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

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Abstract

Natural antioxidants can be derived from plants and vegetables. Among the potentially rich sources of antioxidants, black turmeric rhizome (*Curcuma caesia*) stands out. Traditionally, the extraction of bioactive composites as of plants involves the use of organic solvents despite their known environmental limitations, and an environmentally conscious alternative approach utilises natural deep eutectic solvents (NADES). This study targeted to create and characterise 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 a temperature of 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 considered exhausting the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. The analysis of the research findings 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 for generating NADES 1 and 2 was set at 65 °C, whereas that for NADES 3 and 4 was maintained at 70 °C. The extraction of phenolic content was notably pronounced in NADES 1, 2 and 3 extracts, 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 as of plants.

Keywords: *C. Caesia, NADES, DPPH, FRAP*

Introduction

The human body necessitates 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 [1,2,3].

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) and play a significant role in regulating ROS concentrations under physiological conditions, can be added or removed. Malnutrition and antioxidant deficiency can make people additional expected to involvement oxidative strain, which can raise the risk of cancer. In chronic obstructive pulmonary disease, inflammatory bowel disease, neurodegenerative disorders, cardiovascular disease, and aging, antioxidant mantain may also be compromised. Antioxidant supplementation reduces the depletion of endogenous antioxidants, thereby reducing the associated oxidative damage in several clinical studies [2,4,5].

Exogenous antioxidants can be classified into synthetic antioxidants and natural antioxidants[6]. Natural antioxidants can be obtained from plants and vegetables [7,8]. More than 30 thousand types of plants can be found in Indonesia, and 7000 of them have the potential to become herbal medicines [9]. Black turmeric rhizome (*Curcuma caesia*) has the potential to become herbal medicine and is rich in benefits such as antioxidants.

C. caesia, which is a 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[10]. Studies show that black turmeric rhizome has pharmacological accomplishments such as antioxidant, antibacterial, antimutagenic and cytotoxic activity and has the potential to prevent nuclear factor kappa B activity [11]. Research on *C. caesia* has focused on antifungal[12], antioxidant and antimutagenic [13], anxiolytic, locomotor depressant, anti-convulsant, muscle relaxant [14], antidiabetic, anti-ulcerogenic and antibacterial properties[15]. *C. caesia* contains polyphenols such as flavonoids, phenols and alkaloids polyphenols such as flavonoids, phenols and alkaloids[16]. The hydroxyl groups of flavonoid compounds mediate the antioxidant effect by capturing free radicals and/or by chelating metal ions[17,18].

The withdrawal of a secondary metabolite compound component is observed from the assortment of the category of solvent, and the extraction method is also an important factor in withdrawing secondary metabolite compound components such as flavonoids and phenolics[19,20]. Organic solvents that are either highly flammable or polluting are typically the foundation of conventional extraction methods. In order to recover bioactive compounds that can be used as food and/or nutraceutical ingredients, eco-friendly extraction methods are especially important[21]. One alternative that is the focus of development is the practice of environmentally friendly solvents, namely natural deep eutectic solvent (NADES).

NADES is an assortment of at least two buildings that have a lesser liquefying point than one of its components[22,23] molded through cell constituents like sugars, alcohols, amino acids, natural acids and choline subsidiaries[24] which is a primary metabolite compound that can be used as NADES at a certain mole ratio. It has the advantage of being easy to synthesise without the need for further purification, has good biocompatibility, is non-toxic and cheap [25,26] and can extract phenolic components and flavonoids[27]. Dai et al. (2013) 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., *Asteraceae*), resulting in high yield. NADES sucrose-citric acid was able to extract 4-O-caffeoylquinic acid and 4,5-dicaffeoylquinic acid phenolic group with high abundance and anthocyanin compounds, which are glycosylated flavonoids[12].

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), ChCl: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) 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–Ciocalteus 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 clorida and rhizome *C. caesia* from Pekanbaru, Riau Province, Indonesia.

Produce of NADES

NADES was prepared grounded on the method of Choi et al. (2011), Dheyab et al. (2021) and Mansinhos et al. (2021) [28-30]. 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 ChCl 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. (20112) [28], Dheyab et al. (2021)[29], and Mansinhos et al. (2021)[30] [28–30] by mixing 1 ml each of NADES solvents 1, 2, 3 and 4, by adding powder of each sample 2 ± 0.1 mg hooked on 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

Total phenolic content was restrained consuming colorimetric method with Folin–Ciocalteu reagent by using a preceding method through slight modification. 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

Total flavonoid content was firm referring to the technique of Abu Bakar [31] and by using quercetin as a 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% aluminum 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

DPPH (2,2- diphenyl-1-picrylhydrayl) Free radical activity was analysed based on the linear regression equation, and the inhibitory concentration (IC_{50}) was then determined. 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 result and then incubated intended for 30 min. The absorbance was then restrained at 419 nm wavelength. Free-radical scavenging activity is communicated as percent extremist restraint which can be determined utilizing the accompanying equation:

$$\text{Radical scavenging activity (\%)} = \{(\text{OD control} - \text{OD sample}) / \text{OD control}\} \times 100$$

Antioxidant Activity with FRAP

Iron-reducing power test was directed to decide the antioxidant activity of the four samples following the methods of Abu Bakar et al. (2009)[31]. FRAP reagent was equipped through collaborating 300 mM acetate buffer solution (pH 3.6), 10 mM TPTZ, and 20 mM $FeCl_3.6H_2O$ at the ratio of 10:1:1, and the sample was heated to 37 °C in a water bath. A sum of 3.0 mL of FRAP reagent was added to the cuvette, and a clear perusing was taken at 593 nm frequency by utilizing a spectrophotometer. The cuvette received a total of 100 milliliters of extract and 300 milliliters of condensed water. A subsequent construing at 593 nm was obtained afterward 4 minutes of totalling the sample to the FRAP reagent. The Vitamin C standard curve was compared to the alteration in absorbance afterward 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.

FRAP (mM/gr sample) = (Abs sample × value FRAP from curve standard)/Abs standard

Result and Discussion

Naturel deep eutectic solvent (NADES) has the characteristics of sustainability, biodegradability, low volatility at room temperature, high dissolving capacity, and choosiness, thus assembly NADES a promising alternative for use in nutraceutical extraction and several other alternatives[32]. Based on the outcomes of research for the preparation of NADES solvents, the characteristics of each solvent made were obtained. The outcomes are exposed in the table 1.

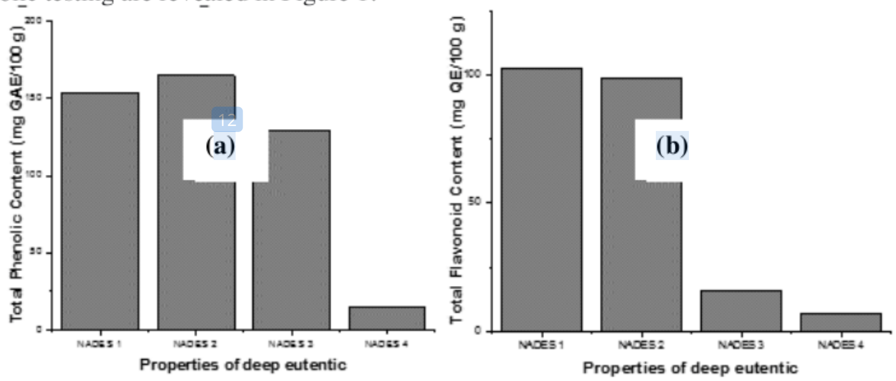
Table 1. Characteristic chemical properties of NADES

Solvent NADES	Density (g/mL)	pH	T (°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

Based on Table 1, the NADES made has three parameter characteristics, namely, density, pH, and shape obtained. 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., (2015)[33] 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. (2021), 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[34].

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 convinced 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. (2020) 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[35]. 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[36].

The extraction of phenolic compounds and flavonoids as of natural materials by exhausting DES solvents involves a solid–liquid extraction process. DES is a assortment of two or extra apparatuses that can arrangement a liquid upon mixing through a melting point well below that of the separate constituents because of hydrogen bonding interactions, and heating helps remove phenolic compounds from plants[37]. In the contemporary study, total phenolic and flavonoid levels were tested for each NADES solvent used. The results of total flavonoid and phenolic testing are revealed in Figure 1.



NADES 1 : Citric acid : Sucrose (1:1)
NADES 2 : Sucrose : Glucose : Fructose (1:1:1)
NADES 3 : ChCl : Glycerol (1:2)
NADES 4 : Glycerol : Urea (1:1)

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

Because glucose is a hydrogen bonding acceptor (HBA), the NADES composition that was chosen for this study—citric acid and glucose—is the ideal combination. HBD properties are found in citric acid. Let's say the two materials fuse at a certain temperature to form a stable green solvent that can be used. These two components are excipients that can be consumed safely. Since there are no harmful organic solvents in the extracted extract, it can be consumed immediately. Combining NADES with non-conventional extraction techniques minimizes the amount of unwanted compounds extracted and effectively extracts the desired target compounds. 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[38], extraction of phenol compounds from *Cajanus cajan* leaves [39], and extraction of polyphenols and caffeine from robusta coffee beans[40,41].

Antioxidant activity testing was passed out exhausting the DPPH and FRAP approaches through quantifying the absorbance through 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. Antioxidant activity is determined by DPPH in terms of IC_{50}, which is the concentration that inhibits free radicals by 50%. The lesser the IC50 rate, the better the antioxidant activity of the compound or extract [42]. Figure 2 shows that the percent inhibition of all NADES solvents tends to increase as the measured concentration increases. The percent inhibition data will be continued to measure the IC_{50} value in the DPPH measurement method.

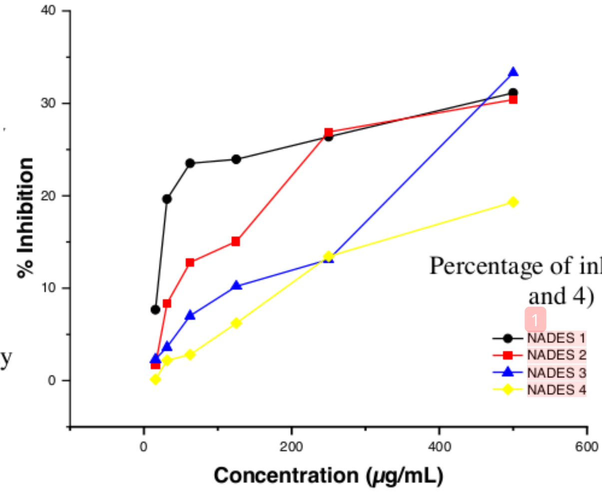


Figure 2. Percentage of inhibition sample NADES (1,2,3 and 4)

Table 2. Activity of antioxidant with 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.

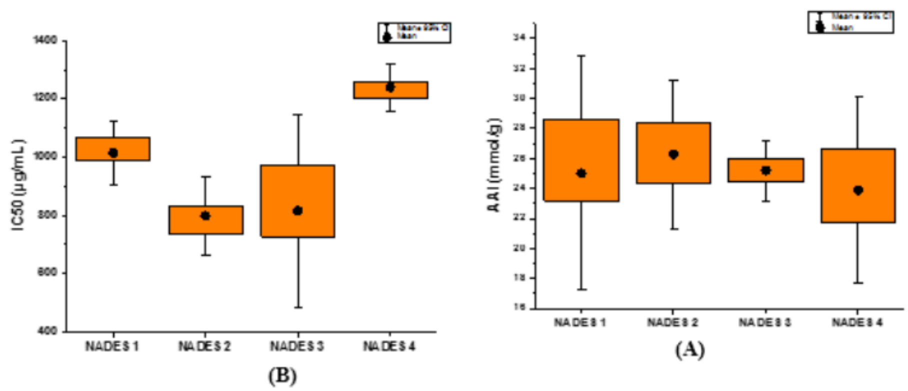


Figure 3. Box plot of antioxidant activity from different samples (NADES 1, 2, 3, 4, and 5). (A) Box plot obtained using the DPPH method, (B). Box plot obtained using the FRAP method.

The 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[43,44]. The FRAP method can be charity toward assessment antioxidant activity in plants. The FRAP method has the advantages of being inexpensive, having simple reagent preparation, and being quick. This technique can be utilized to decide the all out cell reinforcement content of a material in view of the capacity of cancer prevention agent mixtures to diminish Fe^{3+} particles to Fe^{2+} so the cancer prevention agent force of a compound is closely resembling the lessening skill of the compound [45].

Table 2 and Figure 3 show the measurement consequences of antioxidant activity obtained by means of the DPPH and FRAP technique. 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. (2021) conveyed that NADES choline chloride encompassing polyalcohol for instance glycerol and sugar as HBD is supplementary actual in removing flavonoids than 70% ethanol[46]. The antioxidant test against DPPH, in which the bioactive compounds of peppermint excerpt in the NADES that they verified indicated antioxidant activity in contradiction of DPPH radicals and the capacity to condense iron ions, can be affected by NADES that contain sugar[46]. In addition, according to Doldolova et al. (2021), 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 excerpts. 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 committed. 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[47]. NADES supports environmentally friendly technologies and can be functional in the food, pharmaceutical and cosmetic productions. These strippers are confirmed toward competently products plant metabolite extracts through advanced yields than conventional organic solvents. Considering the eco-friendly nature of these solvents, they are experiencing increased claim in a dumpier time matched with conventional organic solvents[48–50].

Conclusion

Based on these studies, NADES is widely used and meets countless criteria as a more environmentally friendly alternate to conventional solvents. The results showed that the extract obtained from NADES 2, namely, the combination of sucrose:glucose:fructose (1:1:1) resulted in higher antioxidant activity results than other extracts both by using the DPPH and FRAP method.

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