic DNA was isolated using CTAB (Cetyltrimethylammonium bromide) extraction protocol [6] from approximately 100 g of leaf tissue. Three DNA fragments were amplified using Thermo scientific phusion high-fidelity DNA polymerase with universal primer pairs (Table 1). PCR products were purified with Thermofisher scientific PCR clean-up purification kit. These DNA were sequenced by Applied biosystems 3500 genetic analyzer system using BigDye™ terminator v3.1 cycle sequencing kit. Additionally, GenBank accessions were downloaded to complete dataset for molecular investigation comprising of 37 sequences of *Ehretia* species, and three sequences of outgroup representatives (Table 2). Sequences were aligned automatically by BioEdit v7.1.9 [7]. Phylogenetic analysis was performed by PAUP*4.0a152 [8]. Likelihood trees were built using heuristic search. Bootstrap analyses (criterion=parsimony, with full heuristic search: PBS; criterion=distance, with neighbor-joining search and maximum

Table 1. List of primer pairs used in the study.

Primer names	DNA regions	Primer sequences (5' → 3')
ITS-AB-101	ITS	ACG AAT TCA TGG TCC GGT GAA GTG TTC G
ITS-AB-102		TAG AAT TCC CCG GTT CGC TCG CCG TTA C
TrnL-F	<i>trnL-trnF</i>	ATT TGA ACT GGT GAC ACG AG
TrnL-C		CGA AAT CGG TAG ACG CTA CG
MatK-F1A	<i>matK</i>	ACY GTA TTT TAT GTT TAC GAC G
MatK-R1A		TCC ATH TDG AAA TCT TGG TTC A

Table 2. Species lists with ID number of DNA sequences on GenBank.

Name	ITS1	<i>trnL-trnF</i>	<i>matK</i>	Name	ITS1	<i>trnL-trnF</i>	<i>matK</i>
<i>E. asperula</i>	KY320205	KY320206	KY320207	<i>E. obtusifolia</i>	AY331401.1		
<i>E. acuminata</i>	AF385799.2	AY376167.1	HQ427413.1	<i>E. macrophylla</i>	AF385802.2	KF673271.1	
<i>E. amoena</i>			JX518091.1	<i>E. microphylla</i>	AY463160.1	KF158204.1	
<i>E. anacua</i>	AF385796.2		DQ197228.1	<i>E. monopyrena</i>	AF385792.2		
<i>E. aquatica</i>	AF385791.2		EU599659.1	<i>E. resinosa</i>	AY463161.1		
<i>E. cortesia</i>	AY463159.1	KF673292.1		<i>E. rigida</i>	AF385789.2		JX518014.1
<i>E. coerulea</i>	KF673249.1			<i>E. saligna</i>	AF385786.2	KF673272.1	KM894705.1
<i>E. cysmosa</i>	AF385790.2			<i>E. tinifolia</i>	AF385793.2	HQ286270.1	
<i>E. grahamii</i>			KU564569.1	<i>E. thyrsoiflora</i>		EU600007.1	EU599655.1
<i>E. laevis</i>	AF385787.2	KF673273.1		<i>E. wallichiana</i>	AY331402.1		
<i>E. latifolia</i>	AF385797.2	KF673282.1		<i>B. succulenta</i>	AF385776.2		DQ197229.1
<i>E. longiflora</i>	AY331400.1	EU600010.1	KJ687555.1	<i>B. petiolaris</i>		KF673275.1	

likelihood setting: DBS) were estimated based on 1,000 replicates, in which each was performed with 100 random-addition-sequence replicates, and the starting tree obtained by neighbor-joining.

Results

All three DNA regions (ITS, *trnL-trnF*, and *matK*) were amplified by using the universal primer pairs (Table 1), and

the products obtained were 848 bp, 950 bp, and 811 bp in length, respectively (Fig. 1). ITS is a nuclear sequence from 3' end of 18S to 5' end of 26S. However, we used a portion of ITS-ITS1 for phylogenetic calculation in this study. While *trnL-trnF* is the intergenic region between two coding regions, *matK* spans from codon 171 to codon 440 of the open reading frame. The nucleotide sequences of each DNA region of the

three samples are identical. Therefore, sequences from only one sample were used for the phylogenetic calculation. DNA sequences of all three DNA regions (ITS, *trnL-trnF*, and *matK*) were deposited on GenBank with ID number KY320205, KY320206, and KY320207, respectively.

The ITS1, *trnL-trnF*, and *matK* datasets were sorted by BioEdit software and then, re-aligned manually to be more precise. The length of these alignment datasets and the number of

DNA sequences in each dataset vary depending on the DNA regions. Despite being the shortest alignment (285 bp), ITS1 database is the largest collection (20 sequences). In contrast, alignments of *trnL-trnF* region (463 bp in length) and *matK* region (678 bp in length) have 12 and 11 sequences, respectively. Indeed, many *Ehretia* species do not comprise all three DNA sequences. Additionally, it was observed from these alignments that nuclear marker ITS1 contains more variable sites (132) compared to the two

plastid markers (*trnL-trnF* of 33 and *matK* of 34) (Fig. 2).

The best likelihood trees with bootstrap support values of all three DNA markers were indicated in Fig. 3. Based on the ITS1 region (Fig. 3A), the phylogenetic relationship of *Ehretia* genus is in agreement with the previous study [5], in which this taxon was divided into 4 main clades: *Ehretia* I (100 PBS, 100DPS), *Ehretia* II (even PBS was low 54, DBS was high 93), *Ehretia* III (96 PBS, 100 DBS), and *Ehretia* IV (BS under 50, but 100 PBS, 100 DPS). The phylogenetic trees derived from the two plastid markers differ to some degree. The species belonging to the same clade at ITS1 tree were still grouped, though with relatively low branch support. Similarly, some species were changed their phylogeny positions. For instance, *E. anacua*, which was initially subjected to *Ehretia* II clade at ITS1 tree, was separated away from other *Ehretia* II species at *matK* tree (Fig. 3C). Analysis of *matK* data also suggested the systematic relationship of three species in which ITS1 sequences were not available. *E. amoena* and *E. grahamii* were aligned with *Ehretia* I species with high bootstrap value of 92 PBS and 94 DPS. Meanwhile, *E. thyriflora* relates to *E. acuminata* with low supported bootstrap in *matK* tree, but with high supported bootstrap in *trnL-trnF* tree (Fig. 3B). In the phylogenetic tree constructed from ITS1 data, which have a significant amount of data so far, *E. resiona* is a close relative of *E. asperula* even with low bootstrap support (54PBS, 69DBS).

Discussions

The molecular phylogenetics has been resolving the evolutionary relationship between related species over the few past decades [9]. Many regions in plant nuclear and plastid genome have been assessed and evaluated, such as ITS, *trnL-trnF*, *matK*, and *rbcL* [10]. ITS from the nuclear ribosomal DNA is obviously the only nuclear region and a proper choice for phylogeny analysis. It

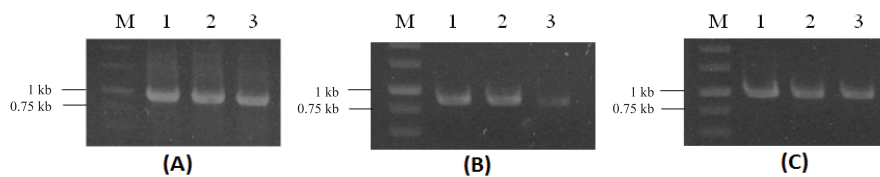


Fig. 1. PCR results of three DNA regions: ITS (A), *trnL-trnF* (B), and *matK* (C). M: 1 kb marker; 1-3: PCR products of three different *E. asperula* DNA samples.

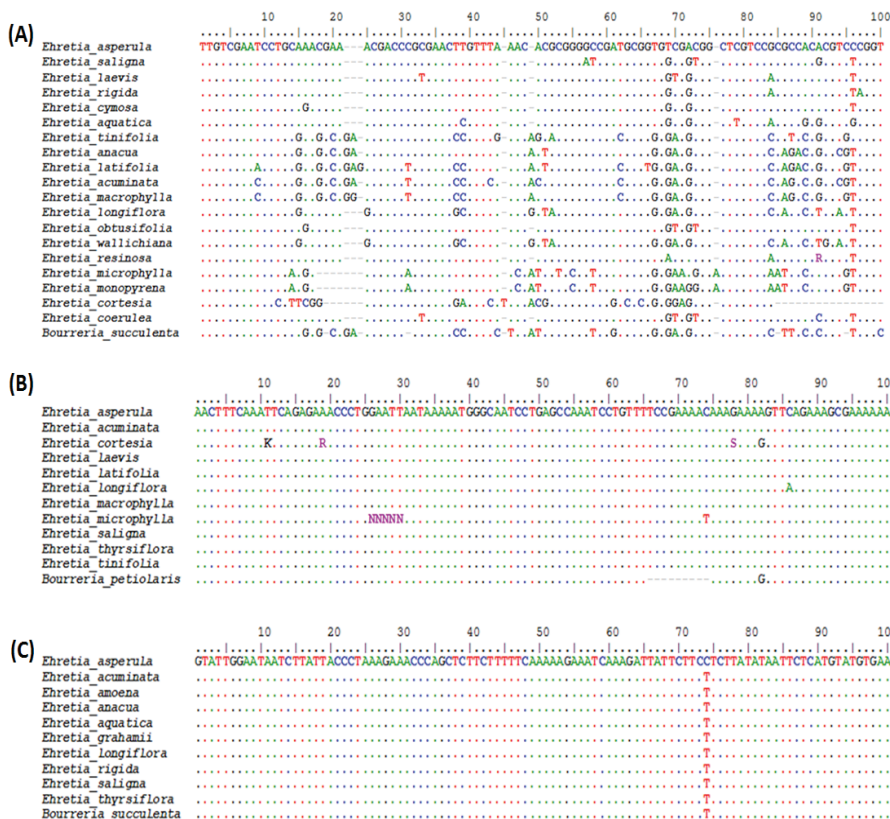


Fig. 2. Alignments (partial) of *E. asperula* with other *Ehretia* species and an outgroup species of three DNA regions: ITS (A), *trnL-trnF* (B), and *matK* (C).

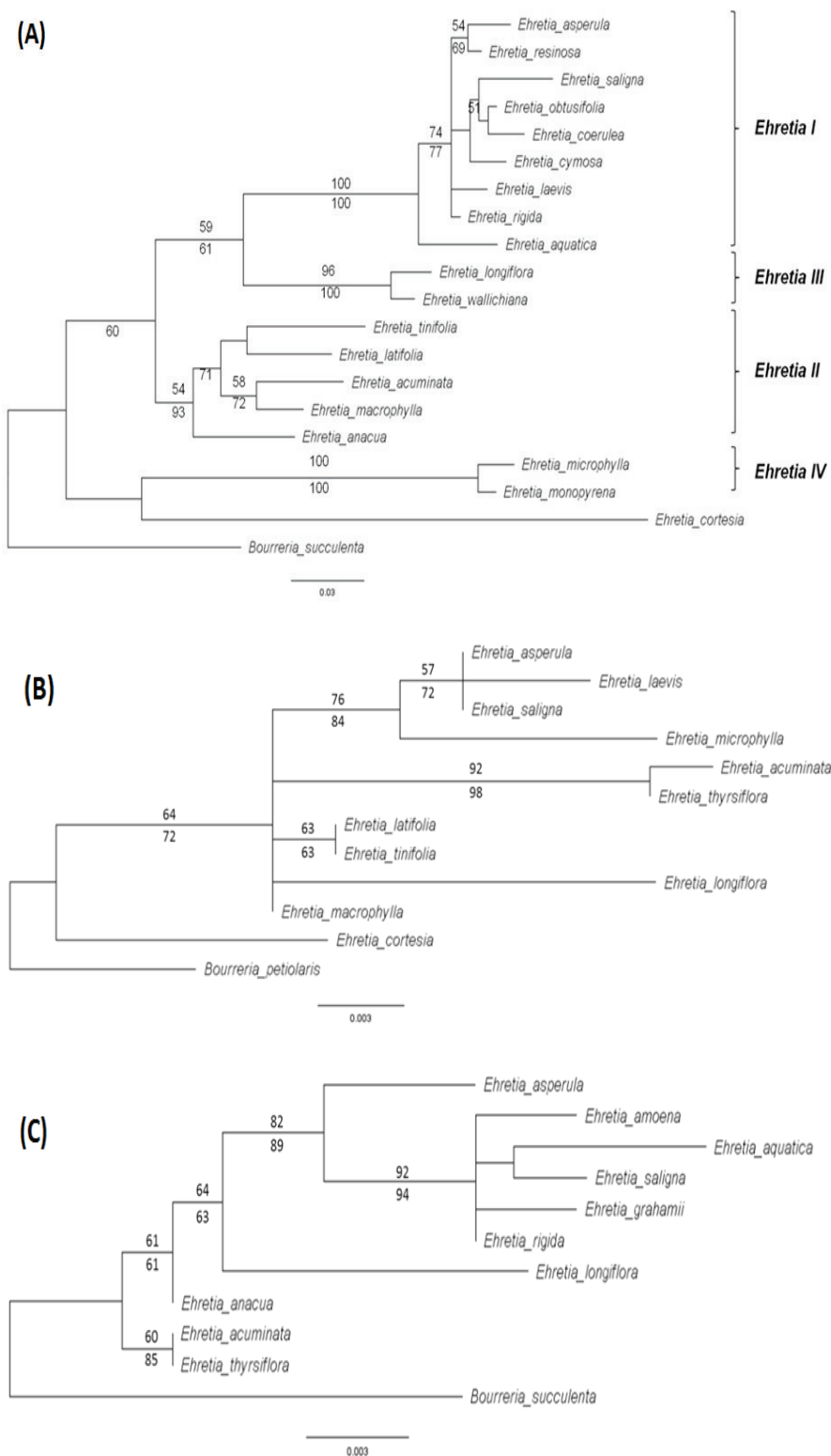


Fig. 3. Maximum likelihood trees of *Ehretia* based on ITS1 (A), *trnL-trnF* (B), and *matK* (C) sequences (-ln = 1733.905, 819.1461 and 1163.316 respectively). Branch lengths are to scale. Major clades at ITS1 tree are indicated. Number on the branches are bootstrap support values, where values under 50% are not shown (above: parsimony, below: distance).

has greater species discrimination than other plastid regions at lower taxonomic levels and has efficiency even at intraspecies levels [10]. In fact, scientists sometimes use a portion of ITS, ITS1, or ITS2 to calculate the distance between species [11]. On the other hand, plastid genome contributes many candidates for phylogeny calculation. They have been used by means of separated sequence or in combination with others [10]. However, it is difficult to pick a combination that meets all requirements: good discriminatory power, good sequence quality, and universality. With regard to the Ehretiaceae family, both primary and secondary structures of ITS1 region were adopted successfully [4, 12, 13]. Similarly, *trnL-trnF* intergenic sequence can discriminate species when being combined with other plastid or nuclear DNA markers [14, 15]. On the other hand, *matK* has never been seen in any phylogeny study of the Boraginales order.

In our study, we used ITS1 to figure out the systematic position of *E. asperula* since ITS1 was analyzed in most researches about Ehretiaceae in general and *Ehretia* species in specific. Our analysis of ITS1 divided the *Ehretia* genus into 4 clades, which were numbered according to a previous report [4]. However, the position of a clade in relation to others differs from the previous studies due to the difference in the kind of data input. Yet, this result was acceptable for our purpose and discussion. Besides ITS1, two other plastid DNA markers were applied to investigate their efficiency on species discrimination. As expected, ITS1 could distinguish efficiently all the 20 species (Fig. 3A). In contrast, as it can be seen in Fig. 1C, *matK* could not differentiate between *E. acuminata* and *E. thyrsoiflora*. A similar situation was observed at *trnL-trnF* maximum likelihood tree (Fig. 3B). Perhaps, *trnL-trnF* and *matK* are effective when dealing with higher taxon level or in combination with other plastid and/or nuclear markers. Inferred from the ITS1 maximum likelihood tree (Fig. 3A), *E. asperula* and *E. resinosa*

are sister species even though the molecular data provides low bootstrap support (54PBS, 69DBS).

Based on three phylogenetic trees, *E. asperula* was grouped with *Ehretia* I species, which also have been used for medical purposes for a long time. For instances, *E. saligna*'s decoction of wood is drunk for aches and pain [16]. Moreover, the paste of the *E. laevis* leaves has remarkably wound healing properties [17]. Furthermore, *E. cymosa* leaves were used to treat toothaches [18] and stomach ulcers [19]. In order to treat many ailments, dried leaves and wood of *E. asperula* were normally added in hot water, and sometimes with other Vietnamese herbs [2].

Many cancer patients in Vietnam cannot afford to get some expensive treatments, such as surgery, radiation therapy, and chemotherapy. Furthermore, these treatments are usually coupled with unwanted side effects [20]. Traditional medicine, which uses many kinds of Vietnamese herbs, offers an alternative potential cost-effective and harmless treatment. It is likely to lead to a high demand for traditional medical herb, in general, and *E. asperula*, in specific, than ever before. Even though scientists utilized tissue culture for the massive production of *E. asperula* [21], most of this herb in medical plant market was exploited from its limited natural habitat. This paradoxical situation brings many concerns as *E. asperula* market is expanding. The concerns are outright substitution, contamination, and adulteration with some non-effective, less effective, or even some allergic, lethal herbs, and mislabeling fillers [22]. Initially, the conventional procedures to identify a plant are morphological and anatomical methods, which are not always successful for some reasons. Fortunately, advance in molecular technology has offered researchers a simple, cost-effective, and rapid approach to species identification based on DNA sequences, and DNA barcoding [22]. According to this study, we suggest that the ITS1 region is a suitable DNA

barcode for the classification of *Ehretia* genus and the identification of *E. asperula*.

Conclusions

In short, we sequenced three DNA markers (ITS, *trnL-trnF*, and *matK*) of *E. asperula* and deposited these sequences on the Genbank with ID number KY320205, KY320206, and KY320207, respectively. *E. asperula*'s systematic position was evaluated by three phylogenetic trees. It is concluded that *E. asperula* belongs to *Ehretia* I clade and has a close relation with *E. resinosa*. Additionally, we presented ITS1 as a potential DNA barcode for identification, which could be further assessed to monitor the non-authentic medical herb market.

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A simple, efficient and universal method for the extraction of genomic DNA from bacteria, yeasts, molds and microalgae suitable for PCR-based applications

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Abstract

The extraction of genomic DNA from microbial cells plays a significant role in PCR-based applications such as molecular diagnosis, microbial taxonomy, screening of genetically engineered microorganisms, and other such PCR-based applications. Currently, many methods for extraction of genomic DNA from microorganisms have been developed. However, these methods either require hazardous chemicals or consist of time-consuming steps for effective execution. In this study, we have established a simple and universal genomic DNA extraction method for different microorganisms including bacteria, yeasts, molds, and microalgae. Our method does not require harmful reagents such as phenol and chloroform for the extraction process to minimize the generation of hazardous wastes. The obtained genomic DNA products displayed high concentrations and represented a good purity level with the average 260 nm/280 nm absorbance ratios ($A_{260/280}$) that range from 1.6 to 2.0. The DNA molecules further remained considerably intact when analyzed on agarose gels. More importantly, these DNA products were qualified through successful PCR amplifications of 16S rRNA gene, rDNA internal transcribed spacer (ITS), or 18S rRNA gene from genomes of bacteria, fungi, and microalgae respectively. Furthermore, with the extracted genomic DNA products, the processes of the identification of the haploid and diploid states of the *Saccharomyces* yeast strains or detection of putative strains of *Aspergillus oryzae* and *Aspergillus flavus* that have been isolated from infected food materials through PCR analyses are facilitated. The genomic DNA extraction method established in this study is easy to manage, time saving and cost-effective, and environmentally friendly.

Keywords: bacteria, microalgae, molds, PCR, simple genomic DNA extraction, yeasts.

Classification number: 3.5

Introduction

Across natural processes, microorganisms play important roles in nutritional cycles that are involved in the maintenance of the balance in ecological systems. In the context of applied microbiology, numerous microbial species are utilized for the production of foods, beverages, drugs,

biofertilizers, or for the applications of environmental pollution treatment [1, 2]. The accurate identification of these microorganisms for specific purposes is usually performed on the basis of barcode ribosomal DNA sequences that include bacterial 16S rRNA, fungal ITS (internal transcribed spacer) region, and microalgal 18S rRNA

[3-6]. Furthermore, the selected useful microorganisms can be subjected to further genetic improvement to enhance beneficial traits [1, 2, 7]. Consequently, the development of efficient genomic DNA extraction methods with respect to different microbial species is always considered a central step in PCR-based molecular biology applications that include molecular taxonomy, molecular diagnosis, recombinant DNA cloning studies, etc. These DNA extraction methods were developed according to either chemical reagents or commercial kits [8-12]. However, commercial kits employed for microbial genomic DNA extraction are expensive for large-scale screening experiments in laboratories, while conventional genomic DNA extraction methods are usually developed for a specific microbial group or require hazardous reagents such as phenol and chloroform for the cleanup step [8]. In this study, we have successfully established a simple and universal method for the rapid extraction of genomic DNA from different microbial species including bacteria, yeasts, molds, and microalgae. The extracted genomic DNA samples displayed superior quality and were determined as suitable for specific PCR-based applications.

Materials and methods

Microbial strains and cultivation conditions

All of the microbial strains and PCR primers are listed in Table 1 and Table 2

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Table 1. Microbial strains used in this study.

Name	Description	Source
<i>Escherichia coli</i> DH5 α	The laboratory Gram-negative bacterial strain	Our collection
<i>Agrobacterium tumefaciens</i> AGL1	The laboratory Gram-negative bacterial strain employed for genetic transformation of plants and fungi	Our collection
<i>Burkholderia vietnamiensis</i> LU4.4	A Gram-negative bacterial strain isolated from rice rhizosphere displaying antifungal activity	Our collection
<i>Lactobacillus fermentum</i> H7	A Gram-positive lactic acid bacterial strain isolated from a fermented pickle	Our collection
<i>Bacillus subtilis</i> PY79	The laboratory Gram-positive bacterial strain	Our collection
<i>Saccharomyces cerevisiae</i> BY4741	The laboratory haploid yeast strain (<i>MATa</i>)	Euroscarf
<i>Saccharomyces cerevisiae</i> BY4742	The laboratory haploid yeast strain (<i>MATα</i>)	Euroscarf
<i>Saccharomyces cerevisiae</i> BY4743	The laboratory diploid yeast strain (<i>MATa/MATα</i>)	Euroscarf
<i>Saccharomyces boulardii</i> NOM	A probiotic yeast strain isolated from the commercial product Normagut (Germany)	Our collection
<i>Saccharomyces boulardii</i> PE	A probiotic yeast strain isolated from the commercial product Perenterol (Germany)	Our collection
<i>Saccharomyces boulardii</i> BIO	A probiotic yeast strain isolated from the commercial product Bioflora (France)	Our collection
<i>Candida albicans</i> JCM2070	An opportunistic yeast-causing candidiasis in human	JCM, Japan
<i>Candida glabrata</i> RN4	A yeast strain of <i>Candida glabrata</i> isolated from a fermented sticky rice product	Our collection
<i>Pichia anomala</i> BMH9	A yeast strain isolated from a traditional yeast cake	Our collection
<i>Hanseniaspora thailandica</i> Y39	A yeast strain isolated from the peel of a red apple fruit	Our collection
<i>Aspergillus oryzae</i> RIB40	The laboratory strain used for the research of food production	Our collection
<i>Aspergillus flavus</i> NRRL3357	The laboratory strain used for the research of mycotoxin biosynthesis	Our collection
<i>Aspergillus niger</i> N402	The laboratory strain used for the research of production of enzymes and organic acids	Our collection
<i>Penicillium chrysogenum</i> VTCC-F1172	A fungal strain used for the research of penicillin production	VTCC, Vietnam
<i>Magnaporthe oryzae</i> MN1	A fungal pathogen that causes the rice blast disease isolated in Southern Vietnam	Our collection
<i>Aspergillus</i> sp. A1	The fungal strains isolated from mold-infected rice seeds in Hanoi	Our collection
<i>Aspergillus</i> sp. A2		
<i>Aspergillus</i> sp. A3		
<i>Aspergillus</i> sp. A4		
<i>Aspergillus</i> sp. A5		
<i>Chlorella</i> sp. PT01	A freshwater microalgal strain	VTCC, Vietnam
<i>Chlorella</i> sp. PT02	A marine microalgal strain	VTCC, Vietnam

respectively.

Four bacterial species that include *Escherichia coli*, *Bacillus subtilis*, *Agrobacterium tumefaciens*, and *Burkholderia vietnamiensis* were grown in the LB medium (1% peptone, 0.5% yeast extract, 0.5% NaCl). One lactic acid bacterium *Lactobacillus fermentum* was cultivated in the MRS medium (1% sucrose, 1% peptone, 1% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4$, 0.5% CH_3COONa , 0.2% K_2HPO_4 , 0.2% NaH_2PO_4 , 0.5% $CaCO_3$, 0.1% Tween 80, pH 6.5).

Six yeast species that include *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Candida albicans*, *Candida glabrata*, *Pichia anomala*, and *Hanseniaspora thailandica* were cultivated in the YPG medium (1% yeast extract, 1% peptone, 2% glucose, 1.8% agar). A single colony of each microbial strain (Table 1) was grown in a conical flask that contained 10 ml of a suitable medium at 30°C, 200 rpm until the OD_{600} value reached 1.5-2.0 and the respective cell biomass was then harvested.

Five different mold species including *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Magnaporthe oryzae*, and five putative strains of *A. oryzae* and *A. flavus* that were isolated from mold-infected rice seeds and mold-infected peanut seeds (Table 1) were cultivated in the potato dextrose medium (Himedia, India) or Czapek-Dox medium (comprising 3% sucrose, 0.3% $NaNO_3$, 0.1% KH_2PO_4 , 0.05% $MgSO_4$, 0.05% KCl, 0.001% $FeSO_4$) at 30°C for 3-7 days.

Two microalgal strains *Chlorella* sp. PT01 and PT02 were cultivated in 100 ml conical flasks that contained 50 ml BBM (Bold's Base medium) [13]. The flasks were incubated at room temperature under white light of 2,000 lux intensity, subjected to a lighting cycle of 12 h/12 h (light/dark).

Preparation of the extraction buffer

This genomic DNA extraction protocol requires only a unique extraction buffer referred to as the

GX buffer (2.5% SDS, 200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.2% β -mercaptoethanol). The buffer composition was adapted from certain published reports [8, 10, 14-17]. It is observed that the stock solutions, including 1 M Tris-HCl (pH 8.0), 0.25 M EDTA (pH 8.0), 2.5 M NaCl, can be autoclaved and stored at room temperature for subsequent use. Further, SDS (sodium dodecyl sulfate) should be added to the buffer after the other components. This buffer provided better results for genomic DNA extraction when freshly prepared. Alternatively, the ready extraction buffer can also be stored in the dark at room temperature for 2-3 weeks, and it requires to be heated at 60°C for 10 min before its application.

Genomic DNA extraction

The genomic DNA extraction method was adapted from some previously published protocols for fungi [7, 10, 14]

with suitable modifications for each microorganism employed in this study.

For bacterial cells, the following procedure was performed: 2 ml of each bacterial culture with the OD_{600} values of 1.5-2.0 was centrifuged at 12,000 rpm for 1 min to harvest the cells. The cell pellet was resuspended in 70 μ l TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)] and the tube was strongly vortexed for 15 s. Subsequently, 30 μ l of lysozyme (10 mg/ml) was added to the tube. The resultant mixture was incubated at room temperature for 10 min. In the subsequent step, 600 μ l of GX buffer and 3 μ l proteinase K (20 mg/ml) were added to the tube. The tube was gently vortexed for 15 s and incubated at 60°C for 30 min. To achieve neutralization, 300 μ l of a 3 M sodium acetate solution (pH 5.2) was added to the tube. The supernatant phase (600-700 μ l) obtained from a

Table 2. Primers used in this study.

Name	Sequence (5'-3')	Target sequence	Reference
16SfD1 16SrP1	AGAGTTTGATCCTGGCTCAG ACGGTTACCTTGTTACGA	Bacterial 16S rRNA gene	Weisburg, et al. (1991) [4]
ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Fungal rDNA ITS	White, et al. (1990) [6]
18S1 18S12	TACCTGGTTGATCCTGCCAG CCTTCCGCAGGTTACCTAC	Microalgal 18S rRNA gene	Honda, et al. (1999) [6]
ScMAT ScMATa ScMAT α	AGTCACATCAAGATCGTTTATGG ACTCCACTTCAAGTAAGAGTTTG GCACGGAATATGGGACTACTTCG	<i>Saccharomyces</i> mating-type genes <i>MATa</i> , <i>MATα</i>	Illuxley, et al. (1990) [18]
AO-ITS- uni-F	ATGGCCGCCGGGGGCTCT	Specific to the rDNA ITS1 of <i>A. oryzae</i> and <i>A.</i> <i>flavus</i>	Chiba, et al. (2013) [19]
AFB-F AFB-R	AAGCAAACCAAGACCAACAAG AACAAGTCTTTTCTGGGTCTA	Specific to aflatoxin biosynthesis gene cluster in <i>A.</i> <i>flavus</i>	Chiba, et al. (2013) [19]

centrifugation at 12,000 rpm, 4°C for 20 min was transferred to a new 1.5 ml microcentrifuge tube. The genomic DNA was precipitated with 700 µl of cold isopropanol before it was subjected to centrifugation at 12,000 rpm, 4°C for 20 min. The obtained pellet was washed with 500 µl of 70% ethanol and recollected by centrifugation. The DNA pellet was subsequently dried in a SpeedVac machine (Thermo Scientific, USA) and dissolved in 50 µl of TE buffer. This genomic DNA product was treated with 3 µl of RNase A (10 mg/ml) at 60°C for 30 min for the removal of RNA and stored at -20°C for ensuing applications.

For yeasts and microalgae, the following processes were performed: Yeast cells were collected from 2 ml of each culture obtained through a centrifugation at 4,000 rpm for 5 min, while microalgal cells were harvested at 8,000 rpm for 15 min. To break the cells, 600 µl of GX buffer and 150 mg of 0.1 mm diameter glass beads (Carl Roth, Germany) were added to the tube. The tube was strongly vortexed for 30 s and subsequently added with 3 µl of proteinase K (20 mg/ml). Subsequently, the tube was incubated at a temperature of 60°C for 30 min. The remaining steps of the extraction procedure were performed as those described above for the extraction of genomic DNA from bacteria.

For molds: 1 ml of each fungal spore suspension (10^6 spores/ml) was added to a 250 ml conical flask containing 100 ml of potato dextrose broth or Czapek-Dox liquid. The flask was subjected to a shaking incubator at 200 rpm, at a temperature of 30°C for 3 days. Fungal mycelium was collected by filtration through Miracloth (Calbiochem, Germany), and 200 mg of the obtained biomass was distributed to a 2 ml microcentrifuge tube. The fungal biomass was crushed directly in the

tube for 1 min using a clean glass rod. Subsequently, 600 µl of GX buffer and 3 µl of proteinase K (20 mg/ml) were added to the tube. The tube was vortexed for 15 s and incubated at 60°C for 30 min. The next steps of the extraction procedure were performed as described above for the extraction of genomic DNA from bacteria.

Analysis of the extracted genomic DNA products

The genomic DNA products were analyzed on 0.7% agarose gels through electrophoresis and the DNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Scientific, USA) for the 260/280 nm absorbance ratios ($A_{260/280}$).

Verification of genomic DNA quality by PCR

All genomic DNA products were diluted to the concentration of 100 ng/µl as DNA template for PCR. Taq DNA polymerase as GoTaq® Green MasterMix (Promega, USA) was utilized for all PCR amplifications in accordance to the manufacturer's instruction. The universal primer pairs including 16SfD1/16SrP1 [4], ITS1/ITS4 [6], and 18S1/18S12 [5] (Table 2) were employed for specific amplifications of bacterial 16S rRNA gene, fungal rDNA ITS, and microalgal 18S rRNA gene, respectively. The thermal cycling parameters were determined as follows: 94°C (6 min); 30 cycles of 94°C (30 s), 58°C (30 s), 72°C (40 s to 1.5 min); 72°C (10 min); 4°C (∞). The obtained PCR products were analyzed on 0.7% agarose gels and visualized under UV light of the Gel Doc XR System (Bio-Rad, USA).

Determination of haploid and diploid states in *Saccharomyces* yeast strains: Three strains of the baker's yeast *S. cerevisiae*, including BY4741 (haploid, *MATa*), BY4742 (haploid, *MATa*), BY4743 (diploid, *MATa/MATa*),

and three strains of the commercial probiotic *S. boulardii*, including NOM, PE, BIO (Table 1) were cultivated in the YPG liquid medium for genomic DNA extraction. The yeast ploidy states were determined through the PCR by employing the primer pairs ScMAT/ScMATa and ScMAT/ScMATa (Table 2) that are known to specifically amplify the mating-type genes *MATa* and *MATa* respectively [18]. The thermal cycling parameters are as follows: 94°C (6 min); 30 cycles of 94°C (30 s), 58°C (30 s), 72°C (30 s); 72°C (10 min); 4°C (∞). Each yeast strain was examined separately for the genes *MATa* and *MATa*. Thereafter, the obtained PCR products were mixed together for a comparative analysis on a 0.7% agarose gel.

Detection of *A. oryzae* and *A. flavus* strains by singleplex and multiplex PCR:

The genomic DNA samples extracted from the fungal isolates including *A. oryzae* RIB40, *A. flavus* NRRL3357, and *Aspergillus* sp. (A1, A2, A3, A4, A5) were employed for singleplex PCR using five different primers in pairs that include ITS1/ITS4, AO-ITS-uni-F/ITS4, and AFB-F/AFB-R (Table 2). The universal primer pair ITS1/ITS4 is widely employed the amplification of the ITS region of rDNA in fungi [6], whereas the primer pair AO-ITS-uni-F/ITS4 was designed for specific amplification of the rDNA ITS in *A. flavus* and *A. oryzae* [19]. The primer pair AFB-F/AFB-R was designed to amplify the specific sequence located between *aflR* and *aflJ* of the aflatoxin biosynthesis gene cluster in *A. flavus* [19]. For multiplex PCR, five primers were applied simultaneously in a single reaction with the thermal cycling parameters as follows: 94°C (6 min); 30 cycles of 94°C (30 s), 60°C (30 s), 72°C (1.5 min); 72°C (10 min); 4°C (∞). Two standard strains *A. oryzae* RIB40 and *A. flavus* NRRL3357 were employed as the reference controls. The obtained PCR products were analyzed on 1.2% agarose

gels.

Results and discussions

Establishment of a universal genomic DNA extraction method for different microorganisms

In this study, a unique procedure has been established for the extraction of genomic DNA from several microbial species including bacteria, yeasts, molds, and microalgae. Only the first step of the microbial biomass treatment is specific for each cell type. For lysis of bacterial cells, lysozyme was utilized to break down the peptidoglycan layer of the bacterial cell wall. Since this enzyme works more effectively in the presence of EDTA [20, 21], the bacterial cells in our procedure were treated with lysozyme in the TE (Tris-EDTA) buffer. The cells of yeasts and microalgae were broken mechanically in the extraction buffer (GX buffer) with glass beads, while the mycelia of the molds were crushed by hand with a glass rod. The overview of the genomic DNA extraction procedure is illustrated in Fig. 1.

The results revealed that the established method worked effectively for both Gram-positive and Gram-negative bacteria, including *Escherichia coli*, *Agrobacterium tumefaciens*, *Burkholderia vietnamiensis*, *Lactobacillus fermentum*, and *Bacillus subtilis* (Fig. 2A). To test the efficacy of this method for yeasts, five different yeast species including *Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata*, *Pichia anomala* and *Hanseniaspora thailandica* were employed. Since the yeast cell wall is easily disrupted with glass beads through the process of vortexing [22], we added glass beads with a diameter of 0.1 mm and GX buffer to a 2 ml microcentrifuge tube containing the yeast biomass, and subsequently, the tube was vortexed strongly to break the cells. Following the subsequent steps for the genomic DNA extraction (Fig. 1), the results indicated

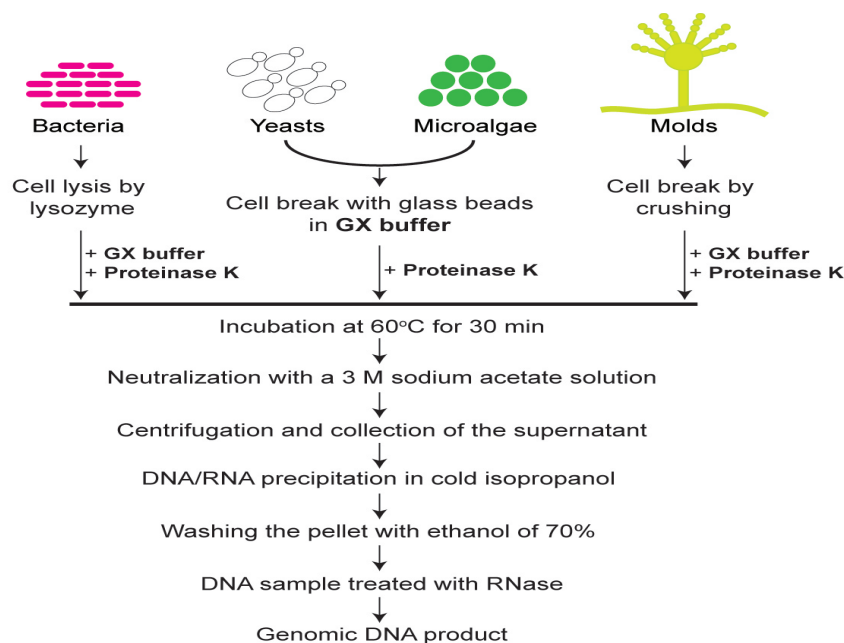


Fig. 1. The universal procedure of genomic DNA extraction for different microorganisms.

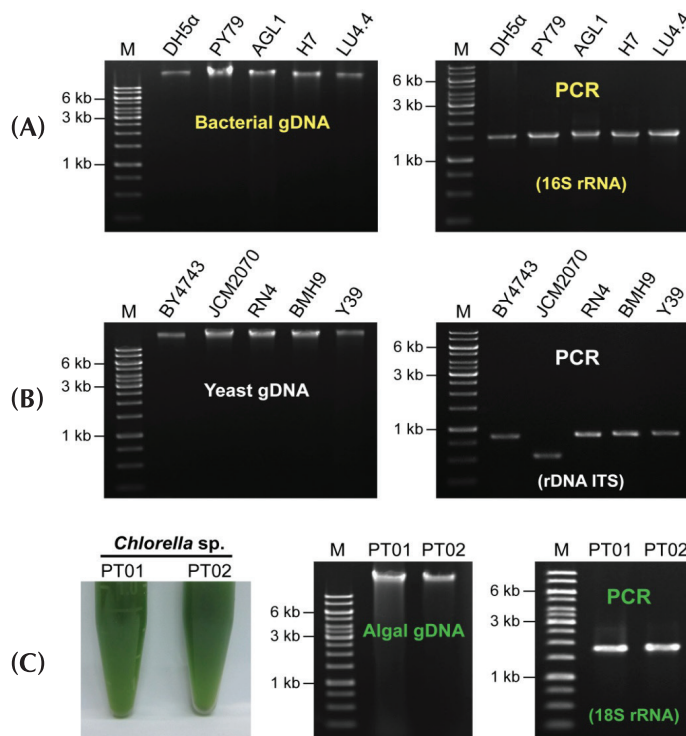


Fig. 2. Extraction of genomic DNA from bacteria, yeasts and microalgae. (A) The genomic DNA (gDNA) products extracted from five bacteria and the PCR products of the 16S rRNA genes on agarose gels. (B) The genomic DNA samples extracted from five yeasts and the PCR products of the rDNA ITS on agarose gels. (C) The analysis of the genomic DNA products extracted from two microalgae and the respective PCR products of the 18S rRNA genes on agarose gels.

that the genomic DNA products extracted from the yeasts as well as from the bacteria displayed sharp bands with lesser amounts of smearing of DNA on agarose gels (Fig. 2A, B). Particularly, these DNA products exhibited high concentrations that ranged from 753 to 6,059 ng/μl and superior purity with the $A_{260/280}$ values ranging from 1.81 to 2.02 (Table 3). When the same procedure as that for yeasts was applied to the microalgal strains including *Chlorella* sp. PT01 and PT02, the results revealed that this method also worked suitably for these green microalgae (Fig. 2C). In comparison to the bacteria and yeasts, the genomic DNA products extracted from the microalgae exhibited lower concentrations (99-177 ng/μl) with the $A_{260/280}$ values ranging from 1.57 to 1.87 (Table 3). More importantly, all the extracted genomic DNA products could be employed productively as the DNA template for PCR amplifications of the bacterial 16S rRNA gene, the yeast rDNA ITS sequence or microalgal 18S rRNA gene using the respective primer pair (Fig. 2, Table 2).

For genomic DNA extraction from molds, we crushed fungal biomass directly in a 2 ml microcentrifuge tube with a glass rod (Fig. 1). Five mold species including *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum* and *Magnaporthe oryzae* (Fig. 3A, Table 1) were utilized to test this procedure. The obtained genomic DNA products were superior in quality with the $A_{260/280}$ values ranging from 1.86 to 1.96 and high DNA concentrations of 1,466-6,528 ng/μl (Fig. 3B, Table 3). It is worth mentioning that the crushing of fungal cells in the tubes with a clean glass rod facilitates the prevention of cross-contamination among fungal samples and reduces the cost when compared to the grinding of the fungal biomass in liquid nitrogen using a mortar and a pestle. The obtained fungal genomic DNA products were evaluated for quality by PCR. The universal primer pair ITS1/ITS4 (Table 2) was utilized for

Table 3. The concentration and purity of the extracted genomic DNA products.

Microbial species	DNA concentration (ng/μl)	$A_{260/280}$
Bacteria		
<i>Escherichia coli</i> DH5α	1,183 ± 203	1.81
<i>Bacillus subtilis</i> PY79	2,288 ± 139	1.92
<i>Agrobacterium tumefaciens</i> AGL1	859 ± 170	1.85
<i>Lactobacillus fermentum</i> H7	753 ± 208	1.90
<i>Burkholderia vietnamiensis</i> LU4.4	1,135 ± 52	1.92
Yeasts		
<i>Saccharomyces cerevisiae</i> BY4743	858 ± 61	1.94
<i>Candida albicans</i> JCM2070	6,056 ± 55	1.98
<i>Candida glabrata</i> RN4	2,701 ± 239	1.84
<i>Pichia anomala</i> BMH9	1,762 ± 276	2.02
<i>Hanseniaspora thailandica</i> Y39	2,341 ± 38	1.95
Molds		
<i>Aspergillus oryzae</i> RIB40	4,766 ± 91	1.87
<i>Aspergillus flavus</i> NRRL3357	2,669 ± 291	1.96
<i>Aspergillus niger</i> N402	6,177 ± 543	1.89
<i>Penicillium chrysogenum</i> VTCC-F1172	6,528 ± 711	1.86
<i>Magnaporthe oryzae</i> MN1	1,466 ± 104	1.90
Microalgae		
<i>Chlorella</i> sp. PT01	177 ± 12	1.87
<i>Chlorella</i> sp. PT02	99 ± 23	1.57

amplification of the ITS region of fungal rDNA. The results indicated that the ITS region was successfully amplified from the genomes of all five fungal species (Fig. 3C).

Although the genomic DNA extraction method established in this study works well for numerous microbial species, it does not always work suitably for all microorganisms. In fact, we tested this method for the Gram-positive pathogenic bacterium *Staphylococcus aureus*, but no DNA bands appeared on the agarose gel

(data not shown). The reason behind this is that the cell wall of *S. aureus* is highly resistant to the digestion of lysozyme [23]. Additionally, we tested this method for some other fungal species. It also worked rather well for the citrus postharvest pathogen *Penicillium digitatum*, the antagonistic fungus *Trichoderma asperellum*, and the opportunistic human pathogenic fungus *Aspergillus fumigatus*. However, this method did not prove to work efficiently for the extraction of genomic DNA from the model filamentous

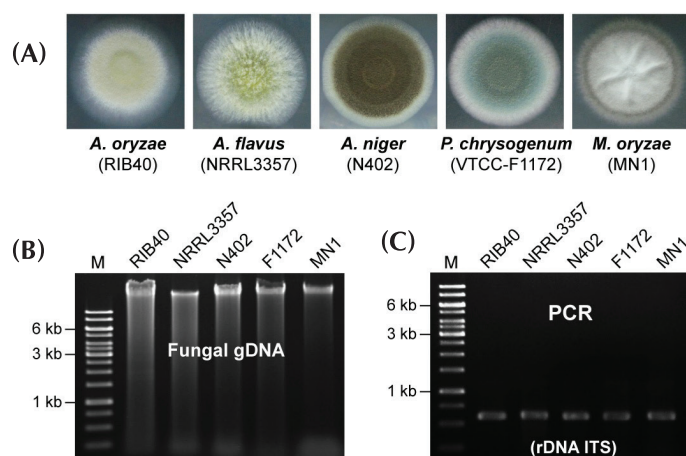


Fig. 3. Extraction of genomic DNA from different molds. (A) The morphology of the tested molds on the PDA medium at 30°C for 3-7 days. (B) The extracted genomic DNA (gDNA) products on a 0.7% agarose gel. (C) Analysis of the PCR products of the ITS on a 0.7% agarose gel.

fungus *Aspergillus nidulans*, the plant pathogen *Curvularia lunata*, and the medicinal mushroom *Cordyceps militaris*, although the obtained DNA products were still functional for successful PCR amplifications (data not shown). Therefore, this method requires to be improved for certain specific microorganisms.

Simple identification of haploid and diploid states in *Saccharomyces* yeast strains by PCR

The baker's yeast *S. cerevisiae* can exist as diploid strains that possess *MATa* and *MATα* mating-type genes or haploid strains that carry only *MATa* or *MATα* gene [24]. The probiotic yeast *S. boulardii* is employed commonly for the treatment of antibiotic-associated diarrhea caused by *Clostridium difficile* infection in human. This probiotic yeast and *S. cerevisiae* share almost identical genomes [25]. In this study, we demonstrated that the ploidy states of three *S. boulardii* strains that were isolated from the commercial probiotic yeast products (Table 1) could be rapidly identified through PCR amplifications. Three standard *S. cerevisiae* strains, including BY4741 (haploid, *MATa*), BY4742 (haploid, *MATα*) and BY4743 (diploid, *MATa/MATα*), were adopted as controls and three *S. boulardii* isolates

named NOM, PE, BIO were cultivated in the YPG liquid medium for genomic DNA extraction adhering to the above established method. The genomic DNA products extracted from all six yeast strains displayed high quality as indicated on an agarose gel (Fig. 4A). The extracted DNA products were utilized as the template for PCR with the specific primer pairs (Table 2); and further, the obtained data indicated that the haploid strains BY4741 and BY4742 possess either *MATa* (544 bp) or *MATα* (404 bp) gene respectively. Conversely, the diploid strain BY4743 carries both *MATa* and *MATα* genes (Fig. 4B). These results are consistent with the results previously reported [26]. Interestingly, all three probiotic strains (NOM, PE, BIO) of *S. boulardii* exist as diploids that carry both the mating-type genes *MATa* (544 bp) or *MATα* (404 bp) like the diploid strain BY4743 of *S. cerevisiae* (Fig. 4B).

Quick detection of *Aspergillus oryzae* and *Aspergillus flavus* strains by PCR

A. oryzae and *A. flavus* play significant roles in the food industry and food safety. *A. oryzae* has been commonly employed for the industrial production of soy sauce, miso, sake, soybean sauce paste in Asian countries,

while *A. flavus* produces the carcinogenic aflatoxins. Since these fungal species are extremely closely related to each other and share similar morphology and genome homology amounting to 99.5%, their recognition is easily confused [2, 27].

In this study, we cultured five isolates *Aspergillus* sp. (A1, A2, A3, A4, A5) that share similar phenotypes of *A. oryzae* and *A. flavus* for genomic DNA extraction. The extracted genomic DNA products were good in quality displaying sharp bands on the agarose gel (Fig. 4C). With the utilization of singleplex PCR with the universal primer pair ITS1/ITS4, we amplified successfully the ITS region of rDNA with the same size of 595 bp from the genomes of all five *Aspergillus* sp. isolates, as well as from the genomes of the standard strains *A. oryzae* RIB40 and *A. flavus* NRRL3357. For the specific amplifications of the ITS region from *A. oryzae* and *A. flavus*, the primer pair AO-ITS-uni-F/ITS4 was utilized. The primer AO-ITS-uni-F was designed to bind only to the ITS1 sequence of *A. oryzae* and *A. flavus* [19]. The PCR with this primer pair resulted in a DNA band of 486 bp for all tested strains that include the reference strains *A. oryzae* RIB40 and *A. flavus* NRRL3357. To discriminate between *A. oryzae* and *A. flavus*, the primer pair AFB-F/AFB-R that specifically binds to the aflatoxin biosynthesis gene cluster of *A. flavus* was utilized [19]. With this PCR, only a DNA band of 116 bp appeared for the *A. flavus* strains (Fig. 4D). From the obtained results, we suggested that the strains A1, A2, A4, A5 belong to *A. flavus* and A3 is *A. oryzae*. Furthermore, these results were additionally confirmed through the performance of multiplex PCR in which all five primers (ITS1, AO-ITS-uni-F, ITS4, AFB-F, AFB-R) were combined in a single reaction. The multiplex PCR resulted in three bands (116 bp, 486 bp, 595 bp) for the *A. flavus* strains (NRRL3357, A1, A2, A4, A5) and only two bands (486 bp, 595 bp) for the *A. oryzae* strains (RIB40, A3) on an

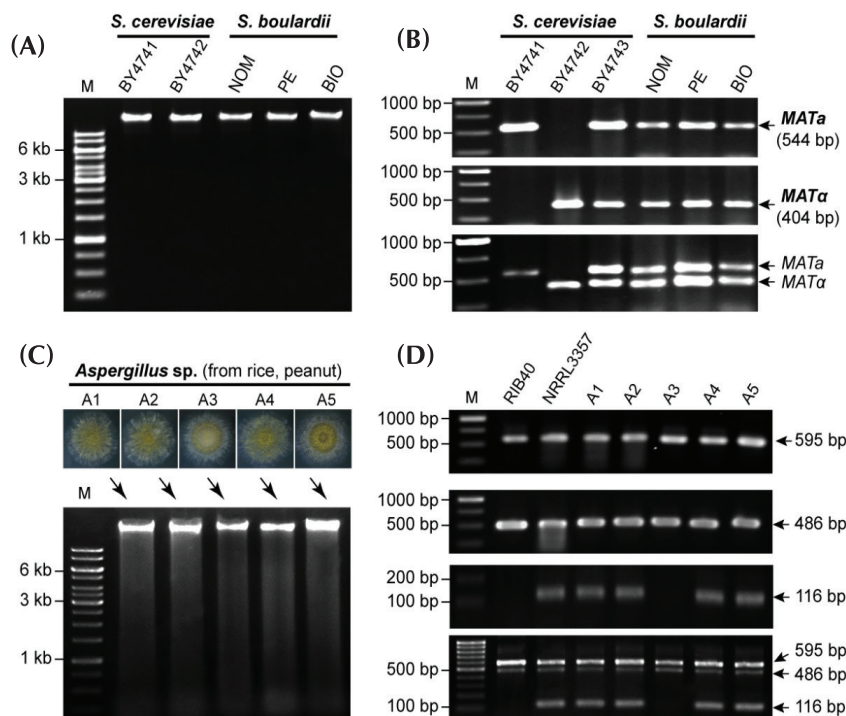


Fig. 4. Genetic identification of the closely related fungal species by PCR. (A) Genomic DNA products extracted from three standard strains of the baker's yeast *S. cerevisiae* and three newly isolated strains of the probiotic yeast *S. boulardii*. (B) Determination of haploid and diploid states in the *Saccharomyces* yeasts with the utilization of singleplex PCR amplifications with the primer pairs ScMAT/ScMATa and ScMAT/ScMATα specific to the yeast mating-type genes *MATa* and *MATα* respectively. (C) The genomic DNA products extracted from the molds *A. oryzae* RIB40, *A. flavus* NRRL3357, and *Aspergillus* sp. (A1-A5). (D) Quick detection of putative strains of *A. oryzae* and *A. flavus* isolated from infected food materials through singleplex and multiplex PCR amplifications.

agarose gel (Fig. 4D).

In summary, the genomic DNA products obtained with our genomic DNA extraction method are completely suitable for PCR-based applications. The method described in this study is significantly uncomplicated in terms of execution and considerably more secure, since it does not employ toxic chemicals such as phenol and chloroform like other DNA extraction methods [17, 28-30]. Our method further provides higher DNA concentrations as compared to the simple method reported by Ceniz (1992) [10], and even the DNA concentrations are 10 times higher in comparison to genomic DNA concentrations obtained

from the phenol-chloroform method employed by Umesha (2016) [30].

Conclusion

The genomic DNA extraction method established in this study is universal, simple to handle, safe and cost-effective for the extraction of high-quality genomic DNA from various microbial cell types. The obtained genomic DNA products can be utilized for different research purposes, especially for PCR-based applications.

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Synthesis and characterization of thermal conductivity of nanofluids based on Ag decorated-CNTs/graphene hybrid materials

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Abstract:

In this work, we present a new nanofluid based on silver nanoparticles decorated on the functionalized carbon nanotubes-graphene sheet (hybrid) materials. Briefly, carbon nanotubes and graphene sheets were first functionalized with a hydroxyl group and carboxyl group respectively. The hybrid material was decorated with silver nanoparticles via chemical reduction method with the assistance of sodium hydroxyl. Finally, the obtained Ag-hybrid material was dispersed in ethylene glycol solution (EG) to form the nanofluid without any surfactant. The thermal conductivity of nanofluid was measured for different weight concentrations at different temperatures. The results showed an increase in thermal conductivity of up to 86% for 0.045% weight concentration at 55°C. This enhancement was due to the high thermal conductivity of graphene, carbon nanotubes (CNTs), and Ag nanoparticles as well as the higher surface area of Ag nanoparticles decorated on graphene and CNTs structures. The results of Transmission Electron Microscope (TEM), X-rays diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR) indicated that the silver nanoparticles were formed on the surface of carbon nanotubes and graphene sheets.

Keywords: CNTs, graphene, nanofluids, silver nanoparticles, thermal conductivity.

Classification numbers: 5.1, 5.5

Introduction

For a decade, the development of nanotechnology has not only minimized the size but also improved the working speed of electronic devices. A serious problem in electronic devices is heat generation during the process of working at high power, leading to a decrease

in their performance and lifetime. In order to solve this problem, there are several methods for heat dissipation, i.e. utilization of fans, thermal grease or fluids. Most of the electronic devices use fluids for heat dissipation such as distilled water or ethylene glycol. However, these basic fluids have poor thermal conductivity, resulting in

the lower efficiency of heat transfer. Therefore, it is enormously important to increase the heat transfer capability of fluids. One of the most promising methods is the addition of solid particles with high thermal conductivity which acts as heat carriers for fluids.

In 1873, Maxwell was the first person who proposed the idea of adding solid particles into fluids to enhance their thermal conductivity [1]. Subsequently, researchers dispersed microparticles into fluids to increase the thermal conductivity of the fluids. Nevertheless, the added microparticles would aggregate and settle down [2]. To address this negative aspect, a great deal of research was carried out by dispersing nanoparticles into fluids.

In 1995, the term “nanofluid” was introduced the first time by S. Choi and J.A. Eastman at Argonne National laboratory [3]. Generally, nanofluid is the fluid having stable suspension of nanomaterials such as nanoparticles, nanofibers, nanorods, nanotubes, nanowires, and nanosheets, which are typically less than 100 nm in size. There are two phases in the system, one phase is a liquid phase and the other one is a solid phase. The nanoparticles used in nanofluids are metals, oxides, carbides, and diamond [4-7].

CNTs and graphene are the materials owning very high thermal conductivity

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(about 3000 W/m.K for CNTs and 5000 W/m.K for graphene) [8-9]. It is reported that by using graphene and CNTs as additives in nanofluids, the thermal conductivity went up significantly. CNTs and graphene are considered as bridges or networks for making heat transfer faster. Rad Sadri, et al. showed that the thermal conductivity rose up to 22.31% for nanofluids containing 0.5wt% of CNTs. This result was obtained after 40 minutes of ultrasonication at 45°C [10]. Zeinab Hajjar, et al. revealed that increasing thermal conductivity depends on the concentration of graphene oxide (GO) dispersing in nanofluids. For example, the thermal conductivity enhancement was 14.75% with 0.05wt% of GO, the thermal conductivity increased by 47.57% with 0.25wt% of GO at 40°C [11]. Mehrauli et al. studied graphene nanofluids with different concentrations of graphene, specifically 0.025, 0.05, 0.075, and 0.1wt%. The result displayed that the maximum thermal conductivity enhanced 27.64% with 0.1wt% of graphene dispersing in nanofluids [12]. Several studies also focused on using metallic or non-metallic nanoparticles decorated on graphene and CNTs to enhance heat transfer capability of nanofluids. For an instant, Baby, et al. studied nanofluids containing copper oxide nanoparticles decorated on graphene. The results depicted that the enhancement in thermal conductivity was approximately 28% with 0.05% volume fraction of CuO-graphene dispersing in DI water-based nanofluids at 25°C and thermal conductivity enhancement was 23% with 0.07% volume fraction in EG-based nanofluids at 50°C [13]. This author group also studied the decoration of silver nanoparticles on graphene and the reported thermal conductivity enhancement was 14% with 0.07% volume fraction of Ag-graphene dispersing in EG-based nanofluids at 70°C [14]. H. Yarmand et al. also showed that the increase in

thermal conductivity was 22.22% with 0.1wt% of Ag-graphene at 40°C [15]. Amiri et al. reported an enhancement of thermal conductivity of 25% with 1wt% of Ag-CNTs dispersing in nanofluids. S.S. Aravind, et al. examined nanofluids containing graphene-multiwall carbon nanotubes (graphene-MWCNTs) nanocomposite based on DI water and EG. The results showed that the thermal conductivity enhancement was 10.5% and 87.9% with 0.04% volume fraction of graphene-MWCNTs at 25°C and 50°C in DI water, respectively. Whereas in EG, thermal conductivity enhancement was 13.7% and 24% at 25°C and 50°C, respectively [16]. Recently, T.T. Baby, et al. studied nanofluids containing silver nanoparticles decorated on graphene-MWCNTs based on EG. The results showed that the enhancement of thermal conductivity was 8% and 20% with 0.04% volume fraction of Ag/graphene-MWCNTs at 25°C and 50°C, respectively [17]. The presence of metallic or non-metallic nanoparticles supposed to avoid the stacking of graphene sheets and CNTs [18].

In this study, we present the results of synthesis and thermal conductivity characterization of nanofluids based on EG containing Ag nanoparticles decorated on functional groups of graphene and CNTs by chemical reaction method with different weight concentrations. The Ag-hybrid materials were synthesized by a simple method and performed the enhancement of high thermal conductivity of nanofluids.

Experiment and methods

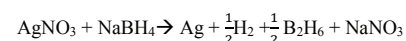
Materials

CNTs were supported by Laboratory of Carbon Nanomaterials, Institute of Material Science, Vietnam Academy of Science and Technology (VAST). Graphite rod (99.99%) was purchased from Aladdin Bio-Chem Technology Company to be used as an electrode to

synthesize graphene sheets. Potassium hydroxide (KOH), ammonia sulfate $(\text{NH}_4)_2\text{SO}_4$, sulfuric acid (H_2SO_4 , 98%), nitric acid (HNO_3 , 68%), thionyl chloride (SOCl_2), tetrahydrofuran (THF), ethylene glycol (EG), and sodium hydroxide (NaOH) were purchased from Shantou Xilong Chemical Factory Guangdong, China. Silver nitrate (AgNO_3) and sodium borohydride (NaBH_4) were purchased from Shanghai Aladdin Bio-Chem Technology Co. LTD, China.

Nanofluid preparation

Schematic of the synthesis of Ag nanoparticles decorated on the hybrid material is shown in Fig. 1. Graphene sheets synthesized by a plasma-assisted electrochemical exfoliation process [19] were functionalized with carboxyl (-COOH) group by treatment in the mixture of acid (HNO_3 : H_2SO_4 , ratio 1:3 respectively) at 70°C for 5 hours under continuous magnetic stirring, then filtered by distilled water and dispersed in EG. CNTs were functionalized with hydroxyl (-OH) group by treatment with SOCl_2 at 60°C for 24 hours under continuous magnetic stirring then filtered by distilled water and washed with tetrahydrofuran. After that, they went through a treatment with EG at 120°C for 48 hours under continuous magnetic stirring and dispersed in EG. CNTs-graphene material was dispersed in EG by ultrasonication for 10 minutes. A specific amount of AgNO_3 (0.05 M) solution was added to the above solution, under continuous stirring. After 30 minutes, 20 ml of reducing solution (a mixture of NaBH_4 and NaOH) was added to the above solution dropwise. The reaction was as follows:



After the reducing process completes, the solution was filtered and washed with distilled water. A calculated amount of Ag-hybrid material was dispersed in EG to generate nanofluid by ultrasonication.

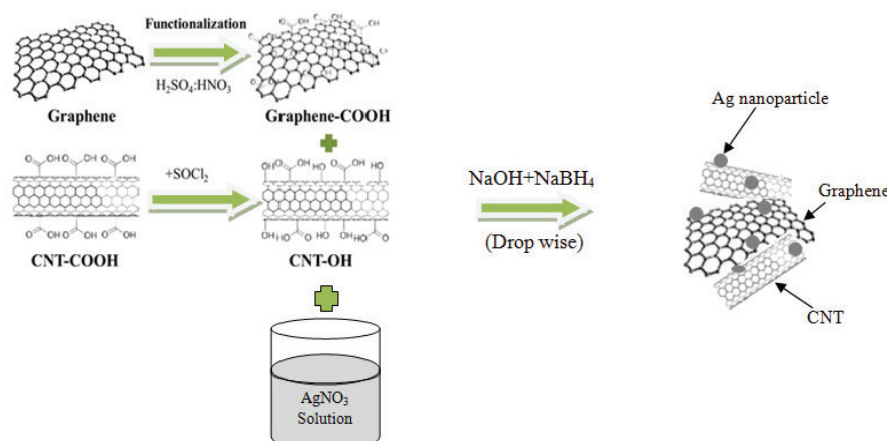


Fig. 1. Schematic of the synthesis of Ag nanoparticles decorated on the hybrid material.

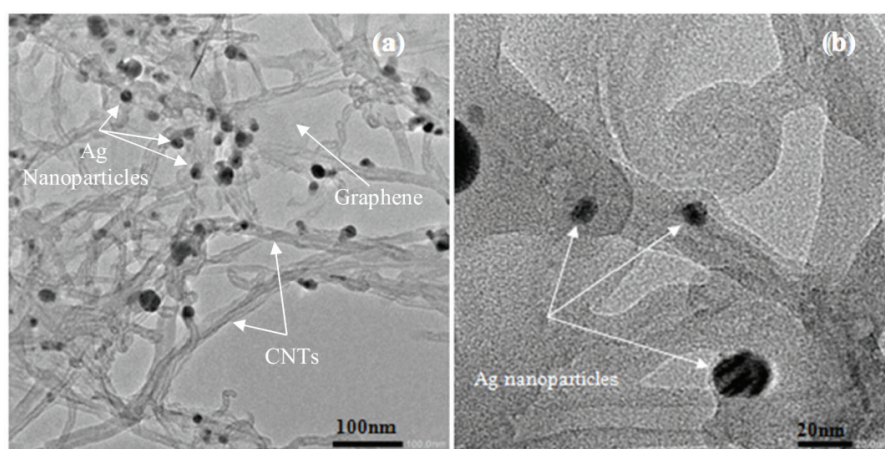


Fig. 2. Transmission electron microscopy (TEM) images of Ag-hybrid materials.

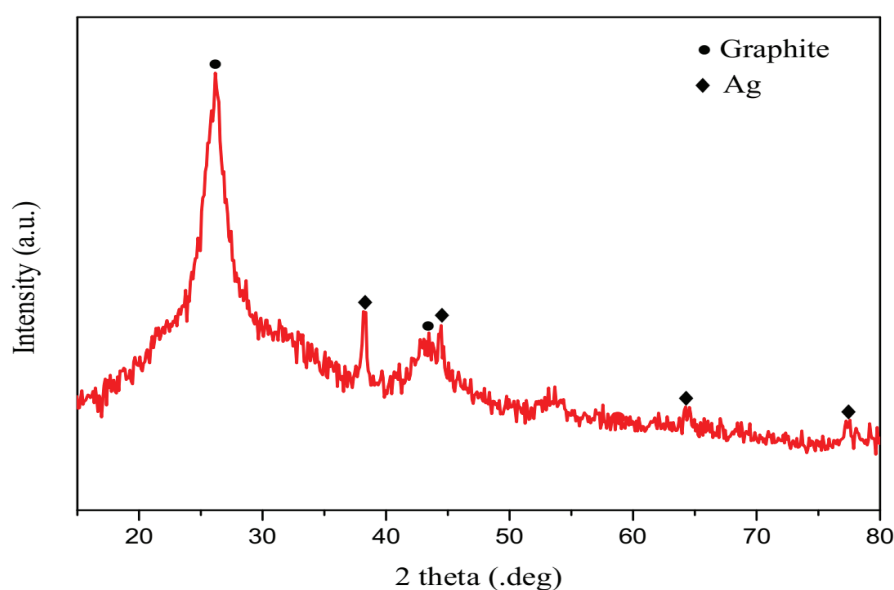


Fig. 3. X-ray diffraction pattern of Ag- hybrid material.

Characterization

The morphology of the samples was characterized by field emission scanning electron microscopy (FESEM, Hitachi S4800) and transmission electron microscopy (TEM, JOEL JEM 2100 microscope). XRD pattern was recorded by an XRD Bruker D8 Endeavor equipped with Cu ($K\alpha$) radiation in a 2θ range of 10° to 90° with a step size of 0.01. The thermal conductivity (K) of the nanofluids was measured by using an HTL-04 thermal conductivity of liquid (Eee, India) in the range from 30° to 60°C . The apparatus for measuring the thermal conductivity of the liquid is designed and developed according to the principle of guarded hot plate method. Detail of measurement method was presented in our previous studies [20, 21].

Results and discussions

The surface morphology of the samples was characterized by TEM images at low and high magnifications as shown in Fig. 2. The distribution of Ag nanoparticles on CNTs and graphene sheets is visible in TEM images. The surface morphology shows that silver nanoparticles were decorated properly on functional groups of graphene sheets and CNTs. The size of silver nanoparticles was estimated from the TEM images and it was smaller than 20 nm.

Figure 3 illustrates the X-ray diffraction (XRD) pattern of Ag-hybrid materials. XRD pattern was recorded by an XRD Bruker D8 Endeavor equipped with Cu ($K\alpha$) radiation in a 2θ range of 10° to 90° with a step size of 0.01. XRD was performed to study the formation of crystallinity and investigate phase compositions of the samples. The result shows some representative peaks of graphite at $2\theta = 26.2^\circ$, 42° , and 53.6° corresponding to (002), (101), and (004) planes of graphite, respectively. Peaks around 38.2° , 44.3° , 64.2° , and 77.5° are responsible for (111), (200), (220), and (311) planes structure of

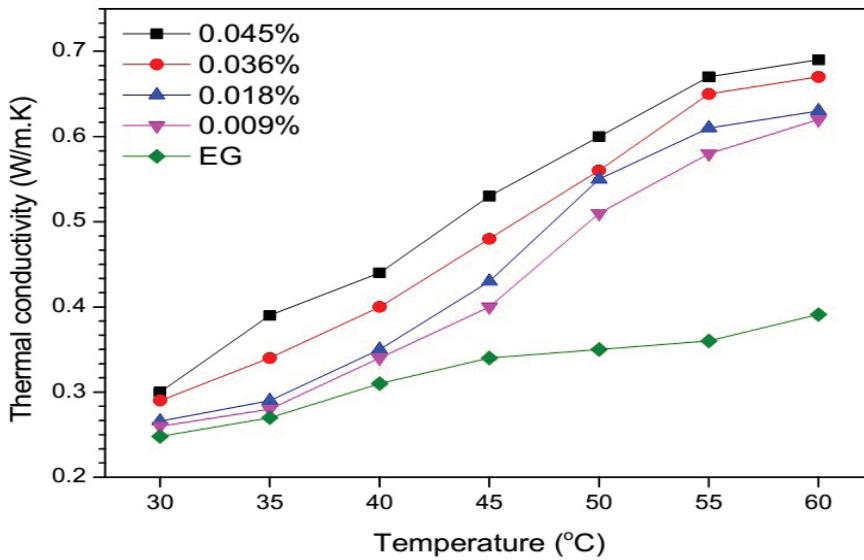


Fig. 4. Thermal conductivity of nanofluids containing EG and Ag-hybrid material at different temperature for different weight concentrations.

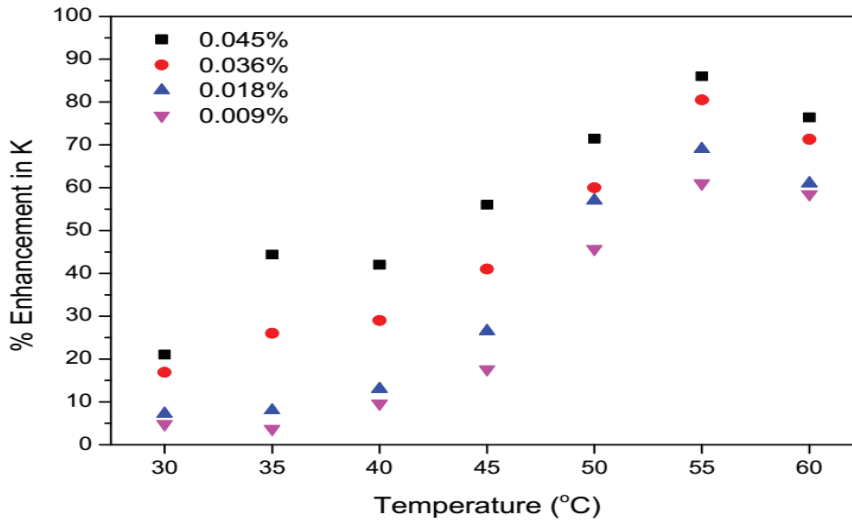


Fig. 5. Thermal conductivity enhancements of nanofluids with different weight concentrations at different temperatures.

Table 1. Summary of experimental results on thermal conductivity of EG based nanofluids.

Ref.	Material type	Material concentration	Temperature	Enhancement
This work	Ag-CNTs/graphene hybrid material	0.009-0.045wt%	30 – 60°C	21-86%
[14]	Ag decorated graphene	0.005-0.07wt%	25-70°C	6-14%
[16]	CNTs-graphene	0.04vol%	25-50°C	13.7-24%
[17]	Ag decorated MWNT-HEG hybrid	0.005-0.04vol%	25-50°C	8-20%
[25]	Graphene oxide nanofluids	2-5wt%	10-60°C	Up to 86%

Ag nanoparticles, respectively. The average size of Ag nanoparticles is approximately 7 nm, which has been calculated with Scherer's formula corresponding to the representative peak of Ag at 38.2°. From both morphology and XRD studies, it is proven that Ag nanoparticles were grown successfully on CNTs and graphene hybrid materials by the chemical reduction method.

The thermal conductivity of the nanofluids was measured for different weight concentrations at different temperatures as showed in Fig. 4. In general, the thermal conductivity of the nanofluids increased together with an increase in the weight concentration or temperature. The enhancement in thermal conductivity is quantified by the following formula:

$$\%K = [(K - K_0) \times 100] / K_0$$

where: K_0 is the thermal conductivity of the base fluid and K is that of the nanofluids.

Figure 5 shows the percentage of thermal conductivity enhancement of nanofluids containing different weight concentrations of Ag-hybrid material at different temperatures. The result shows that the enhancement percentage in thermal conductivity of 0.009wt% Ag-hybrid material at 30°C is ~5% and around 58.5% at 60°C. Whereas, the nanofluids containing 0.045wt% show an enhancement of 21% at 30°C and around 76.4% at 60°C. Especially, the percentage enhancement in thermal conductivity experiences a high rate of 86% at 55°C before decreasing. This could be due to the formation of clusters at high temperatures. The thermal conductivity enhancement is mainly due to the Brownian motion of nanoparticles. According to the theory of Brownian motion, the smaller size of nanoparticles, the faster Brownian motion and the higher temperature, the faster Brownian motion, consequently, heat transfer inside nanofluids is faster [22-24]. However, the formation of

clusters at high temperatures prevents the Brownian motion, causing a decrease in the enhancement percentage of thermal conductivity at high temperatures (Table 1).

The enhancement is due to the high thermal conductivity of graphene, CNTs, and Ag nanoparticles as well as the higher surface area of Ag nanoparticles decorated on graphene and CNTs structures.

Conclusions

The nanofluid containing Ag nanoparticles decorated on CNTs-graphene is synthesized successfully by chemical reduction method. The average size of Ag nanoparticles is smaller than 20 nm. The nanofluid containing 0.045wt% of Ag-hybrid material shows the best performance with the thermal conductivity increase of 21% at 30°C and 86% at 55°C. Experimental results of the thermal conductivity strongly confirm that EG containing Ag-hybrid can be used for heat transfer applications.

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System of environmental - economic accounting for water (SEEA-W): case study of the Nhue - Day river basin

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Abstract:

The system of environmental - economic accounting for water (SEEA-W) is one of efforts by international organizations to address the issue of the lack of water related data; it provides a tool for policymakers to inform decisions in a sustainable manner. In undertaking a pilot study of the Nhue - Day river basin, this research demonstrates that the river basin approach is in accordance with management institutions and available data, as well as being useful for sustainable management of water, in Vietnam.

Keywords: management of water, Nhue - Day river basin, SEEA-W.

Classification number: 6.2

Introduction

Water accounting is a discipline that seeks to provide comprehensive, consistent and comparable policy-relevant information related to water. Based on the experience of more than fifty years of compiling national accounts, the discipline that provides the elements to calculate gross domestic product (GDP), the United Nations Statistics Division (UNSD) developed the System of Environmental - Economic Accounting for Water (SEEA-W), adopted by the United Nations Statistical Commission (UNSC) as a statistical standard in 2012. SEEA-W can assist policymakers in making informed decisions on: (i) Allocating water resources efficiently; (ii) Improving water efficiency; (iii) Understanding the impacts of water

management on all users; (iv) Getting the most value for money from investing in infrastructure; (v) Linking water availability and use; (vi) Making available a standardized information system, which is capable of harmonizing information from different sources, is accepted by stakeholders and is used for the derivation of indicators; (vii) Getting stakeholders involved in decision-making.

Currently, over 50 countries are developing or plan to develop SEEA-W [1]. The application of SEEA-W will gradually improve the quality of data and contribute to develop sustainable policies on water resources. However, the applicability of the framework in practice is much reliant on a country's institutional and organizational structure

and especially the approach to relevant SEEA-W information sources [2]. Viewed from existing conditions and potential of the framework's application in Vietnam, the study has revealed that adopting a river basin approach to SEEA-W is fundamental to implementation of the framework at national level. In this study, the pilot application on the Nhue - Day river basin will prove the relevance and feasibility of implementation of this approach, while demonstrating it has certain impacts on State management on water resources. In addition, the gained results of the pilot study will point out the deficiencies and limitations that need to be improved.

Methodologies

The methodologies adopted in this research were:

(i) Desk study to review, collect data and related information, and search for published data sources.

(ii) Systems analysis to describe the relationship between economic and household activities and other problems related to water resources in the Nhue - Day river basin.

(iii) Consultation survey of views of local authorities in the Nhue - Day river basin and experts from the relevant backgrounds such as water resources

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planning; water pollution; remote sensing and GIS professionals.

(iv) Statistical analysis to gather and process Nhue - Day river basin data. The following table describes the main sources and methodologies used to collected data for Nhue - Day river basin case study (Table 1).

Results and discussions

SEEA-W framework

The framework of SEEA-W is presented in simplified diagrammatic form in Fig. 1, which shows the economy, the system of water resources and their interactions [9]. The economy and the inland water resource system of a territory, referred to as “territory of reference”, which can be a country, an administrative region, or a river basin. The given “territory of reference” includes (i) The inland water resource system of a territory is composed of all water resources in the territory (surface water, groundwater and soil water) and the natural flows between and among them (absorption, evapotranspiration, etc.) which are separately reflected in the form of water assets and volume; (ii) The economy of a territory consists of resident water users that abstract water for production and consumption purposes and put in place the infrastructure to store, treat, distribute and discharge water.

State agencies, enterprises, and households play their particular roles in socio-economic activities in the territory of reference. These entities act as the producers and consumers, and use water resources as a “sink”. In this regard, these entities use water in different ways by abstracting groundwater, surface water and rainwater, and by reusing water, etc. In the other words, economic entities can directly abstract water from the environment to carry on activities involving production and consumption or use water without physically removing it from the environment (recreational uses of water). To depict the relationship of water-related activities within the economy, SEEA-W adopts International Standard Industrial Classification (ISIC)

Table 1. Data collection methodologies.

No	Types of data	Sources or methods to collected
1	Precipitation	Collected from the report of National Center for Water Resources Planning and Investigation (NAWAPI) [3] in 2016 - Monre.
2	Evaporation	Download the average evaporation map from Moderate Resolution Imaging Spectroradiometer (MORIS) and processing for the Nhue - Day river basin by Geographical Information System (GIS) expert.
3	Water use and wastewater	- Wastewater data was extracted from the data system of the Centre for Environmental Monitoring Portal of the Vietnam Environment Administration (VEA), the website Portal managing environmental monitoring of the Nhue - Day river basin. - Water use was estimated according to the guide from the Decree on drainage and wastewater treatment (article 39 - Determination of wastewater volumes, no 80/2014/ND-CP). - In particular, wastewater and water use for agriculture (cultivation and livestock) was collected from data in VEA reports [4].
4	Pollution parameters in wastewater	The study only adopts COD parameters in water to compile the emission accounts table in the Nhue - Day river basin. Due to data restrictions, the following assumptions have been made: (i) Wastewater after treatment meets the standards for surface water in Vietnam at column A1; accordingly, the COD amount after treatment remains at 0.00001 ton/m³ [5]. (ii) The ratio of treated wastewater and wastewater directly discharged into the environment are calculated on the data of Center for Environmental Monitoring 2017 [6]. Additionally, some ratios are referenced by published researches [7] or the National Strategy on environmental protection to 2020 and the orientation towards 2030 [8].
5	Socio-Economic activities	From the provincial statistical yearbooks of Hoa Binh, Ha Noi, Ha Nam, Nam Dinh, and Ninh Binh in 2015.

(Sources: authors, 2017).

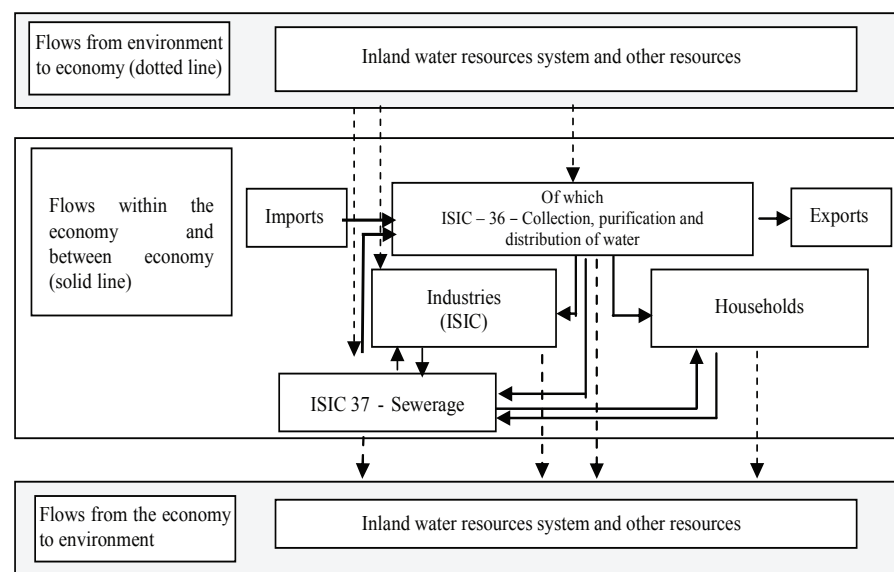


Fig. 1. Detailed description of physical flows within the economy.

(Sources: UN, SEEA-W, 2012).

to develop relevant accounting tables listed in Table 2 [10].

The SEEA-W accounting framework showing interaction between the inland water system and the economy is divided and presented in the five categories corresponding to these accounting tables: (i) Physical water supply and use table (PSUT); (ii) Water emission accounts; (iii) Hybrid accounts for economic activities and water resources; and (iv) Asset accounts. These tables reflect different aspects of water in a given period of accounting. Two important parts of SEEA-W are PSUT and emission accounts. PSUT describes water flows in physical units (m³, million m³, etc.) to reflect water interaction between the environment and the economy and among industries within the economy. Emission accounts seek to show the water pollutants in the economy, the role of the entities to reduce these pollutants through on-site treatment, or through water treatment facilities (ISIC 37).

PSUT and emission accounts tables respectively contribute to:

- (i) Assessment and monitoring of the pressure on water quantities that is exerted by the economy; (ii) Evaluation of alternative options for reducing the pressure on water; (iii) Reflect the information on water collection, treatment, distribution, and reuse of water by the industries; and (iv) Link other economic information such as value added and production yield of each industry, to calculate useful indicators such as intensity, productivity and efficiency of water use by industry, emission situation, water footprint, and water use in households.

- Support policy makers, water managers measure the current situation and control the pollutants in the water resources (e.g. BOD, COD, nitrogen, phosphorus, etc.) based on discharges of water and pollutants into the environment from households and economic activities (Fig. 2 [9]).

Table 2. Classification of socio-economic activities in SEEA-W.

International System Industry Code (ISIC)	Description of Socio - economic activities
1-3	Agriculture, forestry and fishing
5-33, 41-43,	Manufacturing, mining and quarrying and other industry activities
35	Electric power generation, transmission and distribution
36	Water collection, treatment and supply
37	Sewerage, including treatment of wastewater
38, 39, 45-99	Services activities
No code	Households as customer

(Sources: UN, SEEA-W, 2012).

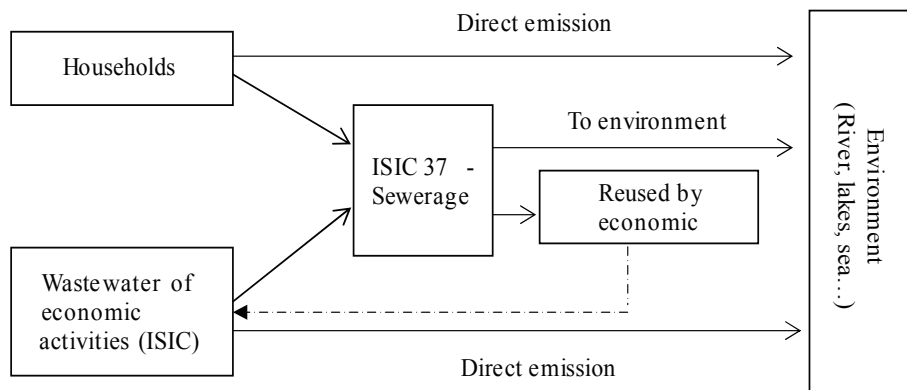


Fig. 2. Wastewater and associated pollution pathway.

(Sources: SEEA-W, 2012).

Table 3. Total GDP at current price of provinces in Nhue - Day river basin by economic activities.

ISIC	2010	2011	2013	2014	2015
Total	361,877	464,685	642,032	735,801	822,582
1-3	43,784	56,024	63,079	65,441	70,642
5- 33, 41-43	181,285	235,740	331,451	382,602	432,358
35	14,537	14,104	19,811	23,658	26,914
36, 37	1,758	2,404	3,484	3,977	4,171
38, 39, 45-99	120,513	156,413	224,207	260,123	288,497

Unit: billions of VND.

(Source: extracted from the provincial statistical year books of Ha Noi [11], Ha Nam [12], Nam Dinh [13], Ninh Binh [14] and Hoa Binh [15]).

Hybrid supply and use tables juxtapose results from PSUT and emission accounts tables with information about related economic activities in the system of national accounts (SNA) such as gross output (GO), intermediate consumption (IC), value added (VA), etc. They contribute development indicators to illustrate the relation between water resources and economic, households' activities in each country, territory [9]. In particular, such types of combination make it possible to review the contribution of water resources to socio-economic development and provide indicators to reflect the efficiency and cost of water use and distribution within the economy, the polluting pressures on the environment created by water, and so on.

Application of SEEA-W in the Nhue - Day river basin

An overview of the Nhue - Day river basin:

- Natural conditions: Being a sub-basin of the Red River, the Nhue - Day river basin is located on the right bank of Red River and accounts for a large part of the southwest Red River delta. The basin encompasses 5 provinces and central cities including: Hoa Binh, Hanoi, Ha Nam, Ninh Binh, and Nam Dinh with total area of 7,388 km² (in which the Day river basin is 6,965 km²). The basin has a variety of geographical structures in which two thirds of the area is delta; the West-to-East geographical picture can be divided into three main regions: Mountainous region, delta, and coastal/mouth region. The basin's river network has a rather high density at 0.7-1.5 km/km² including main rivers, tributaries and distributaries such as Day river, Tich river, Thanh Ha river, and Chau river [4].

- Socio-economic conditions: In 2010, the total population in the basin was 8,191,739 people with average density of 1,108 people/km² [4]. Due to the impact of the strong urbanization process, the population in the basin has increased over time along with positive changes in economic activities. The results of GO in the basin's provinces

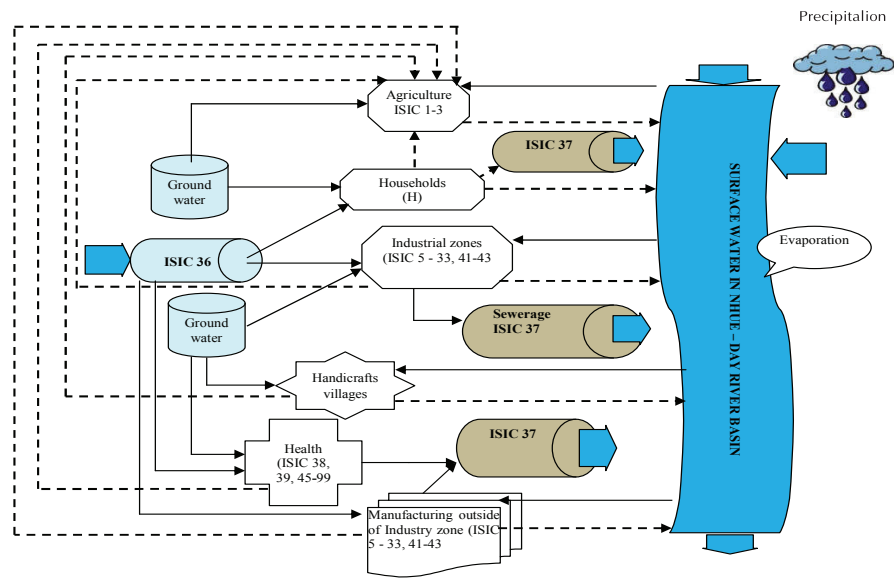


Fig. 3. The relationships between socio-economic activities and water resources in the Nhue - Day river basin.

(Sources: authors, 2017).

Table 4. Estimated wastewater from main wastewater sources in the Nhue - Day river basin from 2009 to 2015.

No	Types of wastewater sources	2010	2012	2014	2015
1	Agriculture	933.34	N/a	N/a	N/a
-	Cultivations	341.67	N/a	N/a	N/a
-	Livestock	591.67	N/a	N/a	N/a
2	Handicraft villages	26.81	128.31	14.92	32.65
3	Industrial zones	21.79	15.85	8.19	17.13
4	Health facilities	91.67	4.21	1.37	0.51
5	Manufacturing outside of industrial zones	86.84	1241.78	4.90	1.27
6	Households	223.03	N/a	N/a	N/a

Unit: millions of m³; N/a: not available data.

(Source: extracted and calculated as described in Table 1).

Table 5. The volume and ratio of water use, water emission of economic activities, households in the Nhue - Day river basin in 2010.

Indicators	Unit	H	Agri (1-3)	Industries ISIC (5-33, 41-43)			Health facilities (38, 39, 45-99)
				Industrial zones	Manufacturing outside of Industrial zones	Handicraft villages	
1. Total of water use	Mil.m ³	223.03	1,166.68	27.23	108.55	33.52	114.59
Ground water	%	33.55	45.63	42.22	5.21	71.27	7.44
Surface water	%	0	40.60	1.01	83.35	24.02	0
From ISIC 36	%	66.46	13.78	56.76	11.43	4.71	92.56
2. Total of wastewater	Mil.m ³	223.03	933.34	21.79	86.84	26.81	91.67
Agriculture	%		0	14.16	0.062	2.83	4.945
To ISIC 37	%	30.00	0	4.23	2.34	15.42	50.48
To Environment (rivers, lakes, sea...)	%	70.00	100	81.61	97.60	81.75	44.58

H: households.

(Source: extracted and calculated as described in Table 1).

has constantly increased, particularly in the sectors of industry, commerce, and service. In 2015, the population of the river basin reached 11,989.3 thousand people (Table 3).

- Pressures on water resources: The

Nhue - Day river basin maintains its important roles in supplying water for socio-economic development activities in the related provinces. Nevertheless, the rapid socio-economic development in the recent years has resulted in

many pressures on the water resources in the basin such as uncontrolled water abstraction and use; increased environmental pollution, which lead to the severe polluted conditions of the water resources in the basin [4].

Table 6. Physical use 2010 in the Nhue - Day river basin.

A. Physical water use table (millions of m ³)	Industry (by ISIC)							H	ROW	Total
	1-3	5-33, 41-43	35	36	37	38, 39, 45-99	Total			
I. From the environment										
I.1. Sources of abstracted water										
1. Total water abstraction (= 1.a + 1.b = 1.2.1 + 1.2.2 + 1.2.3)	1,005.95	139.85	0	481.52	6,865.60	8.52	8,501.44	93.52		8,594.96
1.a. Abstraction for own use	1,005.95	139.85	0	0.00	6,865.60	8.52	8,019.92	93.52		8,113.45
- Agriculture	1,005.95						1,005.95			1,005.95
- Industries		139.85					139.85			139.85
+ Industrial zones		11.77					11.77			11.77
+ Manufacturing outside of Industrial zone		96.14					96.14			96.14
+ Handicraft villages		31.94					31.94			31.94
- Health facilities						8.52	8.52			8.52
- Urban run-off					6,865.6		6,865.60			6,865.60
- Households							0.00	93.52		93.52
1.b. Abstraction for distribution				481.52			481.52			481.52
I.2. Abstraction sources										
I.2.1. From inland water resources	1,005.95	139.85	0	481.52	0	8.52	1,635.84	93.52		1,729.36
- Surface water	532.30	98.808	0	481.52	0	0	1112.63	0		1,112.63
- Ground water	473.65	41.046	0	0	0	8.52	523.22	93.52		616.74
I.2.2. Collection of precipitation	0	0	0	0	6865.6	0	6,865.60	0		6,865.60
1.2.3. From sea	0	0	0	0	0	0	0.00	0		0.00
II. Within the economy										
2. Use of water received from other economic units	21.176	27.87	0	0	120.28	106.07	275.39	185.27	0	460.66
- Reused water	8.363	0	0	0	0	0	8.36	0	0	8.36
- Wastewater to sewerage	0	0	0	0	120.28	0	120.28	0	0	120.28
3. Total use of water (= 1+2)	1,027.13	167.72	0.00	481.52	6,985.88	114.59	8,776.83	278.79	0.00	9,055.63

ROW: rest of the world (outside the Nhue - Day river basin).
(Sources: authors, 2017).

Table 7. Physical supply 2010 in the Nhue - Day river basin.

B. Physical supply table (millions of m³)	Industry (by ISIC)							H	ROW	Total
	1-3	5-33, 41-43	35	36	37	38, 39, 45-99	Total			
Within the economy										
4. Supply of water to other economic units	0	10.99	0	481.52	0	50.81	543.32	66.913		610.23
- Reused water	0	3.90	0	0	0	4.53	8.43	0		8.43
- Wastewater to sewerage	0	7.09	0	0	0	46.28	53.37	66.913		120.28
Into the environment										
5. Total returns (= 5.a + 5.b)	933.34	124.45	0	0	120.28	40.86	1,218.94	156.12		1,375.06
- Agriculture	933.34						933.34			933.34
- Industries		124.45					124.45			124.45
+ Industries zones		17.78					17.78			17.78
+ Manufacturing outside industrial zones		84.75					84.75			84.75
+ Handicraft villages		21.92					21.92			21.92
- Health facilities						40.86	40.86			40.86
- Urban run-offs							0.00			0.00
- Waste water					120.28		120.28	156.12423		276.40
5.a. To inland water resources (= 5.a.1 + 5.a.2)	933.34	124.45	0	0	120.28	40.86	1,218.94	156.12		1,375.06
5.a.1. Surface water	933.34	124.45	0	0	120.28	40.86	1,218.94	156.12		1,375.06
5.a.2. Ground water	0	0	0	0	0	0	0.00	0		0.00
5.b. To other sources	0	0	0	0	0	0	0.00	0		0.00
6. Total supply of water (= 4 + 5)	933.34	135.44	0.00	481.52	120.28	91.67	1,762.25	223.04		1,985.29
7. Consumption (= 3 - 6)	93.78	32.28	0	0	6,865.60	22.92	7,014.58	55.76		7,070.34

(Sources: authors, 2017).

Description of relationship between economy activities and water resources in Nhue - Day river basin:

Figure 3 simulates the relationships between the socio-economic activities and water resources in the Nhue - Day river basin. Accordingly, all entities involved in water abstraction and use in the basin include agriculture production activities (cultivations and livestock activities), industrial activities performed by industrial zones, traditional craft

villages and households, and health services.

Based on these relationships and methodologies for collecting and calculating data in Table 1, wastewater and water use was calculated in Table 4, and Table 5. After considering the completeness of data in the Nhue - Day river basin, the relevant data in 2010 was chosen to calculate and fill in the SEEA-W's account tables.

Compiling physical supply and use table (PSUT):

The PSUT tables compiled and edited based on the standard SEEA-W framework of United Nations and the characteristics of the economic entities and households in the Nhue - Day river basin, describe the relationships between the economy and water resources within the basin (Table 6 and Table 7).

Table 8. The ratios of wastewater which are without treatment or after on site treatment by economic sectors.

Indicators	Agriculture		Industry (CEM-VEA [6])			Health facilities	H [8]
	Cultivation [4]	Livestock [7]	Industrial zones	Manufacturing outside of industrial zones	Handicraft villages		
Without treatment	100	39,9	15,63	7,69	96,9	29,55	70
After on-site treatment	0	61,1	84,37	92,3	0,96	70,45	30

(Sources: authors collected and estimated, 2017).

Table 9. Estimated the average of COD parameter by socio-economic sectors in the Nhue - Day river basin¹.

Socio - economic activities	Average COD per m ³ (Ton/m ³)
Agriculture (1-3)	
Cultivations	0.000024
Livestock	0.00063
Industries, building (5-33, 41-43)	0.00342
Industrial zone	0.00099
Manufacturing outside of industrial zones	0.00584
Handicraft villages	0.00099
Water collection, treatment and supply (ISIC 36)	N/a
Sewerage, including treatment of wastewater (ISIC 37)	N/a
Services activities (38,39, 45-99)	
Health facilities	0.00085
Other services	N/a
Households (non code)	0.001067323

(Sources: calculated from environmental protection planning report to 2020 [4]).

Table 10. Emission account for the Nhue - Day river basin in 2010.

Pollutant chemical oxygen demand (ton of COD per year)	Industry (ISIC)							H	ROW	Total
	1-3	5-33, 41-43	35	36	37	38,39, 45-99	Total			
1. Gross emissions (= 1.a + 1.b)	161.24	206,069.51	N/a	N/a	N/a	23,273.39	23,273.39	16,7304.1	0.00	930,002.99
1.a. Direct emissions to water (= 1.a.1 + 1.a.2 = 1.a.i + 1.a.ii)	161.24	357,912.26	N/a	N/a	N/a	23,273.39	23,273.39	16,6635.0	0.00	929,326.20
1.a.1. Without treatment	157.62	357,368.67	N/a	N/a	N/a	23,266.90	23,266.90	16,6635.0	0.00	928,221.37
1.a.2. After on-site treatment	3.62	541.02	N/a	N/a	N/a	6.49	6.49	0.0	0.00	1,104.83
1.a.i. To inland water resources	161.24	206,061.83	N/a	N/a	N/a	23,273.39	23,273.39	16,6635.0	0.00	777,478.34
1.a.ii. To sea	0	0	N/a	N/a	N/a	0	0	0	0	0
1.b. To sewerage (ISIC 37)	0	7.7	N/a	N/a	N/a	0	0	669.1	0	676.79
2. Reallocation of emissions by ISIC 37	0	7.69	N/a	N/a	N/a	0	0	0.0	0	7.69
3. Net emissions (= 1.a + 2)	161.2	357,919.9	N/a	N/a	N/a	23,273.39	23,273.39	16,6635.0	0	929,333.89

(Sources: authors, 2017).

¹These indicators will be more realistic when there is actual observation data from environmental statistics agencies.

Compiling emission account table:

In this study COD, an important determinant for water quality, was selected to compile the emission account table in the Nhue - Day river basin. Tables 8, 9 and 10 illustrate the ratio of wastewater without treatment by economic sectors, estimated average of COD by economic sectors and areas, and the emission account in the Nhue - Day river basin respectively.

The integration of economic information with water resources and the policy significance:

In Table 11, a hybrid account juxtaposes information from Tables 6 and 7 with the results of socio-economic activities in the Nhue - Day river basin with accounting data gathered from PSUT and emission accounts tables to calculate descriptive indicators illustrating the relationships between the

economy and water resources within the basin. The following table represents the above-mentioned relationship.

Depending on the management purposes of each sector, policy makers can select and calculate necessary indicators from the above account tables to make informed relevant decisions and policies to develop socio-economic conditions and sustainable use of water, for example, by having:

- Calculated the efficiency of water use, the contribution of water to production results of each industry within the river basin, each locality in the basin and in the whole basin.

- Identified the economic agents in water abstraction, water use and emission.

- Assessed different options to reduce the pressures on water resources.

- Reflected relevant information on water collection, treatment, distribution, and water reuse by the economic sectors and households in the economy.

- Applied the indicators in water demand forecast, emission trend when develop the socio-economic planning and water resources management scheme, etc.

In case of the sufficient data, the results of SEEA-W also help to calculate the values of water resources, the changes in asset of water resources in the physical or monetary aspects in the Nhue - Day river basin.

Limitations:

There are some limitations of this case study, which include (i) the lack of available data, with some data sources available but incomplete and fragmented leading to development of estimates and assumptions; (ii) statistical data on water

Table 11. Hybrid account for the Nhue - Day river basin in 2010.

Indicators	Industries (by ISIC category)							Rest of the world	Actual final consumption		Total	
	1-3	5-33, 41-43	35	36	37	38, 39, 45-99	Total		H	G		
A. The results of Economic activities												
1. Total Gross Domestic Products (billions VND)	361,877.51	43,783.66	14,537.25		1,758.38		120,512.91	542,470	N/a	N/a	N/a	542,469.70
2. Value Added (VA)	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
B. Water Resource Information												
3. Total use of water (millions of m³)	1,027.13	167.72	0.00	481.52	6,985.88	114.59	8,776.83	N/a	93.521	N/a		8,870.35
3.a. Total abstraction	1,005.95	139.85	0.00	481.52	6,865.60	8.52	8,501.44				N/a	8,501.44
- Abstraction for own use	1,005.95	139.85	0.00	0.00	6,865.60	8.52	8,019.92	N/a			N/a	8,019.92
3.b. Use of water received from other economic units	21.18	27.87	0.00	0.00	120.28	106.07	275.39	N/a	185.27	N/a		460.66
4. Total supply of water (millions of m³)	0.00	10.99	0.00	481.52	0.00	50.81	543.32	N/a	66.91	N/a		610.23
4.a. Supply of water to other economic units	0.00	3.90	0.00	0.00	0.00	4.53	8.43	N/a	0	N/a		8.43
- Wastewater to sewerage	0.00	7.09	0.00	0.00	0.00	46.28	53.37	N/a	66.91	N/a		120.28
4.b. Total returns	933.34	124.45	0.00	0.00	120.28	40.86	1,218.94		156.12	N/a		1,375.06
5. Total (gross) emissions of chemical oxygen demand (tons)	161.238	357,919.9	N/a	N/a	N/a	23,273.387	381,354.57	N/a	166635	N/a		547,989.57
C. Water resource management and policy indicators (example)												
Average annual GDP per m³ by sectors in the provinces in the Nhue - Day river basin (billions of VND per millions of m³)	352.32	261.05	N/a		N/a		1,051.69	61.807	N/a	N/a	N/a	61.16

resources are incompletely reported by economic sectors. Therefore, to implement this toolkit, it is necessary to develop a comprehensive statistical reporting system in the river basin.

Conclusions

While water problems are increasing, information useful for decision makers within the water sector and related to the water sector appear to be decreasing. Therefore, it is vital that the SEEA-W framework in water-related policies and water resources management strategy in Vietnam be applied. A periodical application of SEEA-W will assist policy makers and water resources managers to reflect the present status of water resources and the relationship between economic activities and water resources. In this way, proper decisions will be made to ensure achievement of water-related sustainable development goals.

Although the pilot study of SEEA-W in the Nhue - Day river basin reveals many difficulties at the initial steps in terms of data and structural organization, it demonstrates the feasibility and significance of the river basin-level approach when viewed from the perception and orientation of water

resources management in Vietnam. In order to improve the results of this pilot study, the following activities need to be undertaken: (i) complete the organization of river basins in Vietnam with special emphasis on environmental monitoring, water-related (water abstraction, water use, emission) statistics and reporting schemes at basin level; (ii) develop a water-related information system and system for sharing information among line ministries, agencies in the river basins.

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Impact of ENSO on extreme temperatures in Vietnam

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Abstract:

This paper presents the results of research on the effects of ENSO to extreme temperature in Vietnam [1] through the frequency deviation of the occurrence extreme temperatures during ENSO and non-ENSO seasons. The results demonstrate that in an El Niño winter, the frequency of absolute maximum temperature decreased over mountainous areas of the temperature in comparison to those under the influence of a non-ENSO winter. In summer, the effects of El Niño and La Nina generally led to a reduction in the frequency of maximum temperatures in comparison to the non-ENSO condition. For minimum temperatures, the effect of El Niño winter led to a decrease in the appearance of the temperatures, while the effect of La Nina led to an increase in the appearance of this characteristic. In contrast, the effects of El Niño and La Nina led to a reduction in the frequency of minimum temperatures during summer. Generally, in both El Niño and La Nina conditions, significant changes were observed in the distribution of frequency deviation with regard to both patterns and values of seasons, in which the South obviously exhibited more changes than the North.

Keywords: effects of ENSO, extremes temperature, winter.

Classification number: 6.2

Introduction

Research on weather and climate extremes is of particular importance for both scientific and practical purposes. Most climate extremes occur under conditions of abnormal variability in terms of atmospheric circulation or solar radiation. In the context of global climate change, some of the weather and climate extreme events are likely

to occur more frequently in the 21st century [2]. In fact, the variation in extreme weather and climate has been exhibited in many places; further, even the moderating climate of local climate conditions also causes to extreme changes [3]. The climate extreme has occurred increasingly more, especially the El Niño and La Nina (ENSO) phenomenon [4] that effects a change

in the frequency distribution and intensity of climatic extremes that affect production, and more importantly, can cause severe natural disasters such as heat waves, cold weather, floods, prolonged droughts, unusually strong typhoons, among others.

This study investigates the distribution of extreme temperature in the seasons of El Niño (E), La Nina (L), and non-ENSO (N) during winter and summer across regions spread throughout the country. The impacts of ENSO on extreme temperatures in Vietnam have been assessed, an evaluation that facilitates the creation of forecasting and early warning methods that can contribute to the prevention and reduction of damage caused by natural disasters.

Data and methods

Data

To investigate the distribution of extreme temperature, we have utilized the average maximum and minimum temperature and monthly absolute maximum and minimum temperature data from 38 meteorological stations from the period 1961-2000 and ENSO data from the period 1951-2000.

Methods

The periods of ENSO (El Niño and

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La Nina) that occurred during the period 1951-2010 are determined with red to the following regulations:

- El Niño (La Nina) forms a continuous period of no less than 6 months with a 5-month moving average of the monthly mean sea surface temperature anomalies in NINO.3 (5°N-5°S, 150°W-90°W) that is greater than or equal to 0.5°C (less than or equal to - 0.5°C) [3].

- Define months and seasons as (3 months) El Niño (E), La Nina (L), and non-ENSO (N):

From the El Niño and La Nina periods defined as above, the months E, L, and N are determined. To determine the ENSO seasons (El Niño and La Nina), each season is defined as constituting 3 months, wherein the spring stretches from March to May, summer from June to August, Autumn from September to November, and winter from December to February, while the average sea surface temperature anomaly (SSTA) in the NINO.3 area is in accordance to the following criteria:

$SSTA \geq 0.5^{\circ}\text{C}$ El Niño (E)

$-0.5^{\circ}\text{C} \leq SSTA < 0.5^{\circ}\text{C}$ neutral or non-ENSO (N)

$SSTA < -0.5^{\circ}\text{C}$ La Nina (L)

Each season must comprise at least two consecutive months to satisfy one of the above provided criteria. In the case where there are no two consecutive months that fulfill the above criteria, the average SSTA of 3 months must meet that criterion. The frequency and frequency deviation of temperature extremes in ENSO (El Niño, La Nina), and non-ENSO seasons are calculated as follows:

- Calculates the 5th, 10th, 25th, 50th, 75th, 90th, 95th percentile of the extreme

temperature in non-ENSO seasons).

Determine the extreme temperature values for the 95th, 90th, 5th, and 10th percentile utilized as the “threshold” values of the “warm” (“cold”) events with the following rules: The “extreme” events occur when maximum/minimum temperature in the ENSO seasons are higher (lower) than the “threshold” values that correspond to the 90th, 95th (5th, 10th) percentile for the non-ENSO (normal) seasons.

- Calculate the frequency of occurrence of extreme temperatures in ENSO seasons, with the extreme temperature as higher (lower) than the “threshold” of extreme temperatures with percentiles 90th, 95th (5th, 10th) in non-ENSO seasons of the corresponding seasons.

- Calculate the frequency deviation (P_{anom}) of extreme temperatures in ENSO seasons that correspond to the percentiles 90th, 95th (5th, 10th) (“threshold”) in non-ENSO seasons of the corresponding seasons through the application of by the formula given below [5]:

$$P_{anom} = 100\left(\frac{p}{100-m} - 1\right)$$

where:

p forms the frequency of extremes for ENSO seasons higher (lower) than the “threshold” values for the m percentile in the non-ENSO seasons (base seasons) of the corresponding seasons;

m represents the number of percentiles.

Thus, the frequency anomaly reflects the increase or decrease in the frequency of extremes during ENSO seasons in comparison to their occurrences during non-ENSO seasons for the corresponding seasons.

Results and discussions

For the above definition, the years and seasons of El Niño (N), La Nina (L), and non-ENSO (N) are presented in Table 1

Mean winter, summer of E, L, N seasons’ extreme temperature distribution (spring and autumn cases are omitted)

Monthly average maximum temperature ($\overline{T_x}$):

- Winter:

In the North-West, the values of monthly $\overline{T_x}$ in E seasons were found to be higher in N and L seasons with 0.5-2°C. The difference between the highest value and the lowest value of the average monthly maximum temperature (Δx) in E and L seasons was determined to be nearly 8-10°C, values that are comparatively lesser than that in N seasons (about 12°C).

In the North-East, monthly $\overline{T_x}$ in E seasons was found to be greater than that in L seasons, but at the same level as that of N seasons. In contrast, the maximum value of monthly $\overline{T_x}$ in E seasons was found to be greater than L and N seasons of 2-3°C, while the minimum value for the monthly $\overline{T_x}$ in E seasons was determined as being greater than that in L seasons and less than that in N seasons of about 1-2°C. Δx in E seasons is 10-11°C higher than that in L seasons (9-10°C) and N seasons (7-9°C).

In North central and South central regions, monthly $\overline{T_x}$ in E seasons was found to be higher in N and L seasons, especially in the North central region (2-5°C) (in the South central, it was only 0.5-1°C). Δx in E seasons in the North central reached 9-10°C, while for N and L seasons it was only 7-8°C. In the South central region, the value of this

Table 1. ENSO (E, L) and non-ENSO (N) seasons in the period 1950-2000.

Spring (III-V)			Summer (VI-VIII)			Autumn (IX-XI)			Winter (XII-II)		
<i>E</i>	<i>L</i>	<i>N</i>	<i>E</i>	<i>L</i>	<i>N</i>	<i>E</i>	<i>L</i>	<i>N</i>	<i>E</i>	<i>L</i>	<i>N</i>
1953	1950	1951	1951	1954	1950	1951	1954	1950	51/52	49/50	50/51
1957	1954	1952	1953	1955	1952	1953	1955	1952	57/58	64/65	52/53
1958	1955	1956	1957	1964	1956	1957	1964	1956	63/64	67/68	53/54
1969	1964	1959	1963	1970	1958	1963	1967	1958	65/66	70/71	54/55
1972	1968	1960	1965	1971	1959	1965	1970	1959	68/69	73/74	55/56
1982	1971	1961	1969	1973	1960	1968	1971	1960	69/70	75/76	56/57
1983	1985	1962	1972	1975	1961	1969	1973	1961	72/73	84/85	58/59
1987	1988	1963	1976	1985	1962	1972	1975	1962	76/77	85/86	59/60
1991	1999	1965	1979	1988	1966	1976	1984	1966	79/80	88/89	60/61
1992		1966	1982	1999	1967	1979	1985	1974	82/83	98/99	61/62
1993		1967	1983		1968	1982	1988	1977	86/87	99/00	62/63
1997		1970	1987		1974	1986	1998	1978	87/88		66/67
1998		1973	1991		1977	1987	1999	1980	91/92		71/72
		1974	1993		1978	1991		1981	97/98		74/75
		1975	1997		1980	1997		1983			77/78
		1976			1981			1989			78/79
		1977			1984			1990			80/81
		1978			1986			1992			81/82
		1979			1989			1993			83/84
		1980			1990			1994			89/90
		1981			1992			1995			90/91
		1984			1994			1996			92/93
		1986			1995			2000			93/94
		1989			1996						94/95
		1990			1998						95/96
		1994			2000						96/97
		1995									00/01
		1996									
		2000									

E: El Niño, L: La Nina, N: non-ENSO.

characteristic was merely about 3-6°C.

In the Central highlands and the South, the difference between the monthly $\overline{T_x}$ in the E, L, and N seasons were not significant, although it can be seen that the $\overline{T_x}$ in E seasons was slightly higher than in the others (not more than 1°C). The values of Δx also fluctuated between 2 and 5°C.

- Summer:

In general, the difference in the monthly $\overline{T_x}$ for the E, L, and N seasons in all of the aforementioned areas was significantly smaller than that observed in winter. In the case of E seasons, the monthly $\overline{T_x}$'s maximum and minimum values were found to be generally higher than those for L and N seasons by about 0.5-1°C. The value of Δx fluctuated along the range 3-5°C, except in the Central highlands where it varied in the range 5-8°C.

Monthly average minimum temperature ($\overline{T_m}$):

- Winter:

In the North-West, the $\overline{T_m}$ in the E seasons was found to be higher than that for the L and N seasons that ranged around 1°C. However, the maximum and minimum values of the $\overline{T_m}$ were dissimilar between Lai Chau (in the north) and Son La (in the south). In Lai Chau, the maximum values of $\overline{T_m}$ in E seasons were higher than that for the N and L seasons that had a value of about 1°C. In contrast, in Son La, the maximum value of $\overline{T_m}$ in N seasons was greater than that observed in E and L seasons, which were around 1°C. For the minimum value of $\overline{T_m}$, the temperature value for the L seasons was smaller than that for E and N seasons that had values around 2°C. The difference between the maximum value and the minimum value of $\overline{T_m}$ (Δm) for E and N seasons was

found to be less by about 5-8°C in comparison to L seasons (6-9°C).

In the Northeast, the $\overline{T_m}$ in E seasons was higher in N and L seasons by about 0.5-1°C. The maximum value of $\overline{T_m}$ was at the same level with a difference of 1.5-2°C. Particularly for the minimum value, a difference was observed for the northern mountains, where in the N seasons the values were lower than those for the N and L seasons by about 1-2°C; while in the Northern delta, the values in the L seasons were lower than those for N and E seasons by about 0.5-1.5°C. The (Δm) values in E and L seasons were in a range of 7-8°C, while the ones in N seasons were in the range 6-9°C.

In the North central, $\overline{T_m}$ in E and N seasons was higher than the one in L seasons of around 0.5°C; while in the South central region, the $\overline{T_m}$ in E and L seasons was slightly higher than that observed during the N seasons. The peak of $\overline{T_m}$ in E seasons and in the North central was higher than the one noted in the L seasons and the N seasons of 0.5-1°C. Further, in the South central region, the values in the L seasons were found to be greater than the ones observed in E seasons and similar in the N seasons. The minimum values of $\overline{T_m}$ in N seasons of the North and the South center were slightly lower than the ones observed for E and L seasons. The Δm for all three seasons of E, L, N in the North central region was higher than the one sobered in the South central coast, in a range of 5-10°C for E and N seasons and 3-7°C for L seasons. In the South central region, the values were in a range of 3-4°C.

In the Central highlands, the $\overline{T_m}$ and its maximum and minimum values for the N seasons were lower than the ones in the E and L seasons of 0.5-1°C. The

Δm in E and N seasons were around 3-7°C, whereas the one in L seasons was only 2-6°C.

In the South, the $\overline{T_m}$ and its maximum and minimum values in the N seasons were lower than the values in the E and L seasons, although they were only less than 0.5°C. The Δm values were also small, comprising only 2-4°C.

- Summer:

Similar to the $\overline{T_x}$, the difference in $\overline{T_m}$ in the summer seasons for E, L, and N seasons in all regions was lesser than the one observed in winter, and in general, did not exceed 0.5°C. In all areas, the maximum values of $\overline{T_m}$ in E and N seasons were greater than the values for L seasons of 0.5-1.5°C, for which, the difference in the North-West was the greatest (1-1.5°C), and the difference in the Central highlands was the lowest (0.5°C). The minimum values of $\overline{T_m}$ did not exhibit significant difference. The Δm for summer values was also smaller in comparison to the ones recorded for winter and showed fluctuation ranging between 1-4°C, where Δm for N seasons was usually greater in comparison to that for E and L seasons.

In conclusion, the influence of ENSO on the monthly average maximum temperature ($\overline{T_x}$) and monthly average minimum temperature ($\overline{T_m}$) in Vietnam exhibited the following central characteristics:

- 1) The segregation of $\overline{T_x}$ and $\overline{T_m}$ in winter for E, L seasons and N seasons was found to be greater than the segregation in summer.
- 2) In most cases and across most areas, $\overline{T_x}$ and $\overline{T_m}$ in the E seasons were higher than those in the L and N seasons.
- 3) The difference between the

maximum and minimum value of $\overline{T_x}$ (Δx), as well as the difference between the maximum and minimum value of $\overline{T_m}$ (Δm) in E seasons was higher than that observed in L and N seasons.

4) In addition to the three general characteristics mentioned above, there were some inconsistencies in some places, for certain E, L and N seasons.

Distribution of extreme temperature characteristics corresponding to the 90th and 10th percentile (25th, 50th, and 75th percentile cases are omitted)

Monthly average maximum temperature ($\overline{T_x}$):

- Winter:

For the 90th percentile, in most stations, $\overline{T_x}$ reached the maximum value in E seasons, while the minimum value was attained in the L seasons. However, the minimum values of $\overline{T_x}$ also occurred in about one quarter of the stations in the N seasons, mainly in the northern mountainous areas, the Central coast, and the Central highlands.

For the 10th percentile, almost half of the stations recorded maximum values of $\overline{T_x}$ in the N seasons, mainly in the North and the Central coast. The other stations observed the maximum value of $\overline{T_x}$ in the E seasons, while the minimum value of $\overline{T_x}$ was noted in most stations in the L seasons.

- Summer:

For the 90th percentile, nearly half of the stations exhibit the maximum values in the E seasons and in N seasons.

In the L seasons, the minimum values were observed across most stations of the country. For the 10th percentile, the maximum and minimum values of E, L, and N at stations were relatively uniform (about one third of each).

Monthly average minimum temperature ($\overline{T_m}$):

- Winter:

For the 90th percentile, in most stations, the minimum $\overline{T_m}$ was observed in E seasons, while about half the stations recorded the minimum values of $\overline{T_m}$ in L seasons and nearly half of the stations observed the minimum values in N seasons, mainly in the North East, South central, and Central highlands.

For the 10th percentile, there were about half of stations, mainly in the northern mountainous region, that had a maximum value of $\overline{T_m}$ in the E seasons and N seasons. In the Northern delta and North central coast, about two-thirds of the stations reported the minimum values of $\overline{T_m}$ in the L seasons.

- Summer:

For the 90th percentile, most (about 70%) of the stations noted the maximum values of $\overline{T_m}$ in the N seasons and the minimum values of $\overline{T_m}$ in the L seasons.

For the 10th percentile, about half of the stations reported the maximum values of $\overline{T_m}$ in the N seasons and about half of the stations observed the minimum values of $\overline{T_m}$ in the E seasons. In the L seasons, about one-third of the stations recorded the minimum value of $\overline{T_m}$ and one-third of the stations observed the maximum value of $\overline{T_m}$.

Thus, it can be seen that for the 90th percentile, the effect of ENSO on the distribution of the maximum and minimum values of $\overline{T_m}$ was rather prominent in winter and relatively consistent as well: the maximum values of $\overline{T_x}$ and $\overline{T_m}$ were observed in the E seasons. These findings are consistent with the distribution of $\overline{T_x}$ and $\overline{T_m}$ provided in section 3. For the 10th percentile, the effect of ENSO on the maximum values of $\overline{T_x}$ and $\overline{T_m}$ was

inconsistent, especially in summer.

Absolute maximum temperature (T_x):

- Winter:

Nearly two-thirds of the stations recorded the highest value of T_x that occurred in E seasons with the 90th percentile, while only about one-third of such stations with the 95th percentile. Meanwhile, for the 90th and 95th percentiles, the lowest values of T_x in two-thirds of the stations occurred in the L seasons. Particularly in the North-West, the highest value of T_x corresponds to 90th and 95th percentiles that occurred in the L seasons. The lowest values occurred in the N seasons.

- Summer:

For the 90th and 95th percentiles, nearly two-thirds of the stations reported the highest values of T_x in the E seasons, mostly in the Red river delta, South central coast, Central highlands, and South-West. About one-third of the stations recorded the highest values of T_x that occurred in N seasons, mainly in North East and North central. The lowest value of T_x was observed in most stations in the L seasons, mostly in the North, Central highlands, and South-West.

The highest values of the T_x in the summer season that correspond to 90th and 95th percentiles fall in the range 37-39°C in Lai Chau, 35-36°C in Son La, 37-39°C in the Northern mountainous region, and 38-40°C in the North delta, 39-41°C in the North central, 38-40°C in the South central, 32-36°C in the Central highlands (27-30°C in Da Lat), and 35-37°C in the South.

Absolute minimum temperature (T_m):

- Winter:

In most stations, for the 10th and 5th percentiles, the highest values of Tm were observed in the E seasons; the lowest values were found in L seasons. Further, nearly one-third of the stations observed the highest values of Tm in the N seasons, mainly in the Red river delta and Northern highlands. Therefore, the highest value of Tm was rarely observed in the L seasons. The lowest values of Tm in this season that corresponded to the 5th and 10th percentiles were 3-4°C in Lai Chau, -0.1 to 0.4°C in Son La, -1 to 4°C in the Northern mountains, 0-6°C

in the North delta, 5-10°C in the North central, 10-15°C in the South central coast, 6-12°C in the Central highlands (4-5°C in Da Lat) and 15-18°C in the South.

- Summer:

About half of the stations observed the highest values of Tm for the 10th and 5th percentiles that occurred in the E seasons, and for about one-third of the stations, it occurred in N seasons, mostly in North and North central regions. In contrast, for most of the stations, the

lowest Tm occurred in the L seasons.

The lowest values of Tm in summer that correspond to the 10th and 5th percentiles were 18-19°C in Lai Chau, 15-17°C in Son La, 15-20°C in the Northern mountains, 18-20°C in the North and North central coast, 20-22°C in South central coast, 16-18°C in Central highlands (only 10-11°C in Da Lat), and 18-22°C in the South (Table 2).

Thus, in terms of the highest and lowest values of Tx and Tm both in winter and summer, the effect of El

Table 2. Absolute maximum temperature in summer and absolute minimum temperature in winter corresponding with percentages at some stations in ENSO (E, L) and non-ENSO (N) seasons.

Percentiles (%)	Tx (Summer)						Tm (Winter)					
	90			95			10			5		
Station	N	E	L	N	E	L	N	E	L	N	E	L
Lai Chau	37.78	37.48	38.16	38.47	38.26	38.20	6.33	6.76	3.66	5.22	6.13	3.41
Son La	34.77	34.86	34.94	34.90	35.28	35.00	2.20	3.70	0.41	-0.10	2.25	-0.17
Lang Son	36.77	37.08	36.39	36.99	37.58	36.97	0.13	1.10	-1.60	-1.49	-1.00	-1.70
Bai Chay	36.00	35.89	35.39	37.84	36.19	35.40	5.21	6.32	6.26	1.87	5.92	5.54
Ha Noi	38.68	39.26	38.61	39.58	39.97	38.70	6.31	6.10	5.15	6.20	5.43	5.01
Thanh Hoa	39.35	39.59	38.75	39.69	40.27	39.47	6.71	7.52	5.94	6.32	6.65	5.62
Vinh	39.98	39.34	40.19	40.88	39.69	40.30	7.73	8.41	5.45	7.03	7.25	5.21
Da Nang	39.79	39.37	38.89	40.20	39.50	38.90	11.91	13.21	10.49	10.36	11.20	9.25
Nha Trang	37.18	37.43	37.75	37.80	37.88	37.80	16.06	16.91	15.44	15.80	16.62	15.12
Plei Ku	31.75	31.79	31.33	31.90	33.04	31.40	7.61	7.61	6.15	7.31	6.46	6.01
B.M.Thuot	33.60	34.29	32.29	35.04	34.40	32.30	11.81	11.62	10.02	11.32	11.32	9.15
Da Lat	26.68	26.68	26.29	29.46	27.18	26.30	5.63	5.11	5.36	4.53	5.01	4.92
Can Tho	34.98	36.13	35.22	35.67	37.25	35.30	17.35	16.83	16.10	16.07	16.51	14.86
Ca Mau	35.26	34.88	34.17	35.88	35.00	34.20	16.33	18.42	17.15	15.34	18.02	16.82

Niño was usually observed in the case of maximum values, while the effect of La Nina was usually observed for minimum values (corresponding to the 90th and 95th for Tx and 10th and 5th percentiles for Tm).

Frequency deviation of absolute maximum temperature during ENSO (E, L) seasons exceeded the threshold values for different percentile in non-ENSO (N) seasons

Monthly absolute maximum temperature (Tx):

- Winter:

El Niño: The monthly absolute maximum temperature (Tx) in the El Niño winter months exceeded the monthly Tx that corresponds to the 90th percentile in the winter months of non-ENSO that constituted the frequency deviations of - 45 to - 100% in the North-West and Northern mountains, and - 20 to - 100% in Central coast and Central highlands. This implies that in the El Niño winters, P_{anom} of Tx that corresponds to the 90th percentile in the non-ENSO decreased by 20-100%. In contrast, the frequency deviations were positive (10-40% in the North-East and the Red river delta, 40-100% in the South). This indicates that during the winter months of El Niño, the frequency of occurrence of monthly Tx exceeded the Tx of the month that corresponds to the 90th percentile in the winter months of non-ENSO in these areas increased in comparison to the winter months of non-ENSO.

For the 95th percentile, in most regions, the frequency deviation was less than 0 (- 45 to 100%), especially in the South, with a positive frequency deviation.

La Nina: The monthly absolute maximum temperature during the winter months of La Nina exceeds the monthly Tx that corresponds to the 90th and 95th percentile of the non-ENSO

winter seasons in most areas (except the North-West) had a frequency deviation less than 0, with values of - 70 to - 100% and - 30 to -100%, respectively. In the North-West, there were positive deviations of 0-30%. Thus, the influence of La Nina in winter mainly led to a reduction in the probability of occurrence of the maximum values of Tx in comparison to the winter months of non-ENSO.

- Summer:

El Niño: The frequency deviation of monthly Tx in El Niño summer months exceeds the monthly Tx that corresponds to the 90th percentile of the summer months of non-ENSO that was less than 0 in most stations. Out of these, P_{anom} were - 20 to - 70% in the Northwest and the Northern mountains, - 40 to - 100% in the Central region, and + 10 to - 70% in the Central highlands. In contrast, in the Northern and Southern delta regions, mainly, the frequency deviation was positive (+ 10 to + 40%). Thus, for the 90th percentile, the influence of El Niño in summer mainly led to a reduction in the probability of occurrence of the maximum values of Tx per month in most regions (except for the North and the South) in comparison to the normal summer months. Similarly, for the 95th percentile, the frequency deviation in most stations ranged from - 45 to - 100%.

La Nina: At most stations, the frequency deviations of Tx per month in La Nina summer seasons exceeds the Tx value that correspond to the 90th percentile of the non ENSO summer months, which was lesser than zero. Out of these, P_{anom} was - 20 to -50% in the North-West and Northern mountains and the South central coast, - 100% in the Red river delta and the Central highlands, and - 50 to 100% in the South. Similarly, for the 95th percentile, the frequency deviation was - 100% in all regions. It can be observed that the influence of La Nina during the summer

months also reduced the probability of occurrence of the maximum values of monthly Tx in comparison to the normal summer months.

Monthly absolute minimum temperature (Tm):

- Winter:

El Niño: Frequency deviation of monthly Tm in El Niño winter was equal to or less than the monthly Tm that corresponds to the 5th percentile in non-ENSO winter months. For most stations, the P_{anom} of Tm was lesser than 0 (- 100%), especially in the Mid-Central coast, North-West highlands with the values of - 40 to - 100%. Similarly, for the 10th percentile, the P_{anom} was - 20 to - 70% in the North-West and Northern mountains, - 40 to - 70% in the Northern delta and North central coast, - 70 to - 100% in South central coast, - 20 to - 40% in the Central highlands and - 30 to - 40% in the South.

La Nina: In contrast to the El Niño winter, in the La Nina winter months, the frequency deviation of monthly Tm was equal to or less than the monthly Tm that corresponds to the 5th and 10th percentile of the non-ENSO winter months. In most stations, the P_{anom} of Tm was positive and ranged from - 30 to 200%, with the largest values observed in the North-West and North-West highlands, the smallest in the North, the South central coast and South, in which, 100-130% of the largest value were determined in the Northern mountain, while the smallest was observed in the North central coast. Particularly in the South-West highlands, the frequency deviation is negative, from - 20 to 100%. The result reveals that the influence of La Nina during winter months led to an increase in the probability of occurrence of the lowest values of the minimum temperature in most parts of Vietnam in comparison to normal winters.

- Summer:

El Niño: Frequency deviation of monthly T_m values in the El Niño summer months was equal to or less than the monthly T_m that corresponds to the 5th and 10th percentile of the non-ENSO summer months. The P_{anom} in most stations was less than 0 and showed fluctuation in the range of - 40 to - 100% for the 5th percentile and - 40 to - 70% (except in the Central highlands with - 100%) for the 10th percentile.

La Nina: Frequency deviation of monthly T_m values in La Nina was equal to or less than the monthly T_m that corresponds to the 5th percentile of non-ENSO summer months. P_{anom} of monthly T_m in most stations was less than 0 and fluctuated between - 20 and - 100% (except Southern central highlands with 90-120%). Similarly, for the 10th percentile, most stations showed negative frequency deviations (about - 60%, except - 100% in North central highlands). In the North-West and Northern mountains, the P_{anom} was from - 60% to + 25%. It can be observed that the influence of La Nina in summer mainly led to a reduction the probability of occurrence of the minimum T_m compared to the normal summer months.

The statistical prominence of the ENSO signals provided above was assessed through the conduction of tests at two tails with a prominent level of 0.05.

The results reveal that the above conditions are acceptable; this indicates that the ENSO signal (frequency deviation) is prominent.

Conclusions

1) Monthly maximum temperature ($\overline{T_x}$) and monthly minimum temperature

($\overline{T_m}$) during El Niño in both winter and summer were higher than the ones observed in La Nina and non-ENSO conditions. The changes that took place in winter were greater than the changes that occurred in summer.

2) With regard to the 90th percentile, the impact of El Niño on $\overline{T_x}$ and $\overline{T_m}$ was considerably prominent and consistent: both $\overline{T_x}$ and $\overline{T_m}$ attained the highest values.

In response to the 10th percentile, the impact of ENSO on $\overline{T_x}$ and $\overline{T_m}$ was inconsistent across regions and no significance, especially in the summer, was observed.

3) For the absolute maximum temperature (T_x) and absolute minimum temperature (T_m), El Niño generally displayed the highest values, both in winter and summer, while La Nina generally exhibited the lowest values corresponding to the 90th and 95th percentile (for T_x) and 10th and 5th percentile (for T_m).

- The effect of El Niño on the absolute maximum temperature led to an increase in the probability of occurrence of extremes values that exceed the value of the 90th percentile in non-ENSO conditions in the Red river delta and the South in winter and summer, and led to a reduction in the possibility of occurrence in mountainous areas. The influence of La Nina caused a reduction in the extreme values that were recorded across all regions, both in winter and summer.

- For absolute minimum temperature, the effect of El Niño led to a decrease in the extreme values for the 10th and 5th percentile, both in winter and summer,

while the effect of La Nina led to an increase in the probability of common occurrence in all regions of the country compared to non-ENSO regions.

4) The ENSO information (obtained through the characteristics of frequency deviation of extreme events that occur in seasons under ENSO conditions in comparison to non-ENSO conditions) is prominent. The results demonstrate that the effect of ENSO on temperature and extreme precipitation is evident that indicates the existence of the possibility of prediction of the occurrence of extreme temperatures based on ENSO information.

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