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Antioxidant Properties of *Curcuma caesia* Extracted Using Natural Deep Eutectic Solvent

Sri Kartini[1,2], Mohd F.A. Bakar[1]*, Fazleen I.A. Bakar[1], Susi Endrini[3], Yan Hendrika[2], Siti Juariah[1,2]

[1]*Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia (UTHM), Hub Pendidikan Tinggi Pagoh, KM1, Jalan Panchor, 84600 Muar, Johor, Malaysia*
[2]*Faculty of Pharmacy and Health Sciences, Abdurrab University, Jl. Riau Ujung No. 73, Tampan, Air Hitam, Payung Sekaki, Pekanbaru, Riau 28291, Indonesia*
[3]*Faculty of Medicine, Abdurrab University, Jl. Riau Ujung No. 73, Tampan, Air Hitam, Payung Sekaki, Pekanbaru, Riau 28291, Indonesia*

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ABSTRACT

Plants serve as viable sources for obtaining natural antioxidants. Among the potentially rich sources of antioxidants, black turmeric rhizome (*Curcuma caesia*) stands out. Traditionally, the conventional method for extracting bioactive chemicals from plants has relied on the utilisation of organic solvents, despite its recognised environmental drawbacks. However, an alternate technique that aligns with environmental consciousness involves the application of natural deep eutectic solvents (NADES). This study targeted to create and characterize four types of NADES designated as follows: NADES 1-citric acid:sucrose (1:1); NADES 2-sucrose:glucose:fructose (1:1:1); NADES 3-choline chloride:glycerol (1:1:2); and NADES 4- glycerol:urea (1:1) at 70°C. The investigation involved the determination of the physical properties of these NADES, including pH, temperature, and density. All formulated NADES were used to ascertain the entire phenolic and flavonoid content of *C. caesia* rhizomes, and their antioxidant potential was determine using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and ferric reducing antioxidant power (FRAP) methods. The results revealed a pH sequence of NADES 1<NADES 2 <NADES 3 <NADES 4, in which NADES 1 exhibited the highest density among the formulations. The temperature of NADES was obtained at 65°C and 70°C. The phenolic content was notably pronounced in NADES 1, 2 and 3 and NADES 1 and 2 yielded high flavonoid content. Remarkably, NADES 2 demonstrated the most potent antioxidant activity among the formulated solvents, as determined using both the DPPH and FRAP methods. In conclusion, NADES is an encouraging tool aimed at the extraction of secondary metabolites from plant.

Keywords: *C. caesia, Deep Eutectic Solvent, Antioxidant, Phytochemicals, Physical properties*

Introduction

The human body requires a balanced interplay of oxidants and antioxidants to ensure regular metabolism, signal transmission and proper cellular function regulation. Accordingly, each cell strives to maintain a state of equilibrium between oxidants and antioxidants. Diminished levels of antioxidant enzymes can serve as an indicator of elevated free radical levels within the body. An adverse consequence of heightened free radicals in the body is the release of reactive oxygen species (ROS). The unbridled excessive production of ROS, stemming as of an inequity between ROS creation and elimination, culminates in the emergence of vascular disorder.[1–3]

Numerous chronic and degenerative diseases, including cancer, respiratory, neurodegenerative, and gastrointestinal disorders, have been linked to the excessive production of ROS. Antioxidants, which can be produced endogenously or externally (exogenously) play a significant role in regulating ROS concentrations under physiological conditions, that can be added or removed.

*Corresponding author. E mail: fadzelly@uthm.edu.my

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The presence of malnutrition and antioxidant insufficiency in individuals can potentially increase their susceptibility to oxidative stress, hence elevating the likelihood of developing cancer. Antioxidant maintenance may also be disturbed in chronic obstructive lung disease, inflammatory bowel disease, neurological illnesses, cardiovascular disease, and ageing. Antioxidant supplementation reduces the depletion of endogenous antioxidants, thereby reducing the associated oxidative damage as shown in several clinical studies[1–3]. Exogenous antioxidants can be classified into synthetic antioxidants and natural antioxidants.[3] Natural antioxidants can be obtained from plants and vegetables.[4,5] More than 30 thousand types of plants can be found in Indonesia, and 7000 of them have the potential to become herbal medicines.[6] Polyphenolic compounds found in plants provide many benefits in preventing infectious and non-communicable diseases.[7–9] Curcuma has been well studied and is known to have antioxidant effects such as *Curcuma longa*, *Curcuma domestica*, and *Curcuma xanthoriza*.[10,11] Black turmeric rhizome (*Curcuma caesia*) has the potential to become herbal medicine and is rich in benefits such as antioxidants.

C. caesia, which is an affiliate of the *Zingiberaceae* family, is a perennial erect rhizome herb through bluish-black rhizomes that is of great economic prominence for its medicinal value. The plant is native to Northeast and Central India. The rhizome of the plant is aromatic through a strong camphoraceous odour and is typically functional on sprains and bruises.[12] Studies show that black turmeric rhizome has pharmacological activities such as antioxidant,[13] antibacterial,[14] antimutagenic and cytotoxic activity and has the potential to prevent nuclear factor kappa B activity.[15] Research on *C. caesia* has focused on antifungal,[16] antioxidant and antimutagenic,[17] anxiolytic, locomotor

depressant, anti-convulsant, muscle relaxant,[18] antidiabetic, anti-ulcerogenic and antibacterial properties.[19] *C. caesia* contains polyphenols such as flavonoids, phenols and alkaloids polyphenols such as flavonoids, phenols and alkaloids.[20] The hydroxyl groups of flavonoid compounds mediate the antioxidant effect by capturing free radicals and/or by chelating metal ions.[20,21]

The extraction of a secondary metabolite component is observed from the assortment of the category of solvent, and the extraction method is also play an important factor in extracting secondary metabolite compounds such as flavonoids and phenolics.[22,23] Conventional extraction procedures commonly rely on the use of organic solvents, which possess properties of high flammability or environmental pollution. Eco-friendly extraction techniques are crucial for recovering the bioactive components that can be utilised as food and/or nutraceutical supplements.[24] One alternative that is now being emphasised in research and development is the utilisation of environmentally friendly solvents, specifically natural deep eutectic solvents. (NADES). The term "NADES" refers to a mixture comprising a minimum of two components, whereby the melting point of at least one component is lower than that of another component[25,26] moulded through cell constituents like sugars, alcohols, amino acids, natural acids and choline subsidiaries.[27] It has the advantage of being easy to synthesise without the need for further purification, has good biocompatibility, is non-toxic and cheap[28,29] and can extract phenolic components including flavonoids.[29] Dai *et al* utilized seven sorts of NADES from lactic corrosive glucose, proline-malic corrosive, sucrose-choline chloride, glucose-choline chloride, sorbitol-choline chloride, 1,2-propanediol-choline chloride, and fructose-sucrose-glucose for the extraction of phenolic from safflower (*Carthamus tinctorius* L.), resulting in high yield of the targeted compounds. The utilisation of NADES comprising of sucrose and citric acid shown a notable capacity to extract phenolic compounds, specifically 4-O-caffeoylquinic acid and 4,5-dicaffeoylquinic acid, along with anthocyanin compounds. These compounds are classified as glycosylated flavonoids and were found to be present in substantial quantities.[16]

Based on this information, this research focused on *C. caesia* rhizomes extracted using NADES solvents. In this study, four types of NADES were used, namely citric acid:sucrose (1:1), sucrose:glucose:fructose (1:1:1), choline chloride:glycerol (1:2) and glycerol:urea (1:1) to extract bioactive compounds (flavonoids and phenols) in the rhizome of *C. caesia*. Moreover, the antioxidant activity was determined exhausting 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and ferric reducing antioxidant power (FRAP) methods.

Materials and Methods

Chemicals and Reagents

The chemicals used include DPPH (Merck), ethanol (Sigma), methanol (Sigma), sodium hydroxide, gallic acid, quercetin, Follin–Ciocalteu reagent, phenol reagent, Na_2CO_3 (Merck), sodium nitrite, 2,4,6-tripyridyl-striazine (TPTZ Sigma), $FeCl_3{\cdot}6H2O$ (sigma), acetate buffer solution (pH 3), aluminium nitrate (Merck), citric acid (Merck), sucrose (Merck), glucose (Merck), fructose (Merck), glycerol (Sigma), urea (Merck) and Cholin chloride. *C. caesia* sample was obtained from KH Garden Pekanbaru, St. Keliling/Gn Gayo Gg. Nurul Salam, East Tangkerang, Tenayan Raya District Pekanbaru, Riau, 28289 on December 22, 2022. Identification was carried out at the Department of Biology, Riau University, Indonesia.

Production of NADES

NADES was prepared based on the method of Choi *et al*., Dheyab *et al*. and Mansinhos *et al*.[30–32] Four NADES solvents were prepared with different materials and compositions. NADES 1 was prepared using a mixture of citric acid and sucrose (molar ratio, 1:1), NADES 2 was prepared with a mixture of sucrose, glucose, and fructose (1:1:1), NADES 3 was prepared with a mixture of choline chloride and glycerol (1:2), and NADES 4 was made by using a mixture of glycerol and urea (1:1). Each mixture was placed in an Erlenmeyer flask and heated at 60–70 °C, then water was added to reduce its viscosity. Water (≤60% by weight) was then supplementary to the assortment. Afterward forming the elucidation, it was transferred into bottles and stored in the freezer and did not settle.

Production of Extract C. caesia by using NADES

Bioactive compounds were extracted using NADES following the methods of Choi *et al*,[30] Dheyab *et al*,[32] and Mansinhos *et al*,[31] and Abu Bakar *et al*[33] by mixing 1 ml each of NADES solvents 1, 2, 3 and 4, with powder of each sample 2 ± 0.1 mg in a glass container. The sample mixture was stirred at 60–70 °C by using a hot plate and magnetic stirrer. Stirring was carried out for 60 min, the sample was cooled and centrifuged, and the supernatant was taken.

Determination of Total Phenolic Content

The quantification of total phenolic content was performed using a colorimetric approach with the Folin–Ciocalteu reagent, using a previously established protocol with minor modifications.[33] Absorbance was stately at the wavelength of 725 nm, and the outcomes were obtained from the equivalence to gallic acid (mg GAE/g) based on the linearity of the standard used (0–100 µg/ml).

Determination of Total Flavonoid Content

The total flavonoid content was determined using the methodology described by Abu Bakar *et al*[33] and quercetin was employed as the reference standard. The container was filled with 0.5 milliliters of the sample and 0.3 milliliters of 5% sodium nitrite. 0.6 mL of 10% aluminium nitrate was supplementary to the mixture after it had been permissible to standpoint for five minutes. The blend was then diluted ten times with 2 mL of 1 M NaOH. At a wavelength of 510 nm, the maximum absorption was observed. The quercetin ordinary was used to create the linearity curve at a attentiveness of 0.5–100 µg/mL.

Antioxidant Activity with DPPH

Free radical activity was analysed based on the linear regression equation, and the inhibitory concentration (IC_{50}) was then determined.[34] Elucidations of the extracts obtained were made at concentrations of 31.25, 62.5, 125, 250, and 500 µg/mL, added with 1.5×10^{-4} M DPPH and then incubated for 30 min. The absorbance was then detected at wavelength of 419 nm. The measurement of free-radical scavenging activity is expressed as the percentage of radical inhibition, which may be calculated using the following equation:

$$\text{Radical scavenging activity } (\%) \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \text{x} 100\%$$

Antioxidant Activity with FRAP

The Ferric-reducing antioxidant power test was conducted to determine the antioxidant activity of the four samples, using the procedures described by Abu Bakar *et al*[33] and Okoli *et al*[35]. FRAP reagent was prepared by adding 300 mM acetate buffer solution (pH 3.6), 10 mM TPTZ, and 20 mM $FeCl_3{\cdot}6H2O$ at the ratio of 10:1:1. The mixture was heated to 37 °C in a water bath. The cuvette was filled with a combined volume of 100 millilitres of extract and 300 millilitres of distilled water. After adding the sample to the FRAP reagent, a subsequent measurement was taken at a wavelength of 593 nm, prior a 4 minutes incubation. Vitamin C standard curve was compared to the alteration in absorbance after four minutes from the early blank reading. This standard curve was used to determine the sample's FRAP value. The concentration of an antioxidant that can decrease iron was used as the final measurement.

$$\text{FRAP} = \frac{\text{Abs sample} \times \text{value FRAP from curve standard}}{\text{Abs standard}}$$

Result and Discussion

Natural deep eutectic solvents (NADES) possess several desirable qualities, including sustainability, biodegradability, low volatility at ambient temperatures, high dissolving capacity, and selectivity. Consequently, NADES holds significant potential as a viable option

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for nutraceutical extraction and various other applications.[36] The parameters of each solvent were obtained based on research findings for the production of NADES solvents. The results are displayed in Table 1.

According to the data presented in Table 1, the NADES exhibits three distinct parameter features, specifically density, pH, and form. Density is one of the most imperative physical belongings of NADES. Most of the NADES batches exhibited higher density than water. According Garcia *et al*.,[37] NADES density can be affected by the amount of hydroxyl groups and chain length used. The density of NADES will increase with the increase in hydroxyl groups and the stretch of the carbon chain on the hydrogen bond donor (HBD). The density obtained was 1.0012–1.1160 g/mL, which exceeds that of water, according to Al-risheq *et al* , indicating that temperature affects the density of NADES obtained. Density shows an inversely proportional relationship with temperature, because density decreases linearly as temperature increases. This phenomenon is due to the increased mobility and activity of molecules in the solvent with higher temperatures, causing an increase in volume.[38]

The effect of other characteristics such as pH obtained ranged from 1.5 to 8.7. The difference in pH is based on the composition of the NADES used. NADES 1, a mixture of citric acid and sucrose (1:1), resulted in a low pH. This property may be influenced by the acidity factor of citric acid. Usually, low pH is used aimed at the extraction of certain compounds such as anthocyanin compounds. Anthocyanins are a sub-type of organic compounds from the flavonoid family and are affiliates of a larger assembly of compounds, namely polyphenols. Silva *et al*.[39] explained that the extraction of anthocyanin compounds by using NADES with a mixture of citric acid results in low pH, thus supporting the stability of anthocyanin compounds. They found that the optimum extraction of anthocyanins by using NADES results in a mixture of citric acid with a pH of 1.17.[39] ADES 2 shows a lower pH compared to NADES 3 and 4, with the expectation that NADES should ideally have neutral properties. This pH deviation may be caused by the potential formation of invert sugar during production, due to the heating process, causing a decrease in pH. Research conducted by Wilberta *et al*[40] also showed that the presence of invert sugar caused a decrease in the pH of sap sugar. Additionally, it should be noted that fructose shows its optimal performance at pH 4.5.[41] In contrast, NADES 3 contains alkaline glycerol, resulting in a much higher pH Meanwhile, NADES 4 which has the highest pH, is associated with a mixture of alkaline materials, such as urea. Urea tends to be alkaline, and its pH ranges from 8 to 9.[42]

The process of extracting phenolic chemicals and flavonoids from natural materials using DES solvents comprises a solid-liquid extraction procedure. The Dual Extraction System (DES) is a collection of two or more components that can form a liquid mixture through the process of mixing, facilitated by hydrogen bonding interactions. Notably, the melting point of this mixture is significantly lower than that of its individual elements. Additionally, the application of heat aids in the removal of phenolic compounds from plant materials.[43] In the present investigation, the quantification of total phenolic and flavonoid content was conducted for every NADES solvent employed. The findings pertaining to the analysis of total flavonoid and phenolic content are presented in Figure 1.

Table 1: Characteristic chemical properties of NADES

Solvent NADES	Density (g/mL)	pH	Temperature (°C)	Form
NADES 1 Citric acid: Sucrose (1:1)	1.1160	1.5	65	Liquid
NADES 2 Sucrose: Glucose: Fructose (1:1:1:)	1.0312	2.8	65	Liquid
NADES 3 Choline chloride: Glycerol (1:2)	1.0012	7.4	70	Liquid
NADES 4 Glycerol: Urea (1:1)	1.0264	8.7	70	Liquid

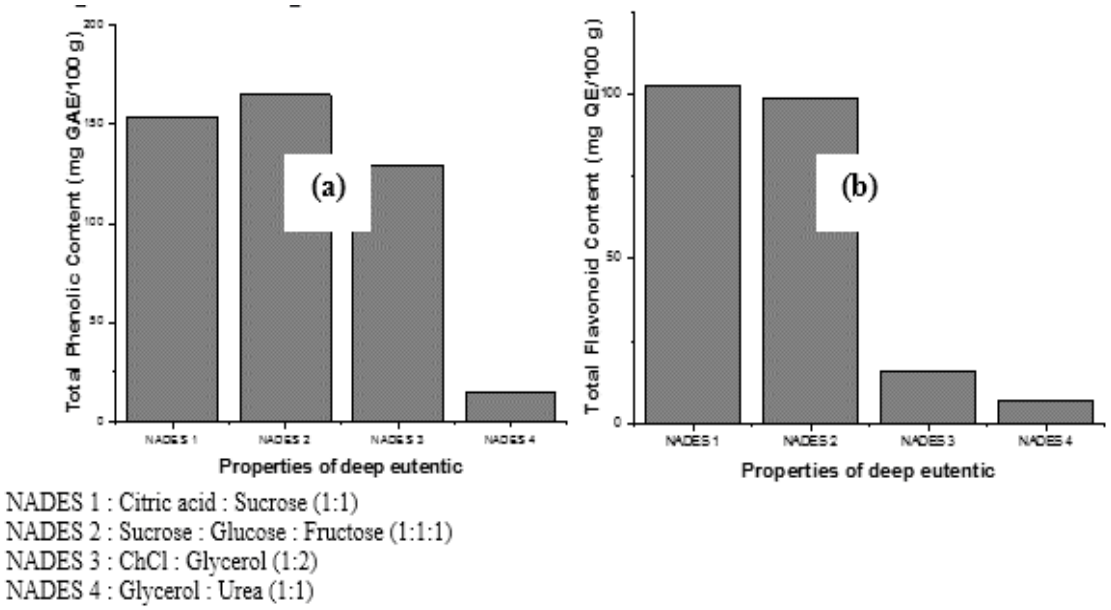

Figure 1: (a) Total phenolic content and (b) total flavonoid content.

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Table 2: Activity of antioxidant using different method (DPPH and FRAP)

Samples	Activity Antioxidant	
	IC_{50} (µg/mL)	AAI (mmol/g)
NADES 1	1014.88[a]	25.03[d]
NADES 2	798.42[ab]	26.27[d]
NADES 3	815.92[b]	25.20[d]
NADES 4	1240.52[c]	23.90[d]

ANOVA test on IC_{50} shows p-value <0.05 then tested further post hoc Turkey obtained p-value>0.05 symbolised according to the code [a,b,c]. ANOVA test on AAI shows a p-value> 0.05 so that it is symbolised in the same group that is interconnected.

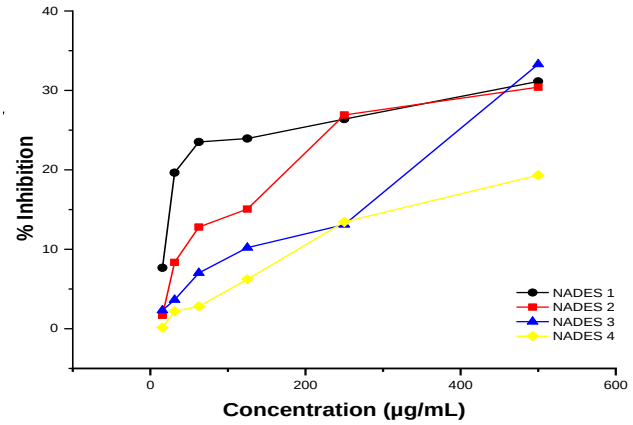

Figure 2: Percentage of inhibition of NADES solvents against DPPH radical

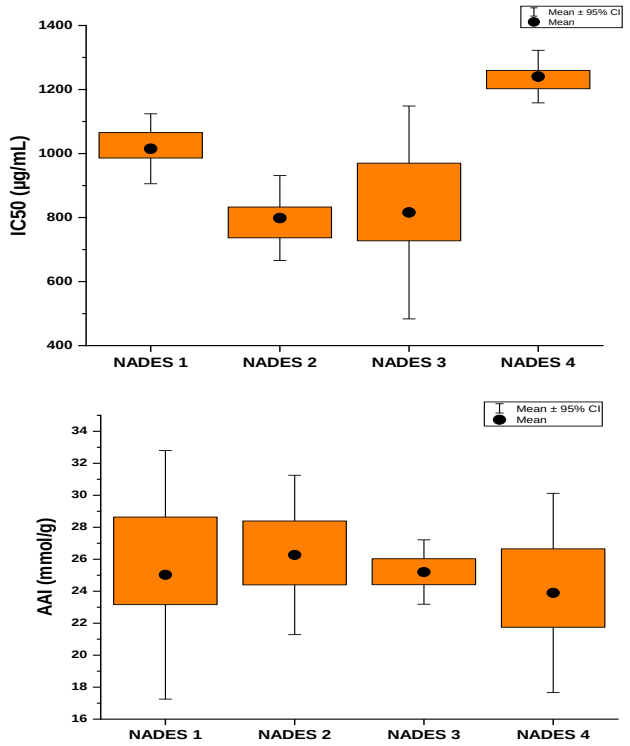

Figure 3: Box plot of antioxidant activity from different samples (NADES 1, 2, 3, 4, and 5). (A) Box plot obtained using the DPPH method, (B). Box plot obtained using the FRAP method.

The selection of citric acid and glucose as the composition for this study is considered an optimal combination due to the fact that glucose acts as a hydrogen bonding acceptor (HBA). The properties of HBD can be observed in citric acid. Assuming that the two materials undergo fusion at a specific temperature, they combine to produce a chemically stable green solvent with potential utility. Both of these components are excipients that can be safely ingested. Due to the absence of any detrimental organic solvents in the extracted extract, it is deemed suitable for immediate consumption. The integration of non-aqueous dispersive extraction systems (NADES) with unconventional extraction methodologies serves to reduce the extraction of undesired chemicals while efficiently extracting the desirable target molecules. Numerous studies have conveyed the accomplishment of NADES as an alternate solvent to substitute conventional organic solvents in experiments on flavonoid extraction from *Radix Scutellariae*,[44] extraction of phenol compounds from *Cajanus cajan* leaves,[45] and extraction of polyphenols and caffeine from robusta coffee beans.[46,47]

The assessment of antioxidant activity was conducted using the DPPH and FRAP methods, wherein absorbance measurements were obtained using a UV-Vis spectrophotometer. The DPPH technique is a simple and easy DPPH radical absorption method that uses a small sample in a short time. The assessment of antioxidant activity is conducted using the DPPH method, wherein the IC_{50} value represents the concentration required to suppress free radicals by 50%. A lower IC_{50} value indicates a higher level of antioxidant activity for the chemical or extract.[48] According to Figure 2, there is a positive correlation between the observed concentration and the percentage of inhibition exhibited by all NADES solvents. The measurement of IC_{50} value in the DPPH measurement method will involve the continuation of percent inhibition data.

The DPPH method works based on oxidation-reduction reactions, where DPPH is a synthetic free radical that can dissolve in polar compounds such as ethanol and methanol. Antioxidant compounds react with DPPH by donating hydrogen atoms to obtain electron pairs. The colour change of DPPH indicates how strong the antioxidant activity in black tea samples when measured in terms of intensity by using a spectrophotometer at a wavelength of 517 nm.[49,50] The FRAP method can be used to determine antioxidant activity in plants. The FRAP method has the advantages of being inexpensive, having simple reagent preparation, and being quick. This method assesses the overall antioxidant content of a substance by measuring the capacity of its antioxidant compounds to convert Fe^{3+} ions into Fe^{2+} ions, equating antioxidant potential with the substance's reducing ability.[51]

Table 2 and Figure 3 show the results of antioxidant activity obtained by means of the DPPH and FRAP techniques. Among the four extracts obtained using the NADES solvent, by using the DPPH antioxidant activity measurement method, the extract with the largest to smallest IC_{50} value is NADES 2 > NADES 3 > NADES 1 > NADES 4. The measurement of antioxidant activity by using FRAP method shows the antioxidant activity strength of *C. caesia* in solvent NADES 2 (AAI =26.27±2.01 mmol/g) > NADES 3 (AAI =25.2±0.81 mmol/g) > NADES 1 (AAI =25.09±3.13 mmol/g) > NADES4 (AAI =23.90±2.51 mmol/g).

Juric *et al*[52] found that NADES choline chloride : glycerol was more effective in extracting flavonoids compared to 70% *Mentha piperita* ethanol.. The antioxidant assay involving the use of DPPH is commonly employed to evaluate the antioxidant properties of various bioactive compounds. In this study, the bioactive constituents of peppermint extract were investigated for their antioxidant activity against DPPH radicals and their ability to chelate iron ions when included into NADES. It was shown that the presence of sugar in NADES might potentially influence the outcome of the antioxidant test.[46] In addition, according to Doldolova *et al*, the copper ion (Cu^{2+}) plummeting antioxidant power assay method (CUPRAC), which only selectively oxidizes antioxidant mixtures, can be used to appropriately test the antioxidant capability of NADES extracts. As a result, the issue of utilizing components containing sugar, citric acid, or amino acids that are prone to interfering throughout the investigation phase can be ommited. As a result, the extraction process and the variety of

eutectic concoction used to disband the mark compound can be optimized for optimal antioxidant activity.[53] The utilisation of NADES demonstrates support for sustainable technology and has the potential to be effectively employed in several industries such as food, pharmaceutical, and cosmetic manufacturing. The strippers in question have been verified to effectively produce plant metabolite extracts with higher yields compared to traditional organic solvents. Due to their environmentally favourable properties, these solvents are gaining popularity in comparison to traditional organic solvents during a period of heightened concern for waste management.[54–56]

Conclusion

Based on the findings of these investigations, it is evident that Natural Deep Eutectic Solvents (NADES) have gained significant popularity and are deemed to be a highly suitable alternative to conventional solvents due to their numerous environmentally friendly attributes. The findings indicated that the extract derived from NADES 2, specifically the composition of sucrose, glucose, and fructose in a 1:1:1 ratio, exhibited superior antioxidant activity compared to other extracts, as assessed by the DPPH and FRAP methods.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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