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Lipid Peroxidation Inhibition of Three Choline Chloride-Based NADES Extracts from *Pluchea indica* Leaves: *Ex Vivo* and *In Silico* Approaches

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ABSTRACT

Pluchea indica (L.) Less leaves contain chlorogenic acid, which have antioxidant activity. This study aims to evaluate the ability to inhibit lipid peroxidation of *P. indica* leaf extract obtained from three natural deep eutectic solvents (NADES) based on choline chloride (ChCl). The chlorogenic acid content in the extracts was determined by RP-HPLC. Evaluation of lipid peroxidation inhibitory activity (LPOI) was carried out using the Thiobarbituric Acid Reactive Substances (TBARS) method on rat liver homogenates *ex vivo*. The study was carried out *in silico* against three enzymes, namely cyclooxygenase-2 (COX-2), lipoxygenase (LOX), and cytochrome P450 (CYP450). The ChCl-Urea (1:2) extract of *P. indica* leaves contained the highest levels of chlorogenic acid (0.404%, w/w). The extract at 12.5% (v/v) gave the best LPOI activity with a percentage of inhibition of 22.5%. Chlorogenic acid as an antioxidant in inhibiting lipid peroxidation could be influenced by interaction with COX-2 and CYP450 inhibition *in silico* with binding energy values of –7.27 and –6.47 kcal/mol, respectively. Thus, chlorogenic acid, one of the active compounds from *P. indica* leaves, was responsible for inhibiting lipid peroxidation. However, in the future, it is necessary to investigate other phenolic chemical components from the NADES extract of *P. indica* leaves that play a role in this activity.

Keywords: chlorogenic acid, phenolic acid, natural antioxidant, molecular docking, malondialdehyde

INTRODUCTION

Lipid is a natural compound in the body that acts as energy reserves (Solomons & Fryhle, 2009), controls the physiological state of membrane organelle, and acts as signalling molecules (Ayala et al., 2014). Lipids with a C-C double bond, such as polyunsaturated fatty acids (PUFAs), can be oxidized by free radicals (Ayala et al., 2014). Polyunsaturated fats are distributed in body tissues that have vital functions (Solomons & Fryhle, 2009), one of them is the liver (Rael et al., 2004). Lipid peroxidation in the liver triggered by reactive oxygen (ROS) can cause various liver diseases (Ayala et al., 2014), including non-alcoholic fatty liver disease (NAFLD) (Arvind et al., 2019), alcoholic liver disease (ALD) (Patel & Mueller, 2023), viral hepatitis, and liver fibrosis. PUFA oxidation produces lipid mediators through enzymatic and free radical-related reactions (Massey & Nicolaou, 2013). Three enzymes that play a role in the lipid oxidation process, namely lipoxygenase (LOX), cyclooxygenases (COX) (Salehi

et al., 2020), and cytochrome P450 (CYP450), which produce bioactive lipids, such as eicosanoids, docosanoids, and octadecanoids (Ayala et al., 2014). LOX mediates the production of hydroperoxy eicosatetraenoic acids, lipoxins, leukotrienes, or hepoxilins following the oxidation of arachidonic acid. COX produces prostaglandins. CYP450 produces epoxyeicosatrienoic acids, leukotoxins, thromboxane, or prostacyclin (Massey & Nicolaou, 2013). Lipid peroxidation products from this oxidation chain include lipid hydroperoxides (LOOH), malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE) (Ayala et al., 2014; Devasagayam et al., 2003). MDA is the most mutagenic product of the lipid peroxidation process and is commonly used as a biomarker of the reaction (Ayala et al., 2014). Monitoring of lipid peroxidation can be determined through testing with thiobarbituric acid (TBA) reagents under acidic conditions known as thiobarbituric acid reactive substances (TBARS) assay (Gulcin, 2020). The pink colour formed was then measured spectrophotometrically at 532 nm (Devasagayam et al., 2003).

Under normal conditions, where lipid peroxidation rates are low and antioxidant defence systems are in good condition, it causes an adaptive stress response. In contrast, when the rates of lipid peroxidation are high and the antioxidant defence system is weak, it will cause oxidative damage which facilitates various pathological conditions (Singh & Singh, 2008). Thus, antioxidants are needed from outside the body which can be obtained from natural metabolites (Alam et al., 2013). Plants are one source of natural ingredients that are rich in antioxidant bioactive components (Gulcin, 2020). In general, exploration of natural antioxidants is mostly done on the phenolic group, including phenolic acids, flavonoids, tannins, and others (Dai & Mumper, 2010). Several studies have shown that phenolics from the chlorogenic acid group have the potential to be developed as natural antioxidants (Lu et al., 2020). This compound can be found in various plant species, one of which is *Pluchea indica* (L.) Less. (Kongkiatpaiboon et al., 2018) or Beluntas (common name in Indonesia).

Previously, it has been reported that there were studies on the antioxidant activity of methanolic (Noridayu et al., 2011), ethanol (Andarwulan et al., 2010), and distillate water (Vongsak et al., 2018) extracts of *P. indica* leaves. A study by Vongsak et al. (2018) shows that chlorogenic acid is one of the phenolic compounds that plays an important role in the antioxidant activity of *P. indica* extract (Vongsak et al., 2018). Currently, the development of extraction techniques is in great demand in the use of alternative solvents, such as natural deep eutectic solvents (NADES). This solvent is a mixture of hydrogen bond acceptor (HBA), such as choline chloride (ChCl) and hydrogen bond donor (HBD), such as organic acids/bases, sugar, polyalcohol, etc. (Dai et al., 2013). Several studies reported the ability to extract chlorogenic acid from plant parts, such as *Lavandula pedunculata* with ChCl-urea (1:2), *Achillea millefolium* with ChCl-lactic acid (1:2) (Ivanović et al., 2022), *Lonicera japonica* with ChCl-1,3-butanediol (1:6) (Peng et al., 2016), etc. However, reports on the antioxidant activity of these compounds in the NADES extract of *P. indica* leaves in comprehensively inhibiting lipid peroxidation *ex vivo* and *in silico* have never been reported. Based on this phenomenon, the aim of this study is to evaluate the lipid peroxidation inhibitory *ex vivo*, and to predict the molecular interactions of chlorogenic acid with lipoxygenase, cyclooxygenase-2, and cytochrome P450 *in silico*. In the future, the development of NADES extract as a source of antioxidants with phenolic content, especially chlorogenic acid, can be further deepened.

MATERIALS AND METHODS

Plant

P. indica leaves were obtained from the Tropical Biopharmaca Research Center (Trop BRC), Bogor Agricultural University (IPB) University, Bogor, Indonesia (no. BMK0188092016). Plants were determined at the Herbarium Bogoriense Laboratory, National Research and Innovation Agency, Indonesia (No. B-172/II.6.2/IR.01.02/2/2023). Leaves were washed and dried at room temperature (25-27 °C) for 3 days. The dried leaves were blended and sieved.

Chemicals

Choline chloride (ChCl) ACS grade was obtained from Xi'an Rongsheng Biotechnology Co, Ltd, China. Urea (U) was sourced from Vivantis Technologies (Selangor, Malaysia). Lactic acid (LA) ACS grade, 1,3-butanediol anhydrous (1,3-but) was sourced from Sigma-Aldrich (St. Louis, USA). Deionized water was obtained from Hanna Instruments SRL (Nusfalau, Romania) and water for injection was obtained from PT. Ikapharmindo

Putramas Pharmaceutical Laboratories (Jakarta, Indonesia). Other chemicals used included methanol pro HPLC, phosphate buffer saline (PBS), thiobarbituric acid (TBA), trichloro acetic acid (TCA), butylated hydroxytoluene (BHT), ethanol absolute (≥99%), potassium chloride (KCl), $FeCl_3.6H_2O$, and hydrochloric acid (HCl) were obtained from Sigma-Aldrich (St. Louis, USA) and distillate water. Chlorogenic acid (CA) (≥95%) and gallic acid (GA) (≥ 90%) were used as reference standard from MarkHerb (Bandung, Indonesia).

Preparation of NADES

In this study, three types of NADES were used, viz ChCl-U (1:2) (Mansinhos et al., 2021), ChCl-LA (1:1) (Ivanović et al., 2022), and ChCl-1,3-but (1:6) (Peng et al., 2016). Briefly, each component was weighed according to the specified molar ratio. The NADES component mixture was added with 30% (v/v) water and then heated at 80 °C on a magnetic stirrer (Cimarec+™ Stirring Hotplates Series, Thermo Scientific, USA) with a speed of 1000 rpm. The preparation of NADES was considered complete when a homogeneous and transparent liquid was obtained.

Analysis Fourier Transform Infrared Spectroscopy (FTIR) of NADES

This analysis was carried out on the NADES solvent used in this study in real time using an FTIR spectrometer (Agilent Cary 630 FTIR, California, USA). The liquid samples were scanned from 4000 to 500 cm^{-1} at room temperature.

Extraction using Ultrasonic-Assisted Extraction (UAE)-NADES

Ultrasound-assisted extraction (UAE) with NADES was performed following the method of Kongkiatpaiboon et al., (2018). The ratio of dried leaves powder to solvent is 1:20 (w/v). Extraction was carried out in an ultrasonic bath (Branson 5510, Shanghai, China) at 40 kHz for 15 min at 40 °C. The extract was then centrifuged with a Universal Centrifuge (Gemmy PLC-025, Taipei, Taiwan) for 20 min at 4000 rpm. Next, the extract was filtered again with a 0.45 µm filter membrane. The NADES extract was then identified for chemical content qualitatively following the procedures in the Indonesian Herbal Pharmacopeia (Ministry of Health Republic of Indonesia, 2017). In addition, the pH of the NADES extract was also determined with a pH meter (HI 9124 HANNA Instruments, China). The extract was stored in a tightly sealed dark container in the refrigerator at 4 °C.

Determination of Chlorogenic acid content using RP-HPLC

Determination of chlorogenic acid levels in each extract in this study was carried out using RP-HPLC. The stationary phase used was Column ShimPack GIST C18 (5µm, 4.6×150). The mobile phase system used in this study followed the procedure of Kongkiatpaiboon et al. (2018) and Ernawati et al. (2021) with slight modifications. The injection volume was set to 10 µL. Then, the flow rate was 1.0 $mL.min^{-1}$, and run time was 50 min. Meanwhile, compound detection was performed using PDA detector at 326 nm. Calculation of chlorogenic acid levels used equation 1 (Eq.1), where C_S = standard concentration, A_E = extract area, A_S = standard area, V = volume before dilution, f = dilution factor, and W = sample weight (Ministry of Health Republic of Indonesia, 2017).

$$\text{Chlorogenic acid (\%, w/w)} = \frac{C_S \times \frac{A_E}{A_S} \times V \times f}{W} \times 100\% \quad \text{..... (Eq1)}$$

Antioxidant *ex vivo* study

Animals

The *ex vivo* antioxidant activity test in this study followed the procedure of Rahman et al. (2015). Three of male white Wistar rats (*Rattus norvegicus*), aged 2-3 months with healthy body (±150-200 g), were used in this study. The rats were obtained from the Animal Center for Research, CV. Gelas Kaca, Karanganyar, Solo. For 7 days, rats were acclimated by giving standard feed and drinking ad libitum. Mice were placed in cages in a room with a 12-h light/dark cycle and good air circulation (22-25 °C). The test protocol (No. 23-03-0923) has been approved by Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia (KET-884/UN2.F1/ETIK/PPM.00.02/2023).

Preparation of rat liver homogenate

To obtain the liver homogenate, the rats were previously anesthetized with ketamine (61.67 mg/kg BW) administered intraperitoneally. After becoming unconscious, the rats were dissected to remove their liver organs. The liver was washed, and then weighed. The liver was isolated in cold 50 mM PBS pH 7.4. Liver homogenate was prepared in a concentration of 10% (w/v) in cold PBS. In short, pieces of liver were crushed in cold PBS in a mortar with a cold condition. After homogenization, the homogenate was centrifuged using cool centrifuge (Centurion C2 Series) at 4 °C at 12,000 rpm for 15 min. The supernatant was transferred and collected in a tube

and stored at −20 °C until the antioxidant activity test was performed (Rahman et al., 2015).

Lipid peroxidation inhibition activity (LPOI) assay

Before testing the antioxidant activity, sample preparation was carried out first. NADES extract was made with 3 variations of concentration, such as 3.125, 6.25, and 12.5% (v/v) in PBS pH 7.4. Gallic acid was used as a positive comparator for natural antioxidants made at concentrations of 3.125×10^{-3}, 6.25×10^{-3}, and 12.5×10^{-3} % (b/v). Briefly, 500 µl of the supernatant was added with 1 ml of 0.15 M KCl and 0.3 ml of extract or gallic acid in a test tube with a screw cap. Next, the mixture was added with 0.3 ml of 0.5 mM $FeCl_3$. This mixture was incubated in an incubator at 37±0.5 °C for 30 min. Next, the mixture was reacted by adding 2 ml of cold TBA-TCA-HCl-BHT solution. The TBA-TCA-HCl-BHT solution was prepared following the procedure of Rahman et al. (2015). The mixture was heated at 90±5 °C on a hotplate (Thermo Scientific) for 60 mins. After that, the mixture was cooled in ice water. After being cooled, the mixture was centrifuged with a Universal Centrifuge (Gemmy PLC-025, Taipei, Taiwan) at 3000 rpm for 5 mins. The pink-coloured supernatant was collected, and then its absorbance was measured at 532 nm using a UV-Vis spectrophotometer (Shimadzu UV-1900). Distillate water solution in PBS without sample was used as a control. The percentage of LPOI was calculated based on equation 2 (Eq. 2), where A_o = control absorbance and A_1 = sample absorbance.

$$\text{LPOI } (\%) = \left[\frac{(A_o - A_1}{A_o}\right] \times 100\% \quad \text{..... (Eq. 2)}$$

In Silico Antioxidant Study

The *in-silico* study process was carried out using a computer with Windows 11 Enterprise Evaluation 64-bit with processor Intel(R) Core (TM) i5-4590 CPU @ 3.30GHz, 8048 MB RAM.

Preparation of receptors

The receptors were downloaded from RCSB Protein Data Bank. (https://www.rcsb.org/). Lipoxygenase (LOX) (PDB ID: 1N8Q, lipoxygenase in complex with an inhibitor, protocatechuic acid as a native ligand); Cytochrome P450 (CYP450) (PDB ID: 1OG5, structure of human cytochrome P450 CYP2C9 with S-warfarin as a native ligand) (Kritsi et al., 2022); Cyclooxygenase-2 (COX-2) (PDB ID: 1CX2, cyclooxygenase-2 (prostaglandin synthase-2) complexed with an inhibitor, 1-phenylsulfonamide-3-trifluoromethyl-5-parabromophenylpyrazole, as a native ligand) (Ali et al., 2023).

Water and non-protein molecules were removed from each receptor. Then, the hydrogen molecules were added to the protein structure. The receptors' preparation process was conducted using Pymol version 2.5.5.

Preparation of ligands

In this study, the chlorogenic acid acted as an active marker compound from *P. indica* leaf extracts. Ascorbic acid (Lee et al., 2008; Mohamed et al., 2014; Van Heeswijk et al., 2005) and gallic acid (Amaravani et al., 2012; Jumpa-Ngern et al., 2022; Kamatham et al., 2015) were used as positive references. 3D structures of chlorogenic acid (PubChem ID: 1794427), gallic acid (PubChem ID: 370), and ascorbic acid (PubChem ID: 54670067) were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Ligands preparation was carried out by minimizing the energy using Avogadro2 version 1.91.0.

Redocking

Method validation was conducted based on the root mean square deviation (RMSD) value (RMSD < 2Å) (Shaker et al., 2021). The process of redocking native ligands on each of the receptors was done out using Autodock tools 4.2.6. The grid box in the redocking process with 1CX2 receptors was set at X = 23.947, Y = 21.582, and Z = 15.436. The grid box in the redocking process with the 1N8Q receptor was set at X = 21,287, Y = 1,742, and Z = 19,171. The grid box in the redocking process with the 1OG5 receptor was set at X = -20.236, Y = 86.761, and Z = 38.657. Spacing = 0.375 Å. Genetic algorithm parameters were set at number of GA runs = 50, population size = 300, and number of evals = 2500000 (medium).

Docking

Chlorogenic acid, gallic acid, and ascorbic acid compounds were docked against 1N8Q, 1OG5, and 1CX2 receptors, respectively, which had been prepared using the parameters set from the redocking procedure using Autodock tools 4.2.6. The visualization was performed using the Discovery Studio 2021, Biovia.

Brine Shrimp Lethality Test (BSLT)

The BSLT test using *Artemia salina* L. was implemented following the protocol from Meyer et al. (1982) and Braguini et al. (2018) with slight modifications. Mature *A. salina* (after 48 hours of egg hatching) was used in this test. Each extract and solvent were prepared at five variations of concentration (per cent, % v/v) in 5 ml of seawater.

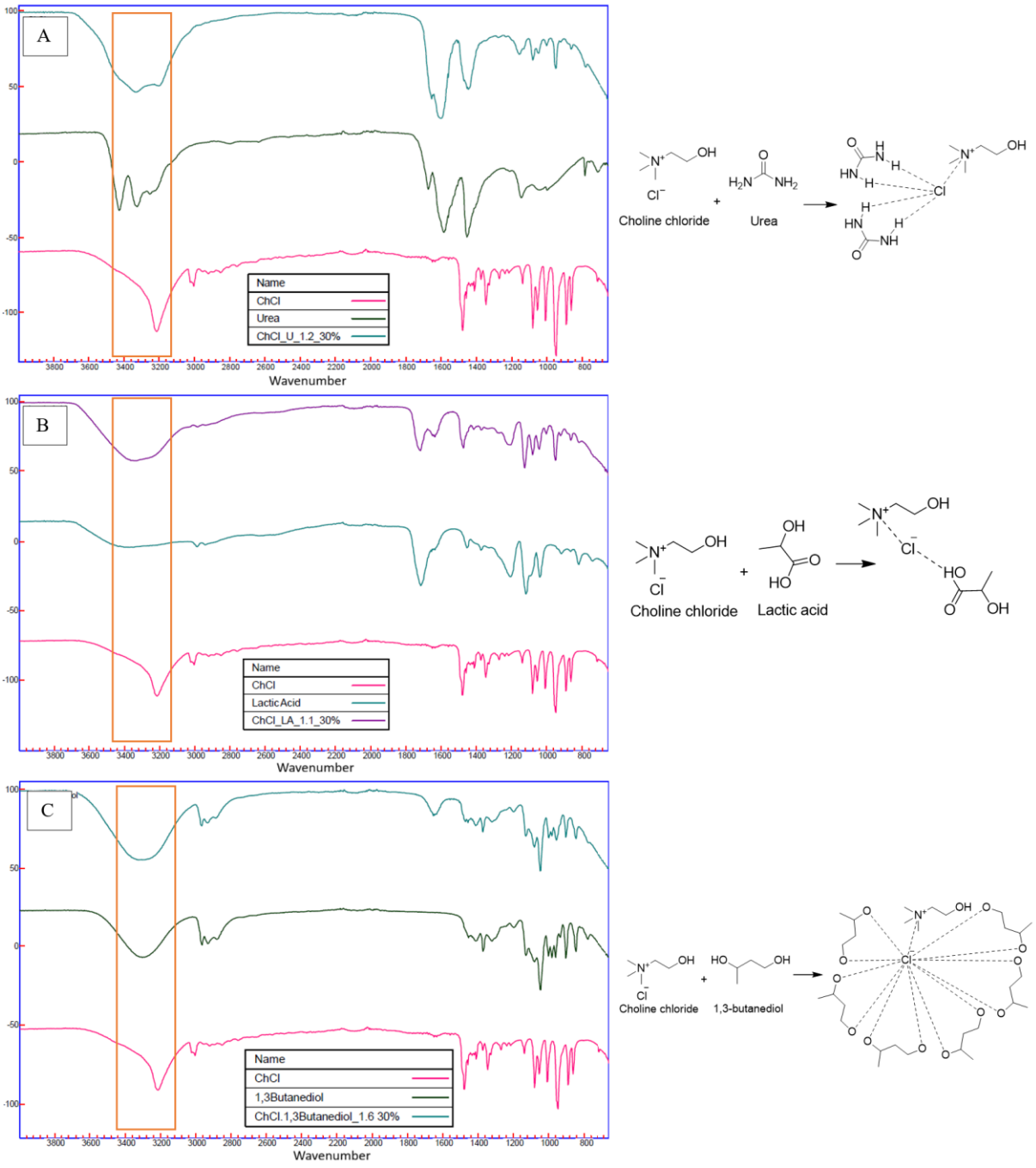

Figure 1. Characteristic FTIR of three NADES in this study and its individual component. (A) ChCl-U (1:2), (B) ChCl-LA (1:1), and (C) ChCl-1,3-but (1:6).
Note: The orange box indicates the shift in the hydroxyl (O–H) vibration.

Each vial containing 10 *A. salina* was added with one drop of suspension yeast (3 mg per 5 mL without sea water) as food. The negative control group (0% extract or solvent) was prepared in 5 mL sea water. After 24 hours of incubation at room temperature under light, the number of surviving *A. salina* was observed. The number of dead *A. salina* is the difference between the initial number of *A. salina* minus the number of surviving *A. salina*. The percentage of *A. salina* deaths was calculated based on the formula: percentage of deaths (%) = (number of dead *A. salina*/initial number of *A. salina*) x 100%. The test was carried out in triplicate.

RESULTS AND DISCUSSION

Several ChCl-based NADES were reported to be able to extract antioxidant polyphenolics from *P. indica* leaves (Hikmawanti et al., 2024). The chlorogenic acid has been reported to be one of the phenolic acids in *P. indica* leaves, which has the potential to act as an antioxidant (Vongsak et al., 2018). In this study, the assessment of the relationship between the amount of chlorogenic acid content in the ChCl-based NADES extracts from *P. indica* leaves and its antioxidant activity through the mechanism of inhibiting lipid peroxidation *ex vivo* and *in silico* was executed.

FTIR Characteristic of NADES

NADES is a solvent that relies on hydrogen bonds between its component molecules (HBA and HBD) in extracting plant metabolites (Hikmawanti et al., 2021). NADES structure can be studied through FTIR analysis. FTIR spectra of each NADES and its components. NADES is known to have the ability to extract plant metabolites due to the hydrogen bonds formed therein (Figure 1). The NADES used in this study was a mixture of choline chloride, ChCl as HBA and HBD, including organic acids (lactic acid, LA), organic bases (urea, U), and polyalcohol (1,3-butanediol, 1,3-but). The differences in the components that make up NADES can cause differences in the physicochemical properties of the three. The increase in solubility of plant metabolites in NADES is related to the hydrogen bonds formed between the NADES and the phytochemical (Hikmawanti et al., 2021).

The three NADES show a shift in hydroxyl (O–H) vibrations in the range of 3200 to 3500 cm^{-1} (Figure 1). The ChCl component of the spectrum is shown in a typical signal at 950 cm^{-1} (C–N). In ChCl-U, the absorption band, which is the stretching vibration of the O–H bond, is shown at signal 3365 cm^{-1}, and the typical signal for urea is demonstrated by the presence of signals at 3324 and 3427 cm^{-1}, which are related to the N–H group (Hikmawanti et al., 2024). In ChCl-LA, there appears to be a minor vibration at 2920-2970 cm^{-1} which representing the C=H double bond. Meanwhile, the absorption band of the alkane group (C–H) of 1,3-butanediol at 2998 cm^{-1} was seen, which shifted to 3035 cm^{-1}. However, water added as much as 30% to the NADES can also affect this signal. It is thought that the hydrogen interactions between the functional groups are what cause these alterations (Fanali et al., 2021).

NADES Extracts

The three extracts are in the form of viscous liquids. The NADES ChCl-U (1:2) and ChCl-LA (1:1) extracts of *P. indica* leaves are dark brown, while the NADES ChCl-1,3-but (1:6) extract is brownish yellow (Supplementary Figure 1). The NADES used in this research consisted of a choline chloride (ChCl)-based component as HBA, while the HBD contained an organic acid (lactic acid, LA), a base (urea, U), and a polyalcohol (1,3-butanediol). The physicochemical properties of NADES are strongly influenced by its constituent components (Fuad et al., 2021). The results of pH measurements and chemical content screening of the three extracts. The ChCl-U (pH = 6.59) and ChCl-1,3-but (5.18) extracts are more alkaline than the ChCl-LA extract (2.27) (Supplementary Table I). The acidity level of NADES extracts may affect their *ex vivo* antioxidant activity and their safety as a medicinal raw material. A study by Salah et al. (2016) reported that the pH of the extraction solvent affected the phenolic content of the extract and its antioxidant activity. In the study, the extraction solvent at pH 5 showed the best conditions for extracting phenolics with the best antioxidant activity. Meanwhile, Ruenroengklin et al. (2008) reported that pH 3-4 conditions in extracted phenolic and antioxidants taken from plants shows relatively higher antioxidant activity. The chemical constituents detected in the NADES extracts include phenolics, alkaloids, and saponins. Only ChCl-LA extract contains tannins.

Chlorogenic Acid Content in NADES Extracts

Chlorogenic acid at a concentration of 1000 ppm yields an area of 39099873 at a retention time (Rt) of 3.431 mins. That the peak of chlorogenic acid appears at an Rt of 3.474 mins in the ChCl-U (1:2) extract (area = 789743), 3.442 mins in the ChCl-LA (1:1) extract (area = 352070), and at 3.430 mins in ChCl-1.3-but (1:6) extract (area = 299411).

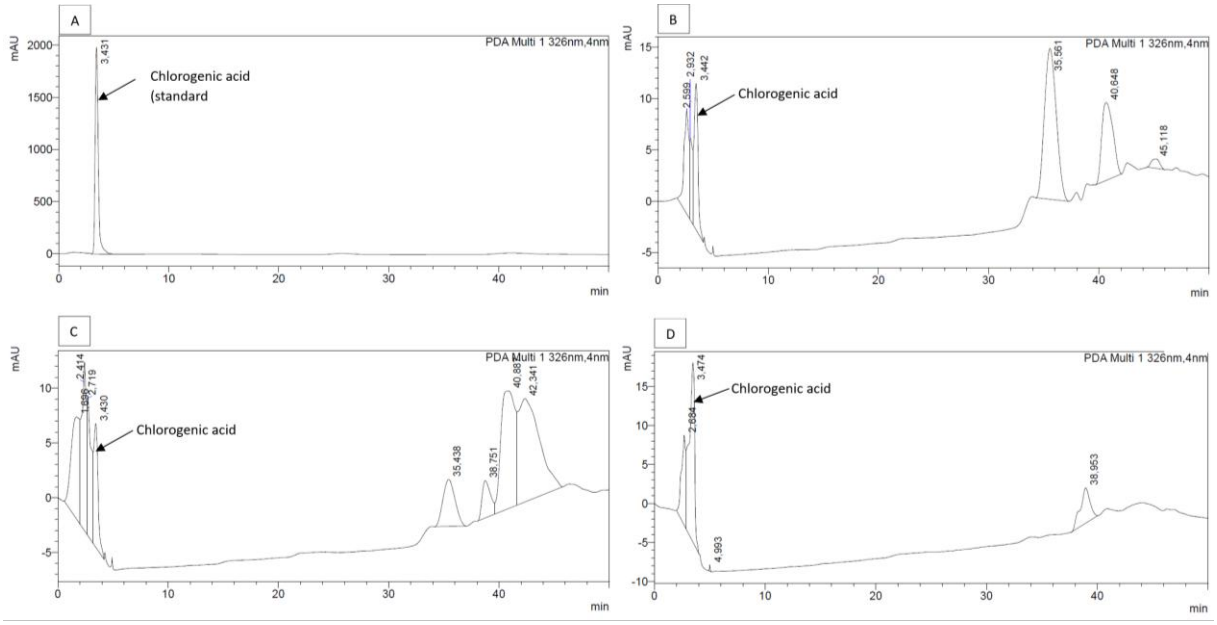

Figure 2. Chromatogram HPLC of chlorogenic acid 1000 ppm (A), ChCl-U (1:2) (B), ChCl-LA (1:1) (C), and ChCl-1,3-but (1:6) (D) NADES extracts from *P. indica* leaves.

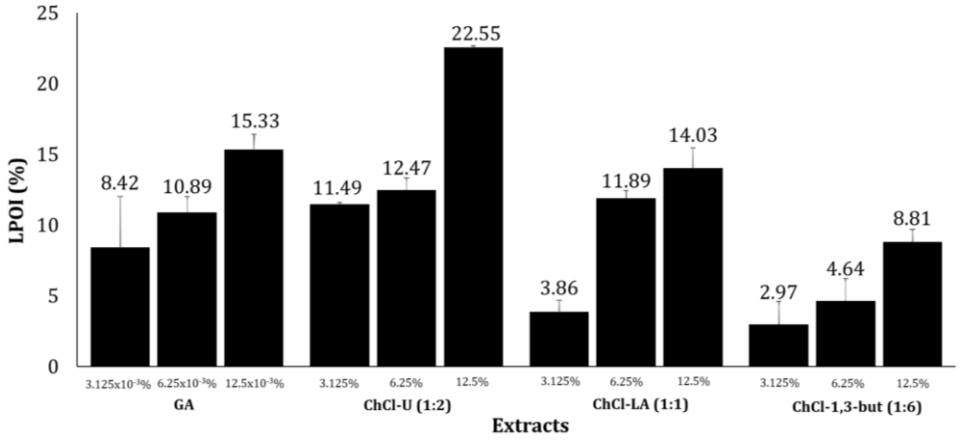

Figure 3. Diagram of LPOI percentage of each *P. indica* leaves NADES extract compared to gallic acid as a positive reference.

Consequently, the chlorogenic acid content in the extracts of ChCl-U (1:2), ChCl-LA (1:1), and ChCl-1,3-but (1:6) of *P. indica* leaves are 0.404%, 0.180%, and 0.153%, respectively.

Antioxidant *ex vivo* study

The ChCl-U (1:2) extract of *P. indica* provides the highest LPOI activity compared to the other two NADES extracts with the order of ChCl-U (1:2) > ChCl-LA (1:1) > ChCl-1,3-but (1:6) (Figure 3). At a concentration of 12.5% (v/v), the ChCl-U extract was able to inhibited 22.55% of lipid peroxidation in rat liver homogenates. In comparison, gallic acid at a concentration of 12.5×10^{-3} % (b/v) was able to inhibited lipid peroxidation by 15.33%. Gallic acid was utilized as a positive reference for natural antioxidants, as it has been reported can reduce lipid peroxidation levels in lipid system (Yen et al., 2002).

In *ex vivo* tests, polyunsaturated fatty acids in rat liver homogenate were subjected to peroxidation using ferric ions, which facilitated the generation of hydroxyl (OH) radicals through Fenton's reaction (Rahman et al., 2015).

Table I. The results of molecular docking

Receptors (PDB ID)	Ligand/ compounds	Binding energy score (S) (kcal/mol)	RMSD (Å)	H bond binding distance	Residues involved in the H-bond interaction
COX-2 (1CX2)	S58[a]	−10.75	1.47	2.36 and 2.25	Arg120
				1.72	His90
				2.00	Gln192
				2.07	Leu352
				2.75 and 3.07	Tyr355
	Chlorogenic acid	−7.27		2.13	His207
				2.18 and 2.16	Ala199
				2.38	Thr212
	Gallic acid [b)]	−3.43		1.76 and 1.76	Ser469
				1.98	Lys492
				1.81	Glu479
	Ascorbic acid[b)]	−4.18		2.44	Ile517
				2.19	Gln192
				2.06	Leu352 and Ser353
				2.26	His90
				2.47	Arg513
				1.86	Arg513
				2.07	Tyr355
				1.99	Tyr355
LOX (1N8Q)	DHB[a]	−4.42	0.96	1.81	His518
				3.02	Trp519
	Chlorogenic acid	+3.00		1.85 and 1.84	Asp766
				2.55	Gln514
				2.28	Ile857
	Gallic acid[b)]	−2.84		2.11	Gln514
				2.29	Ile857
	Ascorbic acid[b)]	−4.30		1.84	His518
				1.86	Gln514
				2.00 and 2.01	Gln514
CYP450 (1OG5)	SWF[a]	−9.19	0.81	2.00	Ala84
				2.40	Phe81
	Chlorogenic acid	−6.47		2.07	Gly79
				2.10 and 2.78	Asn198 and Ala84
				2.20 and 2.42	Gln195
				3.10	Phe457
	Gallic acid[b)]	−3.38		2.07 and 1.92	Gly79
				2.54	Phe81
				2.60	Val94
	Ascorbic acid[b)]	−3.37		2.33	Phe81
				2.07 and 2.27	Gln195
				2.03 and 2.08	Asn198
				1.99	Ala84

Note: [a)] native ligands; [b)] positive reference of natural antioxidant; COX-2 = Cyclooxygenase-2; LOX = Lipoxygenase; CYP450 = Cytochrome P450; S58 = 1-phenylsulfonamide-3-trifluoromethyl-5-parabromophenylpyrazole; DHB = protocatechuic acid; SWF = S-warfarin

This initiator triggers the formation of malondialdehyde (MDA) and other aldehydes, which serve as biomarkers for lipid peroxidation products (Ayala et al., 2014). Following the addition of TBA and subsequent heating, a pink chromogen was formed and its absorbance was measured at 532 nm (Devasagayam et al., 2003; Kumar et al., 2013).

In this study, the ChCl-U extract exhibited the highest chlorogenic acid content and demonstrated the most significant inhibition of lipid peroxidation compared to the other extracts

(ChCl-U(1:2)>ChCl-LA(1:1)>ChCl-1,3-butanediol). This finding suggests that the antioxidant components, including chlorogenic acid, present in the extract can effectively to prevent lipid damage caused by free radicals, which are responsible for lipid peroxidation. However, the method used to assess lipid peroxidation inhibition has limitations, for instance, that TBA can react with other substances in the biological matrix that are unrelated to lipid peroxidation. In addition, the formation of Schiff bases between MDA and amines may result in inaccurate measurements of antioxidant protection (Gulcin, 2020).

Antioxidant *in silico*

According to Ayala et al. (2014), the initiation of lipid peroxidation can be influenced by COX-2, LOX, and CYP450. This research investigated the mechanism by which chlorogenic acid inhibits lipid peroxidation through in silico binding to these three enzymes. Previous studies have reported *in silico* assessments of plant antioxidant activity using the following PDB codes: 1CX2 for COX-2 (Ali et al., 2023), 1OG5 for CYP450, and 1N8Q for LOX (Kritsi et al., 2022).

Initially, redocking was performed for each native ligand of the receptors tested. The structure of each co-crystallized native ligand (with colors representing different elements) aligned with the redocked ligand structure (depicted in purple) for each receptor used (Supplementary Figure 2) illustrates t. The results of each redocking yielded an RMSD value of <2Å (Table I). Specifically, the binding energy for 1-phenylsulfonamide-3-trifluoromethyl-5-parabromophenylpyrazole (S58) as the native ligand against COX-2 (1CX2) was −10.75 kcal/mol, protocatechuic acid (DHB) against LOX (1N8Q) was −4.42 kcal/mol, and S-warfarin (SWF) against CYP450 (1OG5) was −9.19 kcal/mol. These results indicated that the method can be effectively used to dock the tested compounds.

In this study, gallic acid and ascorbic acid were utilized as natural antioxidant comparator compounds. Previously research has indicated that ascorbic acid can inhibit COX-2 (Lee et al., 2008) and LOX (Mohamed et al., 2014). After one week of daily consumption of high-dose ascorbic acid, a decrease in plasma concentrations of the CYP3A4 substrate indinavir was reported (Van Heeswijk et al., 2005). Meanwhile, gallic acid and its analogues have been shown to strongly inhibit COX-2 (Amaravani et al., 2012) and potently inhibit CYP250, particularly CYP1A2, CYP2C9 and CYP2C19 in a non-competitive manner (Jumpa-Ngern et al., 2022).

Chlorogenic acid shows good binding energy compared to gallic acid and ascorbic acid (as a positive comparison of the commonly used natural antioxidants) towards COX-2 (1CX2) and CYP450 (1OG5) receptors with respective values of −7.27 and −6.47 kcal/mol (Table I, Figure 4). Meanwhile, shows a 2D illustration of the interaction between the test compound and the receptor. Redocking results show that hydrogen interactions appear between 1-phenylsulfonamide-3-trifluoromethyl-5-parabromophenylpyrazole (S58) and Arg120, His90, Gln192, Leu352, and Tyr355 residues in COX-2 (1CX2). According to Ali et al. (2023), the pyrazole nitrogen and trifluoromethyl groups of the S58 ligand interact with residues Tyr355 and Arg120 at the binding pocket position. In this study, chlorogenic acid can form hydrogen bonds with residues His207 (2.13Å), Ala199 (2.18Å and 2.16Å), and Thr212 (2.38Å) in the COX-2 (1CX2) receptor binding pocket. Meanwhile, redocking of S-warfarin (SWF) on our work shows that interaction of hydrogen bond occurs with Ala84 and Phe81 in the binding pocket of the CYP450 (1OG5) receptor. According to Kritsi et al. (2022), crucial bindings in the CYP450 pocket are Phe214 and Phe467. In this study, chlorogenic acid is able to form hydrogen bonds with Gly79 (2.07 Å), Phe457 (3.10 Å), Ala84 (2.78 Å), Gln195 (2.20 and 2.42 Å), and Asn198 (2.10 Å) residues (Figure S3). Chlorogenic acid to LOX (1N8Q) demonstrates a positive binding energy value (+3.00 kcal/mol). Based on this finding, the presence of chlorogenic acid as an antioxidant in the process of inhibiting lipid peroxidation may be caused by the mechanism of its interaction with COX-2 and CYP450. In line with the results of the *ex vivo* study, the NADES ChCl-U (1:2) extract of *P. indica* leaves with a high percentage of chlorogenic acid content gave a high percentage of LPOI at the same concentration of other extracts.

In this study, chlorogenic acid provides good molecular interactions with COX-2 and CYP450 in silico. These two receptors participate in the lipid peroxidation process (Ayala et al., 2014). By inhibiting COX-2 and CYP450 by chlorogenic acid as an antioxidant, the metabolism of arachidonic acid as a substance that triggers the production of pro-inflammatory mediators which leads to the formation of excess ROS and lipid peroxidation can be suppressed (Massey & Nicolaou, 2013).

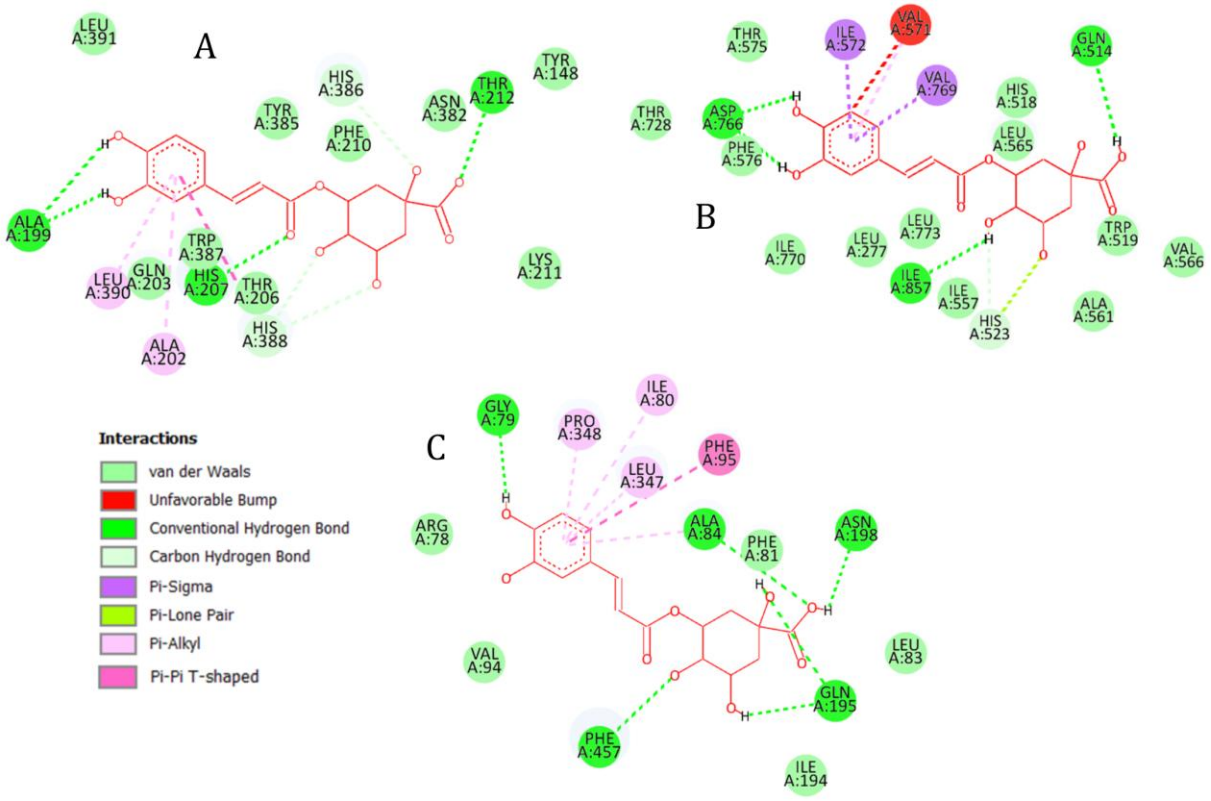

Figure 4. 2D illustration of the interaction between chlorogenic acid and COX-2 (A), LOX (B), and CYP450 (C) receptors. Note: COX-2 = Cyclooxygenase-2; LOX = Lipoxygenase; CYP450 = Cytochrome P450

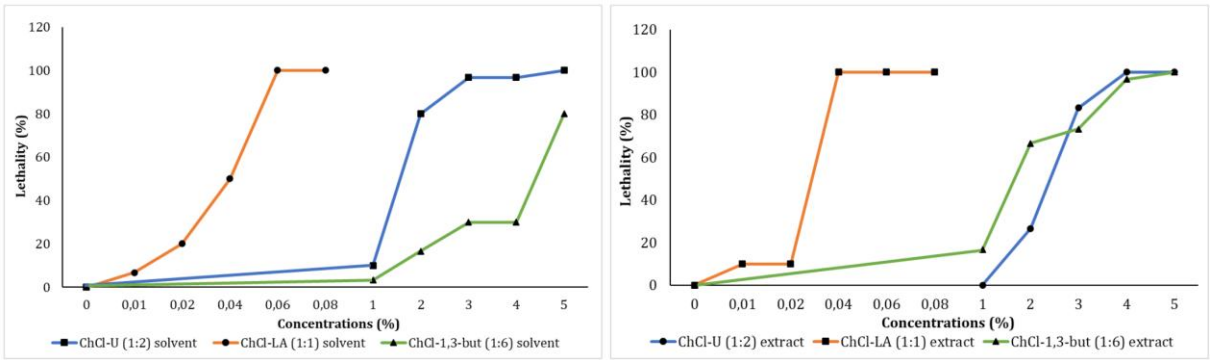

Figure 5. Percentage of lethality of *A. salina* larvae after treatment with each *P. indica* leaves choline chloride based-NADES extracts (right) and its solvents (left). Note: ChCl-U = Choline chloride-urea; ChCl-LA = Choline chloride-lactic acid; ChCl-1,3-but = Choline chloride-1,3-butanediol

When compared with gallic acid and ascorbic acid as references, the activity of chlorogenic acid is considered to be better in inhibiting lipid peroxidation through *in silico* studies. Upadhyay and Rao (2013) review the potential of chlorogenic acid and its derivatives as antioxidants. This compound, like other phenolic acids, is able to prevent oxidative damage. They stated that chlorogenic, from an antioxidant, can be a prooxidant depending on its concentration on the presence of free transition metal ions or their redox status (Upadhyay & Rao, 2013).

BSLT analysis

NADES is claimed to be safe and biodegradable for the environment because of its natural HBA and HBD components (Benvenutti et al., 2019). To study the safety issue of NADES as an alternative extraction solvent, this study also evaluated the toxicity of the NADES solvent used and the extract produced using *A. salina* as a model with the BSLT method (Kudłak et al., 2015).

The higher the concentration of the ChCl-based NADES extract and solvent tested, the higher the percentage of death of *A. salina* (Figure 5). At a concentration of 3%, the solvent ChCl-1,3-but (1:6) (30%) tends to be less toxic than ChCl-U (1:2) (83%). Meanwhile, for ChCl-LA (1:1) at a concentration of 0.04%, the death of *A. salina* is up to 50%. In this study, ChCl-LA (1:1) extract has the most acidic pH compared to ChCl-U (1:2) and ChCl-1,3 butanediol (1:6) extracts. According to Hikmawanti et al. (2024), the ChCl-LA solvent also has the lowest pH value (pH 1.40) compared to ChCl-U (1:2) (pH 9.29) and ChCl-1,3 butanediol (1:6) (pH 2.90). The toxicity and potential contamination caused by DES to the environment can be influenced by the pH, density and water content of the DES. Generally, organic acid-based NADES with high acidity properties shows inhibition of bacterial and fungal growth (Martínez et al., 2022). In addition, the high viscosity of NADES can be one of the causes of *A. salina* death due to low oxygen availability (Kudłak et al., 2015). The toxicity of NADES can also be influenced by the molar ratio of its constituent components (Murador et al., 2019). Further studies are needed to optimize antioxidant extraction from *P. indica* leaves with ChCl-based NADES and its toxicity in cell line cultures or *in vivo* experimental animals.

CONCLUSION

Based on this research, NADES ChCl-U (1:2) extract is selected for further study and development as an antioxidant from *P. indica* leaves. Chlorogenic acid is an important antioxidant component of *P. indica* leaves extract that inhibits lipid peroxidation. The contribution of chlorogenic acid in this mechanism through interactions with proteins/enzymes that play a role in oxidative stress and inflammation can be directed towards the development of therapeutic treatments for chronic diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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