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Ultrasound-assisted depolymerization of kappa-carrageenan and characterization of degradation product

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A B S T R A C T

Degradation of polysaccharides to afford low-molecular-weight oligosaccharides have been shown to produce new bioactivities that are not present in the starting material. The simplicity of ultrasonic treatment in the degradation of a polysaccharide, such as κ-carrageenan, offers practical advantage in producing degraded products with lower molecular weight that may have new interesting potential activities. This study embarked on investigating the effects in molecular weights and structural changes of κ-carrageenan under varying ultrasonic conditions. Molecular weight (MW) monitoring of ultrasonically-treated κ-carrageenan at various conditions were done by gel permeation chromatography. The product formed using the optimized condition was characterized using FTIR and NMR. The decrease in MW has been shown to be dependent on low concentration (5.0 mg mL⁻¹), high amplitude (85%), and long treatment time (180 mins) to afford a degraded κ-carrageenan with average molecular weight (AMW) of 41,864 Da, which is a 96.33% reduction from the raw sample with initial AMW of 1,139,927 Da. Structural analysis reveals that most of the peaks of the raw κ-carrageenan was retained with minor change. 1D and 2D NMR analyses showed that the sonic process afforded a product where the sulfate group at the G4S-4 position was cleaved forming a methylene in the G4S ring. The results would be useful in the structure-activity relationship of κ-carrageenan oligosaccharides and in understanding the effect in the various potential applications of degraded κ-carrageenan.

1. Introduction

When polysaccharides degrade, changes in the physical or chemical properties occur. The degradation is attributed to the formation of intermediate products that affect the original form, fit, and function of the substance [17]. The degradation of polysaccharides has been found to exhibit new promising activities that are absent in its original long polymeric chains. Interesting activities of degraded polysaccharides such as anti-viral, anti-tumor [6], anti-bacterial [22], and plant growth promotion (PGP) [1] properties have been reported. This phenomenon was observed in marine polysaccharides such as ulvan, alginate, fucan, laminarin, chitosan, and carrageenan. The plant growth promoting effect of degraded polysaccharides is very promising because of the economic impact that it brings to farmers. Abad et al. [1] reported that κ-carrageenan degraded by electron beam irradiation when applied to rice field by foliar spraying afforded significant increase in rice production yield from 27% up to 76%. It also showed improvement in the physical attributes that resulted in high resistance to traditional pests. The technology is very promising that it has been demonstrated to be effective in other economically important crops. In the Philippines, it has been adopted by the Department of Agriculture for use by the farmers. Investigations on the degraded κ-carrageenan solution showed that its molecular weight is low due to the radical-based degradation indicative of chain scission [1,14,15]. The general observation is that the lower the molecular weight of the degraded κ-carrageenan, the better its effect in plant growth promotion. Relleve et al. [15] reported that radiolytically degraded κ-carrageenan with molecular weight as low as 24,000 Da showed the highest growth promoting effect on bok choy plant. Aside from the observed enhancement in growth, the inhibition of chlorosis or yellowing of leaves was also observed. In another study using potato plant, Relleve et al. [14] reported that degraded κ-carrageenan having average molecular weight (AMW) of as low as...
10,000 Da showed the highest effect of plant growth promotion on potato plant. Application in peanut plant resulted in an increase in growth attributes and the highest yield of 224% more than the control (tap water) and 98% higher in yield compared to the traditional practices of farmers [1]. The use of low molecular fractions of radiolytically degraded κ-carrageenan with 20,000 Da AMW on mint through foliar spraying afforded growth attributes way better than the control including an increase in essential oil production and menthol content [9]. Despite the observed effects of the degraded κ-carrageenan, the mechanism of the plant growth action has not been fully understood yet. There is a need to characterize the active component in the degraded κ-carrageenan to understand the mechanism of the observed effects. Sonication is the process of applying sound energy to agitate the particles in a sample solution and is a significant procedure for cell disruption, deagglomeration, homogenization, nano-crystallization, nano-emulsification [11] and food processing [12]. Ultrasonication has also been employed to deliberately degrade polysaccharides. Compared to irradiation technique, which requires strict regulations and specialized equipment, ultrasonication can be done easily anytime, anywhere to afford degraded polysaccharides. Aside from irradiation and ultrasonication, other common techniques in polysaccharide depolymerization include acid hydrolysis, enzyme hydrolysis, photo-chemical depolymerization, and microwave-assisted depolymerization. The aim for these polysaccharide degradation processes is to produce low-molecular-weight products because it affords new interesting activities. In general, regardless of the degradation processes, more degraded (lower molecular weight) polysaccharides show higher application effects compared to the original form [16,26]. For ultrasonic degradation of polysaccharides, the obtained low-molecular-weight products were found to exhibit better plant growth, anti-inflammation, and antioxidant activities. The group of Jaulneau et al. [8] reported that ultrasonicated ulvan exhibited plant immunity activity. Cellular and molecular analyses indicate that the ultrasonicated ulvan elicits a jasmonic acid signaling pathway that provided tobacco plant resistance to necrotrophic pathogens. Zheng et al. [25] demonstrated that ultrasonic treatment of polysaccharide konjac glucomannan afforded degradation product having AMW of $5.64 \times 10^5$ Da. The degraded polysaccharide has been shown to increase the immune-suppression capability of konjac glucomannan towards RAW264.7 macrophages. In the study by Ogutu and Mu [10], the MW of pectin was effectively reduced through sonication technique and the antioxidant capacity was enhanced when low-molecular-weight pectin was used. Indeed, ultrasonic degradation of polysaccharides has been demonstrated to have superior agricultural and biological activities compared to its raw forms. However, the characterization of the degraded polysaccharide and the mechanism of agricultural effects have not been fully understood. Importantly, no studies have been done to optimize the potential of ultrasonication as a degradation method. No investigations have been done on the effects in the molecular weights of sonicated polysaccharides and no studies have been reported on the structural changes that occurred in κ-carrageenan after undergoing ultrasonic treatment. It is the aim of this work to investigate the parameters of ultrasonic degradation of κ-carrageenan and explore the structural changes that occurred in the chain.

2. Materials and methods

2.1. Materials description

Refined κ-carrageenan was obtained from Shemberg Philippines. The average molecular weight (AMW) of the raw κ-carrageenan (1,139,927 Da) was determined using gel permeation chromatography (GPC). To obtain a low-molecular-weight carrageenan sample, the raw κ-carrageenan powder was gamma-irradiated using Co-60 (0.5 kGy/h) at an absorbed dose of 50 kGy to afford the irradiated κ-carrageenan having AMW of 117,780 Da (GPC). All of the reagents that were used for the experiments were analytical reagents with purity of more than 98%.

2.2. Degradation through ultrasonication

Ultrasonic degradation was performed in QSONICA Q700, which is equipped with a standard ½ in. horn (5.375 in. length × 0.5 in. Diameter) with an output frequency of 20 kHz. The κ-carrageenan sample was dissolved in water at specified concentration. All ultrasonic degradation experiments were conducted by dipping the probe into a 400 mL beaker containing κ-carrageenan aqueous solution. The probe was dipped 1.5 cm above the bottom of the beaker and was positioned at the center of the beaker. The beaker containing the sample was bathed in cold water to counter the heat given off by the sonication process and the temperatures of all the sonicated κ-carrageenan solutions were maintained at 40–45 °C as monitored using alcohol thermometer.

2.2.1. Effect of ultrasonication at different initial molecular weight

The effect of the initial molecular weight of the precursor material in ultrasonic degradation was investigated using the raw (1,139,927 Da) and irradiated (117,780 Da) κ-carrageenan powders. A κ-carrageenan solution (5.0 g L$^{-1}$; 100 mL) was placed in a 450 mL beaker and underwent ultrasonic degradation for 30 mins at 85% amplitude.

2.2.2. Effect of ultrasonication at different concentration

The raw κ-carrageenan solution (100 mL) in various concentration (2.0, 5.0, 10.0 mg mL$^{-1}$) was placed in a 450 mL beaker and underwent ultrasonic degradation for 30 mins at 85% amplitude.

2.2.3. Effect of ultrasonication at different percent amplitude

The raw κ-carrageenan solution (5.0 mg mL$^{-1}$; 100 mL) was placed in a 450 mL beaker and underwent ultrasonic degradation for 30 mins at different percent amplitude (25%, 55% and 85%).

2.2.4. Effect of ultrasonication with time

The raw κ-carrageenan solution (5.0 mg mL$^{-1}$; 100 mL) was placed in a 450 mL beaker and underwent ultrasonic degradation at 85% amplitude for 30, 60, 90, 150, and 180 mins.

2.3. Gel permeation chromatography (GPC)

Gel permeation chromatography was performed in a Shimadzu HPLC system equipped with degassing unit (DGU-20A3R), high pressure pump (LC-20AD), autosampler (SIL-20A HT), column Oven (CTO-20A), and refractive index detector (RID-10A). Isocratic elution was done using 0.1 M NaCl eluent (flow rate: 0.5 mL min$^{-1}$; column temperature: 40 °C). The mobile phase and sample solutions were filtered using nylon filter with pore size of 0.45 μm. Ultrasonicated κ-carrageenan solutions were analyzed by joining two analytical columns, the SHODEX OHpak SB-805 HQ and SHODEX OHpak SB-804 HQ. The guard column SHODEX OHpak SB-G 6B was also used. The calibration curve was constructed by injecting polyethylene glycol (PEG) and polyethylene oxide (PEO) standards. Samples with concentration of 1.0 mg mL$^{-1}$ dissolved in 0.1 M NaCl and volume of 100 μL was injected in the columns through the autosampler.

2.4. Fourier transform infrared (FT-IR) spectroscopy

FT-IR analysis was performed in PerkinElmer FT-IR frontier, which is equipped with an ATR probe and a crystal stage. The dry powdered sample (10 mg) was placed in the crystal stage and was pressed by the ATR probe up to around 70% force and was scanned in 400–6000 cm$^{-1}$ for 16 times.

2.5. Nuclear magnetic resonance (NMR) spectroscopy

NMR analysis was performed using JEOL 600 MHz NMR
A mass of 5.0 mg of κ-carrageenan was mixed with 0.7 mL of D$_2$O containing 1.0 mM of sodium trimethylsilylpropanesulfonate (DSS) as internal standard and 20 mM Na$_2$HPO$_4$ buffer [21]. $^1$H NMR and $^{13}$C NMR spectra were obtained at 65 °C probe temperature with 90° pulses and relaxation delay of 5 s. Delay Alternating with Nutation for Tailored Excitation (DANTE) pre-saturation was applied at the residual water signal during $^1$H NMR analysis. The $^1$H NMR analysis was performed in 64 scans while for $^{13}$C NMR analysis, 30,000 scans were made. Chemical shifts of $^1$H NMR and $^{13}$C NMR are reported relative to the DSS methyl signal (δ = 0.00 ppm). 2D NMR (HSQC and COSY) experiments were likewise performed.

3. Results and discussion

Ultrasonication is a clean degradation method because no chemicals are added in the process. Moreover, the degradation process is more uniform because the system is constantly agitated by sound waves to affect more homogenous interaction. Since the κ-carrageenan was dissolved in water, the probe sonication system used in this work is appropriate compared to the bath system because volume and viscosity changes during the processing. The ultrasonic probe instrument is designed to deliver a constant amplitude to the liquid sample and will adjust as the liquid sample is processed. As the viscosity, concentration, and temperature of the liquid sample changes during processing, the load on the probe increases and the power supply will adjust due to the increased resistance to the movement of the probe to ensure that the
excursion at the probe tip remains constant. The initial molecular weight and concentration of κ-carrageenan as well as the sonication amplitude and time were investigated for its effects on the resulting molecular weight of the degraded sample.

3.1. Effect of initial molecular weight and concentration

The molecular weight of the starting polymeric material is an important factor in sonication condition as the chains experience various conformational changes in response to the sonic waves. For κ-carrageenan, it is an important factor to consider since its helical structure can untangle and the flexibility of the chains may vary under sonic conditions. Two κ-carrageenans with different molecular weights were investigated. The raw κ-carrageenan has an average molecular weight (AMW) of more than 1 million Da (1,139,927 Da) with a broad polydispersity index (PDI) of 2.65 while the gamma-irradiated κ-carrageenan has an AMW of 117,780 Da with even broader PDI of 3.31 (Fig. 1A). Upon sonication, the AMW of the raw sample reduced to sonicated κ-carrageenan (Fig. 2). Doubling the initial concentration from 5.0 mg mL\(^{-1}\) to 10 mg mL\(^{-1}\), sonic treatment afforded a lower reduction (86.22%) in molecular weight from 1 million Da to 157,026 Da (PDI = 2.05). Doubling the concentration increased the viscosity of the sample, which increased the sonication energy from 1.84 x 10\(^5\) J to 1.91 x 10\(^5\) J. Decreasing the initial κ-carrageenan concentration to 2.0 mg mL\(^{-1}\) decreased the resulting AMW (103,075 Da; PDI = 1.84) upon sonication, which achieved a 90.96% reduction. The energy involved in the sonication of 2.0 mg mL\(^{-1}\) κ-carrageenan reduced to 1.79 x 10\(^5\) J, which is consistent with less viscous solution. The result is attributed to the high probabilities in entanglement of polymeric chains at high viscosity and concentration, which leads to low energy transfer process between the solvent and polymer resulting in low degree of chain scission [18]. Notable is the decreasing trend in the PDI suggesting that more uniform fragments are produced when a dilute solution of the sample is sonicated. Although it was clear that a concentration of 2.0 mg/mL produced the lowest molecular weight product, the succeeding optimization uses the 5.0 mg mL\(^{-1}\) for practical reasons because it affords high enough fragments for characterizations.

3.2. Effect of sonication amplitude and time

The sonication amplitude was investigated for its effect in reducing AMW of the sample since it dictates the intensity of the wave energy. The low (25%), medium (55%), and high (85%) amplitudes were chosen based on the capabilities of the instrument. Further push to 100% amplitude was not done as the transducer heats up rapidly resulting to the overheating of the sonicator. As shown in Fig. 1C, as the % amplitude increases, there is a shift to longer retention time, which translates to the production of lower molecular weight fragments. The AMW of κ-carrageenan was effectively reduced at 85% amplitude. Varying the sonic time showed that as the sonication time increases there is a shift to higher retention time (Fig. 1D). Analysis of the interaction between amplitude and sonication time (Fig. 1E) confirms that higher amplitude at longer time facilitates the reduction of κ-carrageenan, which is attributed to the greater chances for bond scission due to the longer exposure of the polymer at high-intensity wave energy. The result is consistent with the observation that the rate of polymer degradation depends mainly on the duration of the treatment, on the concentration of the solution, and the intensity of ultrasound [2]. Increasing ultrasonic intensity increases the acoustic pressure amplitude, which facilitates the facile formation of a larger number of cavitation bubbles. The acoustic intensity defines the size of the zone of liquid where cavitation occurs and controls the probability of occurrence of cavitation events per unit volume. Depolymerization is caused by the mechanical forces that arise in the liquid due to the propagation of acoustic energy and the collapse of the cavitation bubbles [2]. As the sonication time increases, the sample exposed at 85% amplitude experiences increasing input energy

![Fig. 2. Comparison of FTIR spectra between raw κ-carrageenan (control) and sonicated κ-carrageenan at 180 mins.](image_url)
from $1.84 \times 10^5$ J at 30 mins, $5.49 \times 10^5$ J at 90 mins up to $1.00 \times 10^6$ J (95 W power) at 180 mins, which can facilitate bond breaking. The PDI observation, however, indicates that it is narrow at mid amplitude and at 60 min (Fig. 1E) but begin to widen slightly at higher amplitude and longer time. The molecular weight data of the sonicated samples revealed that at 180 mins sonication time, the molecular weight was reduced from $>1$ million Da ($\text{PDI} = 2.65$) to 41,864 Da ($\text{PDI} = 1.68$), a 96.33% decrease in AMW affording more uniform fragments. The decrease in AMW is significant because it can potentially offer superior effects in various applications such as in plant growth promoting activities. In the study by Jaulneau et al. they identified that ultrasoundication of ulvan polysaccharide afforded fragments with AMW of 40,000–60,000 Da, which was shown to best activate plant immunity against certain pathogens through jasmonic acid signaling pathway [8].

3.3. Characterization of ultrasonicated κ-carrageenan

The FT-IR analysis of degraded κ-carrageenan ultrasonicated for 180 mins revealed a spectrum similar to the raw κ-carrageenan (Fig. 2) indicating that the functional groups and molecular framework is retained. Notable, however, is the decrease in intensity at 843 cm$^{-1}$, which corresponds to sulfate group in κ-carrageenan suggesting the cleavage of some sulfate groups from the chain. Also notable is the observed decrease in the pH of the solution from pH = 8.5 to pH = 5.2 after sonication for 60 mins indicative of the release of protons from the sulfate ester groups into the solution.

The decrease in molecular weight is attributed to the scission along the chain especially at the O-glycosidic bond, which is prone to hydrolytic cleavage. In the hydrolysis studies of κ-carrageenan, Yu et al. [24] reported that κ-carrageenan produced low-molecular-weight products and unraveled the fragmentation pattern of the κ-carrageenan oligosaccharides through negative-ion electrospray ionization (ESI) mass spectrometry. Their results indicated that bond scissions occur at the glycosidic bonds. The reduction of the pH during the sonication process provides the necessary condition for the hydrolysis to occur.

The $^1$H NMR spectrum of the raw κ-carrageenan (Fig. 3A) has nine distinct peaks (Table 1) within the δ 3.0 to around δ 5.2 region. The G4S unit of κ-carrageenan shows resolved $^1$H peaks at δ 4.84 corresponding to sulfated methine (G4S-4), and at δ 3.98 and δ 3.60 corresponding to oxymethine protons located at G4S-3 and G4S-2, respectively. The deshielded peak at δ 5.10 is due to the anomeric proton (DA-1) of the DA unit. Oxymethylene protons at DA-6 appears as non-equivalent geminal protons with $^1$H peaks at δ 4.05 and at δ 4.13, respectively. The oxymethylene protons at DA-8 overlaps at δ 3.89. All $^1$H NMR chemical shifts of κ-carrageenan agrees with previous report as shown in Table 1.

![Fig. 3. $^1$H NMR spectra of (A) raw κ-carrageenan and (B) κ-carrageenan sonicated at 180 min. Condition: D$_2$O, DSS internal standard, 20 mmol of buffer (Na$_2$HPO$_4$). Peaks with marked $\times$ refer to the DSS internal standard.](image)

**Table 1** $^1$H NMR spectral data for raw κ-carrageenan.

| Position* | Chemical Shift δ (ppm) | Reference (ppm) [20] |
|-----------|------------------------|----------------------|
| DA-1      | 5.10                   | 5.17                 |
| G4S-4     | 4.83                   | 4.94                 |
| DA-5, G4S-1, DA-4 | 4.63 | 4.68, 4.70, 4.73 |
| DA-3      | 4.52                   | 4.61                 |
| DA-6      | 4.21/4.05              | 4.30/4.16            |
| DA-2      | 4.13                   | 4.22                 |
| G4S-3     | 3.98                   | 4.15                 |
| G4S-5, G4S-6 | 3.80 | 3.89               |
| G4S-2     | 3.60                   | 3.68                 |

The $^{13}$C NMR spectrum (Fig. 4A) of κ-carrageenan shows 12 peaks (Table 2), which is consistent the structure of G4S and DA units. Notable $^{13}$C NMR peaks are the deshielded anomeric carbons at G4S-1 and DA-1, which appears at δ 104.7 and δ 97.3, respectively. The peak at δ 76.2
corresponds to sulfonated methylene carbon at G4S, and the shielded 13C NMR peak at δ 26.4, which is typical for methyl or methylene carbons. The HSQC spectra (Fig. 5) shows correlation between 1H at δ 1.17 ppm and 13C δ 26.4 indicating that the new peak in 1H NMR spectra (δ 1.17) is directly attached to the new 13C NMR peak at δ 26.4. The 1H-1H correlation between G4S-3 proton (δ 3.98) and new proton peak at δ 1.17 in the COSY spectra of ultrasonicated κ-carrageenan (Fig. 6) revealed that the new 1H NMR peak is correlated three bonds away with G4S-3 1H suggesting that either the G4S-2 or G4S-4 positions have transformed into a methylene (CH2) group. The G4S-4 position is attached to the sulfate ester, which can be cleaved easily because the sulfate can exist as ion in the solution. This is consistent with the observed decrease in FTIR intensity of the sulfate absorbance. Considering the data, ultrasonication of κ-carrageenan converts the sulfated methine at G4S-4 to a methylene via removal of the sulfate group (Scheme 1) affording the ultrasonically degraded κ-carrageenan 2.

The mechanical rupture of polymer chains leads to the formation of free valences on the ends of the chain fragments. In general, ultrasound-assisted degradation of organic polymers takes place via two mechanisms, the homolytic bond breaking and the subsequent reaction with radicals. The homolytic bond breaking is usually caused by mechanical action due to the cavitational collapse of microbubbles. Polymer molecules are distorted and stretched as they enter the area of high velocity gradients generated by collapsing cavitation bubbles. At the final stage of collapse, the shock wave radiated from the cavity generates the stresses within the polymer molecule causing the bond to rupture. Moreover, the collapse of microbubbles has been shown to be accompanied with localized high temperature (up to 5000 °C) and high pressure (about 5 × 10^7 Pa) causing bonds to break [13]. In the case of polysaccharides, the glycosidic bonds can easily be broken. The second known mechanism is the generation of H· and OH· radicals through the ultrasonic decomposition of water at the bubble/water interface especially in the presence of oxygen. The production of reactive free radicals is facilitated by ultrasonic energy or through the creation of intense heat (hot) spots in the systems. During sonication process, microscopic vacuum bubbles form and then collapse into the solution. The local temperature and pressure within a collapsing microbubble can go very high and emission of light of more than 10^7 photons per flash can also be achieved [5]. These conditions cause the water molecules to pyrolyzed forming radicals in the gas phase (Rxn 1). Recombination of radicals may also occur (Rxn 2). Under oxygen atmosphere, there is an improvement of sonochemical activities due to the possibility of thermal dissociation of oxygen molecule leading to production of additional hydroxyl and hydrogen radicals (Rxn 3–8) [23].

![Fig. 4. 13C NMR Spectra of (A) raw κ-carrageenan and (B) κ-carrageenan sonicated at 180 min. Condition: D₂O, DSS internal standard, 20 mmol of buffer (Na₂HPO₄). Peaks with marked × refer to the DSS internal standard.](image-url)
H₂O→·OH + ·H  
·OH + ·OH→H₂O₂  
O₂→2·O  
·O + H₂O→2·OH  
·O + H₂→·OH + ·H  
·OH + H₂O₂→H₂O + HO₂·  
·O + HO₂·→·OH + O₂  
·O + H₂O₂→·OH + HO₂

Both the H₂O₂ and ·OH are strong oxidizing agents that can oxidize organic polymer such as κ-carrageenan causing degradation [7]. In depolymerization reactions, free radicals are formed in the fragment ends and the shear stresses not only dissociate the bonds in the main chain, but also separate the radicals formed preventing their recombination [4]. The radical species can facilitate the homolytic cleavage of the sulfate bond while the ·H can form a new bond at G₄S-4 replacing the sulfate group. The release of sulfate groups would also result in the release of protons into the solution. This bond cleavage mechanism is consistent with the observed increase in acidity (from pH = 8.5 to pH = 5.2) of the resulting ultrasonic solution.

4. Conclusion

The degradation of κ-carrageenan has been demonstrated to be efficient under ultrasonic treatment. A significant reduction in molecular weight (MW) has been observed when the MW of the initial starting material is high. High sonication amplitude and long treatment time promote high degree of MW reduction. FTIR characterization of the ultrasonically degraded κ-carrageenan reveals the retention of absorbance peaks with notable decrease in the peak for sulfate functional group. NMR analysis reveals that most of the peaks of the κ-carrageenan repeating units are retained with notable formation of a methylene peak as shown in ¹H and ¹³C NMR spectra. Heteronuclear 2D NMR analysis (HSQC) showed that the new ¹H peak is attached to the new ¹³C peak. Homonuclear 2D data (COSY) showed that the new ¹H peak is correlated 3 bonds away to G₄S-3 proton. The new methylene peak was assigned to the G₄S-4 proton suggesting that the sulfate group has been cleaved from the G₄S ring to afford the proposed fragment 2 in the κ-carrageenan repeating units. Further studies are recommended to test the effectivity of ultrasonic-treated κ-carrageenan for its effect in plant growth promotion, antiviral, and antibacterial activities.

CRediT authorship contribution statement

Mariel G. Tecson: Conceptualization, Methodology, Investigation, Writing - original draft. Lucille V. Abad: Conceptualization, Methodology, Writing - review & editing. Virgilio D. Ebajo: Methodology, Software, Writing - review & editing. Drexel H. Camacho: Conceptualization, Investigation, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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