Formulation of choline chloride/ascorbic acid natural deep eutectic solvent: characterization, solubilization capacity and antioxidant property

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Abstract

In the present study, natural deep eutectic solvent composed of choline chloride and ascorbic acid (CHCL/AA NADES) was formulated for enhancing the solubility and antioxidant properties of antioxidant extracts from fruit wastes of Mangifera pajang. The solubilities of Mangifera pajang’s antioxidant extracts in water and CHCL/AA NADES at different water contents (0 – 50 wt%) were investigated. It was observed that the antioxidant extracts were most soluble in the CHCL/AA NADES with 10 wt% of water, and the concentration of antioxidant was found to be approximately 15% and 4% as compared to water and pure CHCL/AA NADES, respectively. The positive effect of water on NADES can be related to the reduced viscosity of NADES, where the viscosity decreased up to 74% upon addition of water. Aside from that, all the tested CHCL/AA NADES enhanced the antioxidant capacity of antioxidant extracts by 1.3–14.64% compared to the antioxidant extracts in water. This finding highlights the role of CHCL/AA NADES as an antioxidant capacity enhancer. Noteworthy, the antioxidant extracts solubilized in the CHCL/AA NADES system formed a nano-scale cluster structure, as depicted by the TEM image, suggesting that the CHCL/AA NADES could potentially use in nanoformulation that provides protection to the antioxidant extracts.

Keywords: Antioxidant; Antioxidant activity; Choline chloride/ascorbic acid natural deep eutectic solvent; Mangifera pajang; Solubility.
1.0 Introduction

Over the past two decades, ionic liquids have been regarded as potential solvent due to its unique properties such as low melting point, chemical and thermal stability and low vapor pressure. Considering these properties, ionic liquids are widely used in medicine, chemistry and nanotechnology industries (Zdanowicz, Wilpiszewska, & Spychaj, 2018). Notably, ionic liquids are also exploited in the formulation of Active Pharmaceutical Ingredients (API) and drug delivery. Research has shown that the utilization of ionic liquids could enhance the solubility of drugs and facilitate the delivery of bioactive compounds to the targeted site (Halayqa, Zawadzki, Domańska, & Plichta, 2019). Nevertheless, the toxicity and high cost of ionic liquids often limits their applications (Kudłak, Owczarek, & Namieśnik, 2015).

For these reasons, deep eutectic solvent (DES) which can be prepared by mixing 2 or 3 hydrogen donor (HBD) and hydrogen acceptor compounds (HBA) has been proposed by Abbott, Capper, Davies, Rasheed, and Tambayarajah (2003). Upon mixing, the HBD and HBA components will interact with each other through hydrogen bonding (Jiang et al., 2019; Zhang, Vigier, Royer, & Jerome, 2012). The most commonly employed hydrogen bond acceptor is choline chloride while the hydrogen bond donors are amino acids, alcohol, carboxylic acids and sugars (Dai, van Spronsen, Witkamp, Verpoorte, & Choi, 2013). One of the unique features of the DES solution is that it has a lower melting point than that of its individual HBD and HBA components (Meng, Zhao, Duan, Guan, & Zhao, 2018). DES exhibits the advantages of ionic liquid and not only that, it is low cost and low or non-toxic, renewable and often biodegradable (Tomé, Baião, da Silva, & Brett, 2018). Moreover, the physicochemical properties of DES could be easily altered to suit vast applications (Cunha & Fernandes, 2018; Mbous et al., 2017; Tomé et al., 2018). In particular, Choi et al. (2011) prepared the eutectic mixtures of two or more natural compounds and denominated them as natural deep eutectic solvent (NADES). Since NADES are synthesized by natural compounds, they have no adverse effects and compatible with food, pharmaceutical and cosmetic formulations (Benvenuti, Zielinski, & Ferreira, 2019). Owning to these properties, the number of publications adopting these solvents as a solubilization vehicle for bioactive compounds was exponentially growing (Dai et al., 2013; Faggian et al., 2016; Y. Liu et al., 2018; Morrison, Sun, & Neervannan, 2009; Shamseddin et al., 2017; Silva, Reis, Paiva, & Duarte, 2018; Sut et al., 2017). These reports confirmed the effectiveness of NADES in improving the solubility of bioactive compounds and can be used as solubilization enhancers in the development of drug delivery system. Among these reports, the NADES formulation...
designed by Silva et al. (2018) is found to be interesting as the components used to synthesize
the NADES, choline chloride and ascorbic acid are green from the point of view of green
chemistry. Such that choline chloride is a non-toxic nutrition additive which is approved
under Council Directive 70/524/EEC8 (Radošević et al., 2015), where the benefits of
ascorbic acid (which is also referred to as Vitamin C) as dietary antioxidant are universally
recognized. Predominantly, ascorbic acid readily scavenges reactive oxygen species (ROS),
modulate neurological functions and prevent scurvy (Cheng et al., 2018). It is evident that the
NADES composed of choline chloride and ascorbic acid offer exciting possibility in for
solubilizing vehicle for bioactive compounds. However, it is rather surprising that no study
to-date has provided information regarding the effect of this NADES system on the solubility
of antioxidant compounds.

Antioxidant compounds are known as molecules that are able to stabilize, inhibit,
deactivate and scavenge free radicals, thus protecting the human body against oxidative
damage (Oroian & Escriche, 2015). Knowing the role of antioxidant compounds in reducing
or preventing the oxidative damage, numerous antioxidant sources have been studied. In
particular, Agudo et al. (2007) pointed out that many of the underutilized indigenous fruits
possess good antioxidant properties that might be useful as phytomedicine. Mangifera pajang
is found to be one notable underutilized fruit by the fact that they are locally available but
universally erratic. Previous study performed by Abu Bakar, Mohamed, Rahmat, and Fry
(2009) shown that the fruit wastes of M. pajang (peels and kernels) contain higher level of
antioxidant compared to its flesh, and yet the fruits remain under-utilized. Furthermore, a
recent review paper highlighted its antioxidant properties and economic potential, bringing
about a bright prospect to utilize the fruit wastes as natural sources of antioxidant products
(Jahurul et al., 2019).

Therefore, in this work, we prepared and characterized the choline chloride/ascorbic acid
natural deep eutectic solvent (CHCL/AA NADES). Subsequently, we evaluate the solubility
of antioxidant extracts in the aqueous form and the choline chloride/ascorbic acid NADES
antioxidant compounds of fruit wastes of Mangifera pajang. Moreover, the feasibility of this
eutectic mixture to enhance the antioxidant activity and morphology of antioxidants in
CHCL/AA NADES were studied.
2.0 Materials and methods

2.1 Chemicals and materials

Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent powder and absolute ethanol were purchased from Merck (Germany), where the aqueous ethanol was prepared by mixing the absolute ethanol and distilled water at right composition (54%). Choline chloride was purchased from Sigma-Aldrich (Germany). All chemicals and reagents used were of analytical grade. The fresh fruits of *M. pajang* were collected from a retailer in Sarawak, Malaysia. The fruit wastes of the fruit (peels and kernels) were separated and dried at 60 °C for 24 hours. The dried fruit wastes were grounded into fine powders, kept in an air-tight container and stored in a freezer (-20 °C) until further analysis (Abu Bakar et al., 2009).

2.2 Extraction of antioxidant compounds from fruit wastes of *M. pajang*

The extraction was conducted based on optimum parameters obtained from previous study (Ling, Chan, & Nandong, 2020). Briefly, 0.5 g of peel powders and 0.5 g of kernel powders were mixed with 30 ml of 54% aqueous ethanol and extracted for 2 h at 51 °C using an incubator shaker (SASTEC) which set at a speed of 178 rpm. The mixture was then subjected to centrifugation at 4000 rpm, 25 °C and 20 minutes. The supernatant was collected and the solvent was evaporated using a rotary evaporator (1100S-WD, EYELA). Finally, the antioxidant suspension was freeze dried using freeze dryer (Fisher 1.5L, Labconco). The antioxidant powders were stored in amber storage bottle and kept in a freezer for further analysis.

2.3 Preparation and characterization of choline chloride/ascorbic acid natural deep eutectic solvent

The natural deep eutectic solvent was prepared by using the method of Silva et al. (2018) with modifications. The choline chloride and ascorbic acid were weighted at molar ratio of 1:1, 1:2 and 2:1 by using an analytical balance (Sartorius balance QUINTIX 613-1S, Germany). The mixture was heated at 40°C under constant stirring speed of 100 rpm by using a heating plate (Bibby Scientific, UK) until the formation of a homogenous clear liquid. The mixture was allowed to cool to room temperature before storing in the storage bottle for further analysis.
2.4 Characterization of deep eutectic solvent

2.4.1 Polarized optical microscopy (POM) analysis

A small droplet of natural deep eutectic solvent at molar ratio of 1:1, 1:2 and 2:1 was deposited on a microscopic slide for observation at a magnification of 10x. The polarized light image was observed at room temperature and captured using a Nikon ECLIPSE LV100N polarizing microscope (Nikon Corporation, Japan) coupled with Nikon DS-Fi2 camera and the analyzer software (Silva et al., 2018). When there is no presence of solid crystalline structure, the polarized light image is uniformly black (Aroso et al., 2016).

2.4.2 Proton nuclear magnetic resonance ($^1$H NMR) analysis

The $^1$H NMR analysis has been used to study the ionization states of proton and determine the structure of the molecules. It quantitatively determines the distribution of protons by detecting the proton spectra (Delso, Lafuente, Muñoz-Embíd, & Artal, 2019; Tan et al., 2016). The $^1$H spectra were recorded at 500 MHz on a JEOL ECA 500 spectrometer (JEOL, USA). The solvent used was dimethyl sulfoxide-d$_6$ (DMSO-D$_6$) and tetramethylsilane (TMS) was used as a reference with the chemical shift values ($\delta$) are quoted in part per million (ppm).

2.4.3 Fourier transform infrared spectroscopy (FTIR) analysis

The NADES was scanned by using FTIR spectroscopy (Agilent Technologies Cary 630, USA), at a resolution of 4 cm$^{-1}$ in the wavelength range of 4000 to 400 cm$^{-1}$. The functional group present in the sample was analysed using IRsolution FTIR control software associated with the instrument.

2.4.4 Viscosity measurement

The viscosity of CHCL/AA NADES at different water level were measured using a viscometer (Fann Model 36SA) at a speed of 6 rpm.

2.5 Solubility measurement

The solubility testing was carried out according to the method which described by Soares et al. (2017) with slight modifications. An excess amounts of antioxidant powders were added to the CHCL/AA NADES. The mixture was shaken for 24 h using an orbital shaker (KS 501 digital shaker, IKA WERKE, Germany) at 100 rpm to ensure homogeneity. After the saturation, the samples were subjected to centrifugation for 25 min at 1500 rpm. The supernatant was subjected to dilution and assayed by using a UV-visible spectrophotometer (LAMBDA Bio, PerkinElmer, USA) at the optimum wavelength (212nm and 277nm).
Besides that, aqueous NADES were prepared by adding different concentration of distilled water (10 to 50 wt%) to study the effect of water content on the solubilization capacity of NADES. Each experiment was repeated three times. A calibration curve using the antioxidant extracts and CHCL/AA NADES as the standard was prepared for quantification.

2.6 DPPH Analysis

The antioxidant activity of the antioxidant-CHCL/AA NADES were estimated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as free radical model which adapted from Magalhães, Segundo, Reis, and Lima (2006) and Brand-Williams, Cuvelier, and Berset (1995). An aliquot of 0.1 ml of extract or control (absolute ethanol) was mixed with 3.9 ml of 6 × 10⁻⁵ mol/L DPPH in absolute ethanol. The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark condition. The mixture was measured using a UV-visible spectrophotometer (LAMBDA Bio, PerkinElmer, USA) at 517 nm. A standard of ascorbic acid was prepared according to the method described by Abu Bakar et al. (2009). A standard curve was obtained by plotting the logarithm of absorbance of control/absorbance of sample versus its concentration ranging from 0.02 to 0.5 mg/ml. The slope and intercept of the line provide a relationship between the absorbance and concentration. The final result was expressed as mg ascorbic acid equivalent antioxidant capacity in 1 g of sample (mg AEAC/g).

2.7 TEM analysis

The antioxidants were dissolved in CHCL/AA NADES and was scanned using transmission electron microscopy (TEM) analysis. The morphology and size of the sample was visualized and determined by using TEM. 1 ml of the sample was dispersed in 20 ml of absolute ethanol and sonicated. A drop of diluted sample was dropped on a formvar-coated copper grid. The grid is then fixed over the stage of TEM. The TEM micrograph was observed via a transmission electron microscopy (JEOL Model 1230).

3.0 Results and Discussions

3.1 Characterization of choline chloride/ascorbic acid NADES

3.1.1 POM analysis

In this work, we prepared CHCL/AA NADES at different molar ratio (1:1, 1:2 and 2:1) and the formation of a liquid at room temperature was assessed by using POM analysis. POM images allow the observation of the presence of crystals, where the POM image is uniformly black if there is no crystal-like structure. The POM images obtained are illustrated in Table 1.
The result of the POM analysis corroborates with the visual observations, where among all
the three tested mixture, only the CHCL/AA NADES at molar ratio of 2:1 present the aspect
of clear and transparent liquid without any visible crystals and retain it upon cooling, as
evidence in Table 1. On the other hand, the visual observations and POM image for
CHCL/AA NADES (1:1) CHCL/AA NADES (1:2) denoted the presence of crystals. In the
case of CHCL/AA NADES at molar ratio of 1:1, only small amounts of crystals were
noticed; nonetheless, the NADES still retain as a liquid after 24 hours. Whereas, when the
AA component is in excess, instead of clear liquid, a viscous pasty-like solid was obtained.
The mixtures are very viscous, making them very difficult to manipulate. These observations
show that the molar ratio of the NADES components define the physical structure of
NADES. In fact, in a general ‘eutectic’ solvent, at the eutectic ratio, the intermolecular
hydrogen forces between the two components are balanced. The formation of hydrogen
bonds, which generate significant intermolecular order prevents the mixture from crystallize
and forms a stable deep eutectic mixture (Hammond, 2019). That said, an excess or lack of
either one of the NADES component can make the mixture metastable and results in the
formation of crystalline particles. The POM images obtained in this study are with good
agreement with the previous study conducted by Silva et al. (2018) and therefore, the
CHCL/AA NADES at the molar ratio of 2:1 were prepared for subsequent studies.

3.1.2 $^1$H NMR analysis

The $^1$H NMR analysis has been used to study the ionization states of proton and determine
the structure of the molecules. It quantitatively determines the distribution of protons by
detecting the proton spectra (Delso et al., 2019; Tan et al., 2016). The $^1$H NMR analysis on
the synthesized CHCL/AA NADES and its neat component, choline chloride and ascorbic
acid were performed. Figure 1 presents the proton spectra of CHCL/AA NADES. By
comparing the NMR signals, it was found that the signals for hydroxymethyl groups (R-CH$_2$-
OH) of both choline chloride and ascorbic acid were overlapping, whereas the rest of the
signals can be basically assigned to the molecular groups of choline chloride and ascorbic
acid, manifesting that the high purity of CHCL/AA NADES. The chemical shift for choline
chloride (Figure A.1), appeared at δ (ppm) 3.093 (s, 9H, CHCL-H$_{1,2,3}$), 3.394 – 3.428 (m, 2H,
CHCL-H$_{4}$) and 3.737 – 3.749 (m, 2H, CHCL-H$_{5}$). On the other hand, the chemical shift of
ascorbic acid (Figure A.2) was observed at δ (ppm) 3.737 - 3.749 (m, 3H, AA-H$_{6,7}$), was
overlapped with CHCL and 4.718 (s, 1H, AA-H$_{8}$). These findings explain that the OH groups
of ascorbic acid may interact with choline chloride and then form intermolecular hydrogen
bonds which enhances the solvation capacity of NADES (Huang et al., 2018). Considering the formation of hydrogen bonds, the possible hydrogen bonding interaction between choline chloride and ascorbic acid are also depicted in Figure 1 along with the $^1$H NMR spectra.

### 3.1.3 FTIR analysis

The formation of CHCL/AA NADES and their interaction between the two molecules can be observed by using a FTIR spectroscopy. The FTIR spectra of pure choline chloride, ascorbic acid and their eutectic mixture are illustrated in Figure 2. Figure 2(A) shows the spectra of pure choline chloride. Predominantly, a broad band at 3220 cm$^{-1}$, which belongs to O-H stretching vibration and a vibration band at 3011 cm$^{-1}$, which can be referred to the stretching mode of C-H stretching vibration were observed. Meanwhile, vibration bands at 1483 cm$^{-1}$ refer to an alky group was also observed confirming the characteristic of choline chloride (Delgado-Mellado et al., 2018). On the other hand, a strong absorption at 1751 cm$^{-1}$ and intense band at 1654 cm$^{-1}$, which can be ascribed to the C=O stretching and C=C stretching vibrations, respectively were noticed in Figure 2(B), corresponding to the characteristics of ascorbic acid (Yohannan Panicker, Tresa Varghese, & Philip, 2006).

It was reported that the formation of NADES are relies on hydrogen bonding (Vanda, Dai, Wilson, Verpoorte, & Choi, 2018; Wang, Liu, Zhao, Wang, & Yu, 2019). In infrared spectroscopy, the changes of bond length and corresponding vibrations of hydrogen bonds can depict the establishment of hydrogen bond (Maréchal, 2007). It can be noticed that the spectrum of natural deep eutectic solvent which presented in Figure 2 was roughly an overlap of the choline chloride and ascorbic acid, especially the vibration band that correspond to the amino group (864 – 1200 cm$^{-1}$). As can be seen, the vibration bands which appeared at 3220 cm$^{-1}$ on spectrum of choline chloride was shifted to 3250 cm$^{-1}$ in the CHCL/AA NADES. The shifting of the O-H stretching vibration suggests the formation of hydrogen bonding between the pure choline chloride and ascorbic acid when the NADES is formed. Moreover, the C-H stretching vibration which was observed at 3011 cm$^{-1}$ disappeared when the NADES is formed, indicating the existence of hydrogen bonding (Liu, Zhang, Chen, & Yu, 2018). In addition, comparing Figure 2(B) and Figure 2(C), it can be noticed that the absorption bands of ascorbic acid in the range of 3200 cm$^{-1}$ to 3600 cm$^{-1}$ become broader and wider bands in Figure 2(C). This could once again ascribe to the formation of more hydrogen bonds between ascorbic acid and choline chloride (Yue, Jia, Yao, Sun, & Jing, 2012).
### 3.2 Solubility testing

The solubility of bioactive compounds is one of the key parameters to ensure sufficient bioactivity level for health benefit effects. Here, we studied the solubility of-* M. pajang*’s antioxidant compounds in aqueous and CHCL/AA NADES at different water contents (0 – 50 wt%). Prior to the solubility testing, the optimum wavelength of antioxidant compounds were accessed to be 212 nm and 277 nm by using UV-visible spectrophotometer, which was found to corroborate to the structure of antioxidant compounds (Friedman & Jürgens, 2000).

Considering the solubility of bioactive compounds is a key parameter to ensure sufficient bioactivity level for health benefit effects, the solubilization capacity of CHCL/AA NADES system was evaluated and the results were shown in Table 2 and Figure 3. It was found that the solubility of antioxidant extracts in CHCL/AA NADES were increased by approximately 11% as compared to water, indicating that the CHCL/AA NADES system exhibit outstanding solubilization capacity. With respect to this finding, the solvation capacity of DES was also evidenced in the study reported by Duarte et al. (2017), where the solubility of ibuprofen was increased up to 12-fold when incorporated in a choline chloride-based DES. Additionally, Li and Lee (2016) found that DES based on choline chloride, glycolic acid and oxalic acid significantly improved the solubility of itraconazole up to 53600-fold, as compared to their aqueous solubility. Remarkably, the increment of solubility in this study is rather low as compared to these studies, which is reasonable, considering that the bioactive compounds they studied are hydrophobic and have relatively low solubility. In our case, the antioxidant extracts used in this study are mainly phenolic acid which is hydrophilic in nature (generally favors the hydrophilic interaction with water) (El Riachy, Priego - Capote, León, Rallo, & Luque de Castro, 2011; Jahurul et al., 2019; Materska, 2010). Since the antioxidant extracts can interact well with the water, the magnitude of increment is smaller. Even so, the solubility of the antioxidant extracts in the CHCL/AA NADES is still substantially higher compared to its solubility in water. This suggests that the CHCL/AA NADES plays an important role as a liquid phase for solubilizing the hydrophilic bioactive compounds.

It was reported that the addition of water could increase the solubilization capacity of the NADES (Dai et al., 2013). So, in a further step, the effects of different percentage of water (10 - 50 wt%) on the solubilizing capacity of CHCL/AA NADES system was also studied. Based on Table 2 and Figure 3, the solubility of antioxidant extracts in aqueous CHCL/AA NADES displayed the same behavior at both optimum wavelengths. It was found that antioxidant extracts are most soluble in CHCL/AA NADES system with 10 wt% of water,
where the solubility was increased by approximately 15% and 4% as compared to water and pure CHCL/AA NADES, respectively. This finding is in good agreement with the study reported by Dai, Witkamp, Verpoorte, and Choi (2015), where they found that addition of water improved the solvation ability of DES. However, it was observed that the solubility decreased dramatically when the water content increased from 20% to 50%, reaching nearly the same solubility values in water. It is known that the formation of NADES relies on intermolecular hydrogen bonds between the constituent components. As water is introduced into the system, hydrogen bonds between the components are broken and new hydrogen bonds are established between them and water. In this condition, the physicochemical property of the NADES change (Gabriele, Chiarini, Germani, Tiecco, & Spreti, 2019). In this context, upon dilution with water, the hydrogen bonding between the components of NADES broken and this may lead to disappearance of NADES supermolecular structure. The hydrogen bonds between the choline chloride and ascorbic acid are likely to break and the dilution of water creates an aqueous solution of the free forms of the individual components (Meng et al., 2018). At this stage, the CHCL/AA NADES system losses its unique properties and exists like a liquid with individual choline chloride and ascorbic acid. This is in agreement with previously reported experiments (Dai et al., 2013; Gutiérrez, Ferrer, Yuste, Rojo, & del Monte, 2010). A simple illustration of the rupture of hydrogen bonds is presented as Figure 4.

Another interesting finding is that the solubility of antioxidant compounds in the CHCL/AA NADES system with 10 and 20 wt% water was much higher than that in the pure CHCL/AA NADES system. The positive effect of water on NADES can be related to the reduced viscosity of NADES. Referring to Figure 5, the viscosity of CHCL/AA NADES system is affected by the water level. It was observed that the viscosity reduced from 51570 cP to 8.92 cP with the addition of water. It was reported that high viscosity of NADES is correlated to the presence of extensive hydrogen bonding interaction between the components of NADES (Dai et al., 2015). Clearly, pure CHCL/AA NADES which possess extensive hydrogen bonds network are relatively viscous (51570 cP). High viscosity then hinders the solubilization capacity as there is no space to dissolve the solutes. When 10 wt% of water added to the CHCL/AA NADES system, the viscosity decreased up to 74% as compared to pure NADES. The reduction of viscosity then causes changes in the structure of NADES, where the H-bonded structure is loosened, this provides more spaces for the solubilization of the antioxidant extracts, and consequently increase the solubility (Dai et al., 2015).
Another potential explanation could be attributed to the polarity of antioxidant extracts and the CHCL/AA NADES system. Our study showed that the CHCL/AA NADES system with 10 wt% of water resulted in highest solubility of antioxidant extract, whereas the antioxidant extracts in both CHCL/AA NADES system with 50 wt% of water and water exhibited a similar solubility result. This finding suggests that the solubility of antioxidant extracts is influenced by the polarity of CHCL/AA NADES system, where the polarity can be affected by the addition of water. From this finding, one conclusion can be drawn, the polarity of *M. pajang*’s antioxidant compounds are identical to the polarity of CHCL/AA NADES system with 10 wt% of water. It is also interesting to point out that upon dilution of water, the polarity of CHCL/AA NADES increases to a polarity index similar to the polarity of water, hence, results in similar solubility value in water. The influence of polarity on the solubility is supported by study reported by Dai et al. (2013), where they found that the addition of 5% of water to the DES increase the polarity and further enhance the solubility of rutin. As demonstrated in this section, it is noteworthy that CHCL/AA NADES system can indeed enhances the solubility of bioactive compounds which makes that the nutraceutical and pharmaceutical applications of CHCL/AA NADES system more feasible.

### 3.3 Antioxidant assay

Following the solubility testing, the antioxidant extracts that solubilized in water and CHCL/AA NADES system with different percentage of water (10 – 50 wt%) were prepared and subjected to DPPH radical scavenging assay to investigate the antioxidant capacity. DPPH assay is selected as it is a promising way to indicate the presence of antioxidant compounds. The assay is easy and low cost since the radical compounds is relatively stable and need not be generated (Kedare & Singh, 2011). The DPPH assay results are illustrated as Figure 6. The DPPH assay showed that the highest antioxidant activity was obtained at aqueous CHCL/AA NADES system with water inclusion of 10 wt% (12.53 mg/g). Remarkably, all the tested antioxidant-loaded CHCL/AA NADES solution exhibited a higher antioxidant potential (1.3-14.64%) than the antioxidant in aqueous form. Greater antioxidant activity can be attributed to one of the components of CHCL/AA NADES system, ascorbic acid. Ascorbic acid is well known natural antioxidant due to its capability in scavenging free radical species (ROS) (Putchala, Ramani, Sherlin, Premkumar, & Natesan, 2013). The inclusion of ascorbic acid may contribute to the increased antioxidant activity of antioxidant-CHCL/AA NADES system. This fits into our hypothesis of the role of NADES. Another notable finding is that the antioxidant activity of CHCL/AA NADES system with 10 wt%, 20
wt% and 30 wt% of water content slightly outperformed the pure CHCL/AA NADES system. This phenomenon is coherent with the above-mentioned effect of viscosity on the solubility of antioxidant compounds. It implies that the physicochemical properties of NADES may exert a big effect on the antioxidant activity. Given this information, it can be concluded that CHCL/AA NADES system with 10 wt% water shows an excellent ability in improving the antioxidant activity, which once again highlights its potential in food, nutraceutical and pharmaceutical industry.

3.4 Morphology of antioxidants in the CHCL/AA NADES system

The morphology of antioxidants in the CHCL/AA NADES system was observed using transmission electron micrographs (TEM), as depicted in Figure 7(A) and (B). From the Figure 7(A), it was observed that the sample of particles were spherical in shape with average diameter of 50.05 nm and all the particles are within the range of 15.78 – 101.78 nm, indicating that the fabricated system has the potential to be formulated into nano-formulation system (Manuel et al., 2019). Aside from that, Figure 7(B) clearly shows that the antioxidants dissolved in CHCLC/AA NADES system formed cluster structure, where an amount of antioxidant extracts aggregated and well-rounded in the center of CHCL/AA NADES (lighter color). With this observation, it can be said that the CHCL/AA NADES may act as a carrier system which provide an enhanced stabilization effect (Shin, Kim, & Park, 2015). The agglomeration is possible when the antioxidant come into contact with the CHCL/AA NADES droplets, where they enter the droplet until the droplet internal volume is saturated with the particles and finally lead to formation of aggregates with spherical shape (Maghsoodi, Derakhshandeh, & Yari, 2012). Another explanation for the formation of aggregated clusters of antioxidants could be ascribed to the heterogeneity of mixture, where Hakkinen, Alshammari, Timmermann, D’Agostino, and Abbott (2019) stated that the addition of water improved the heterogeneities in the mixture of alcohol-DES and found that self-aggregated clusters of molecules become more significant at heterogenous mixture. Since 10 wt% of water was added to the CHCL/AA NADES, it is possible that the water acts as a liquid solute and distribute the heterogeneity in the mixture. Consequently, the diffusion of antioxidants become slower and the aggregates are formed. Taking into account that some degree of agglomeration of antioxidants which are not incorporated in the center of CHCL/AA NADES may be attributed to the inadequate amount of CHCL/AA NADES, where the NADES is insufficient to contain all the antioxidant extracts inside. With these
observations, CHCL/AA NADES system show a clear protective effect and can be denoted as a potential carrier to protect the antioxidants or other bioactive compounds.

4.0 Conclusion

In this study, a NADES system composed of choline chloride and ascorbic acid (CHCL/AA NADES) was prepared and characterized. The solubility behavior and antioxidant capacity of M. pajang’s antioxidant compounds in the synthesized CHCL/AA NADES was demonstrated. The molar ratio of choline chloride and ascorbic acid at 2:1 was found to be the optimum composition ratio as it allows the formation of a complete liquid solvent. The $^1$H NMR analysis indicated the high purity of CHCL/AA NADES was obtained and the hydrogen bonding interaction was confirmed by FTIR analysis. CHCL/AA NADES with addition of 10 wt% of water system displayed outstanding solubilization capacity, where it increased the solubility of antioxidant extracts by 11% as compared to water. The addition of water (10 wt%) to the natural deep eutectic solvent reduced the viscosity of CHCL/AA NADES and as a result improved its solubilization capacity by 4%. Since the CHCL/AA NADES successfully enhanced the solubility, the effects of temperature and the shaking speed on the solubility of antioxidants are of great interest and shall be studied in future works. Aside from that, the antioxidant extracts that dissolved in CHCL/AA NADES system exhibited a higher antioxidant potential (1.3-14.64%) as compared to that dissolved in the aqueous form. It is also interesting to found that the antioxidant molecules were found to agglomerate and well-rounded in the center of CHCL/AA NADES system, as depicted in the TEM. Based on the TEM, it can be observed that nano-sized cluster was formed and it can be denoted that the CHCL/AA NADES system showed great promising in protecting the bioactive compounds, which further studies such as stability and protectability of the CHCL/AA NADES nanoclusters should be considered. These findings in this work highlight the potential of CHCL/AA NADES system as a novel formulation for improved solubility and therapeutic properties, allowing them to fit for nutraceutical and pharmaceutical applications.

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Conflict of Interest
There is no conflict of interest with materials discussed in this manuscript.

References


Table 1 Polarized optical image CHCL/AA NADES system at different molar ratio of choline chloride/ascorbic acid

<table>
<thead>
<tr>
<th>Molar ratio of choline chloride: ascorbic acid</th>
<th>Polarized optical microscopy</th>
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<tr>
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<tr>
<td>1:2</td>
<td><img src="image3.png" alt="Image 3" /></td>
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Table 2 Solubility and viscosity of pure NADES and NADES with different water content

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solubility (mg/ml) (Absorbance at 212 nm)</th>
<th>Solubility (mg/ml) (Absorbance at 277 nm)</th>
<th>Viscosity (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.8041 ± 0.0111</td>
<td>0.8017 ± 0.0130</td>
<td>-</td>
</tr>
<tr>
<td>Pure NADES</td>
<td>0.9035 ± 0.0064</td>
<td>0.8935 ± 0.0142</td>
<td>51570</td>
</tr>
<tr>
<td>Aqueous NADES (10 wt%)</td>
<td>0.9428 ± 0.0128</td>
<td>0.9535 ± 0.0071</td>
<td>13290</td>
</tr>
<tr>
<td>Aqueous NADES (20 wt%)</td>
<td>0.9271 ± 0.0064</td>
<td>0.9235 ± 0.0142</td>
<td>588.5</td>
</tr>
<tr>
<td>Aqueous NADES (30 wt%)</td>
<td>0.8669 ± 0.0098</td>
<td>0.8535 ± 0.0071</td>
<td>53.5</td>
</tr>
<tr>
<td>Aqueous NADES (40 wt%)</td>
<td>0.8118 ± 0.0098</td>
<td>0.8185 ± 0.0187</td>
<td>17.83</td>
</tr>
<tr>
<td>Aqueous NADES (50 wt%)</td>
<td>0.8065 ± 0.0037</td>
<td>0.7934 ± 0.0187</td>
<td>8.92</td>
</tr>
</tbody>
</table>
Figure 1 1H NMR spectra of CHCL/AA NADES (X: parts per Million: 1H)
Figure 2 FTIR spectrum of (A): Choline chloride (B): Ascorbic acid and (C): CHCL/AA NADES
Figure 3 Effect of water inclusion in the CHCL/AA NADES on the solubility of antioxidant compounds

Figure 4 Schematic diagram illustrating the rupture of hydrogen bond between choline chloride and ascorbic acid upon addition of water
Figure 5 Viscosity of pure CHCL/AA NADES and added water percentage (wt %)

Figure 6 Effects of water inclusion in the antioxidant-CHCL/AA NADES on the antioxidant activity
Figure 7 (A) and (B) TEM images of antioxidant in CHCL/AA NADES system.