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Extraction of caffeine and polyphenols from kola nuts by ultrasound-assisted extraction and natural deep eutectic solvent

Simamora, I. A. P., Budiastra, I. W. and *Purwanti, N.

Department of Mechanical and Biosystem Engineering, IPB University,
PO BOX 220, Bogor, 16002 West Java, Indonesia

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Abstract

The present work aimed to evaluate the use of ultrasound-assisted extraction (UAE) and a natural deep eutectic solvent composed of lactic acid and sucrose (NADES-LAS) as food-grade solvents for kola nut extraction. The results were compared with the observation from conventional maceration extraction and UAE using ethanol. The yield of kola nut extract obtained by UAE-ethanol for 20 min (7.73 - 8.28%) was similar to the yield obtained over two days of maceration with ethanol (7.83 - 9.10%). A higher yield was obtained using UAE-NADES-LAS because the extract contained not only kola nut extract, but also lactic acid and sucrose. The caffeine and total phenolic contents obtained by UAE-ethanol were higher, which confirmed the superiority of UAE over maceration. Particle size affected the caffeine and total phenolic contents when extraction was performed using UAE. However, the caffeine and total phenolic contents obtained using UAE-NADES-LAS was much lower than the quantity obtained from UAE-ethanol or maceration process.

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Introduction

Kola nut (*Cola nitida*; family Sterculiaceae) is believed to originate from the tropical rainforests of West Africa, specifically Nigeria, Ghana, and Sierra Leone (Adelusi *et al*., 2020). Native Africans use kola nut in various cultural, social, and traditional practices. Kola nut has gained global recognition due to the association with the original formulation of cola drinks (Kanoma *et al*., 2014). It receives further attention due to its functional properties in the food, beverage, and pharmaceutical industries (Benjamin *et al*., 2022). The Food and Drug Administration (FDA) and the Flavor and Extract Manufacturers Association (FEMA) label kola nut extract as a flavouring agent that is Generally Recognized As Safe (GRAS) (Burdock *et al*., 2009). Kola extract has been used over a long time in combination with coca leaves in the formulation of the soft drink Coca-Cola® as a remedy for headaches (Kiple and Ornelas, 2000), and the caffeine content is associated with this remedial effect.

The caffeine content varies depending on the nut variety and geographical location of production (Atanda *et al*., 2007; Dah-Nouvlessounon *et al*., 2015; Yalwa and Bello, 2018), post-harvest treatment (Lowor, 2008; Olaniyan *et al*., 2018), and extraction methods (Nyamien *et al*., 2013; Umeda *et al*., 2020). The reported caffeine content of kola nut ranges from 1.0 to 3.0% (Atanda *et al*., 2007; Yalwa and Bello, 2018). Kola nut has become a great interest for pharmaceutical industries due to its phenolic content. A study showed that the total phenolic content of kola nut was higher than the caffeine content, *i.e*., 3.4 - 6.5% (Lowor, 2008). The phenolic content of kola nut also depends on the nut variety, geographical location of production, post-harvest treatment, and extraction methods.

Ultrasound-assisted extraction (UAE) is a method to accelerate extraction of active compounds from biomass by damaging the integrity of the biomass cells and the photosynthetic system (Hardiningtyas *et al.*, 2022). The selection of solvent to be used in UAE may lead to different extraction yields (Pradana *et al.*, 2024). Natural deep eutectic solvent (NADES) is a green solvent with advantages over conventional solvents due to being environmentally friendly and food-grade, which contributes to safety for consumption (Ahmad *et al*., 2018). This is considered as a greener extractive

*Corresponding author.
Email: nanik_purwanti@apps.ipb.ac.id ; nanik_mp@hotmail.com

reagent used for extracting organic or inorganic species from vegetables and fruits than ionic liquids (Bağda *et al*., 2017a).

Solvent extraction using maceration is the most commonly reported method for extracting kola nut caffeine or polyphenol compounds (Lowor, 2008; Nyamien *et al*., 2013; Dah-Nouvlessounon *et al*., 2015; Umeda *et al*., 2020). Various solvents such as water, ethanol, methanol, chloroform, dichloromethane, and recently introduced flavour-charged water (Swiss Water Process) have been reported for extracting the kola nut, including the caffeine and polyphenol compounds. The use of organic solvents such as methanol, chloroform, and dichloromethane may not be appropriate for kola nut extract used in food and beverage formulations, while the maceration method requires a long processing time. The application of food-grade solvents and green extraction technologies that shorten the extraction process is required to address the stated challenges. Therefore, the present work aimed to evaluate (1) kola nut extraction using UAE, and compare the results with the maceration results, and (2) the use of NADES as a food-grade solvent to extract kola nut, and compare the NADES performance with ethanol.

Materials and methods

Materials

Kola nut samples were obtained from the Industrial Plant and Refreshment Instrument Standard Testing Center (BSIP TRI), an Indonesian government institution in Sukabumi, West Java. The reagents used included ethanol (CH_3CH_2OH), lactic acid, hydrochloric acid (HCl), ammonia, Parry reagent, hydrogen peroxide (H_2O_2), ferric chloride hexahydrate ($FeCl_3.6H_2O$), Folin-Ciocalteu reagent (Merck, Germany), caffeine standard and gallic acid (Sigma Aldrich, USA), and sucrose and sodium carbonate (Na_2CO_3) (Himedia, Indonesia). Distilled water or aqua pro-analytic (OneMed, Indonesia) was used for preparing solutions, and all reagents were of analytical grade.

Instruments

The instruments used were 40- and 60-mesh sieves, digital balance (Fujitsu, Japan), tray dryer, oven (Termaks, Sweden), desiccator, disk mill, ultrasound homogeniser UP200st (Hielscher, Jerman) with 14 mm diameter sonotrode, glassware, 20 - 200 µL and 100 - 1,000 µL micropipettes (Gilson, France), and Pasteur pipette. Others included water bath (IKA, Germany), hot-plate magnetic stirrer (IKA C-MAG HS7, Germany), vortex mixer, UV-Vis spectrophotometer (Ultrospec 7500, Biochrom, UK), rotary vacuum evaporator (RV 10, IKA, Germany), and filter paper Whatman no. 3 (Whatman Plc, UK).

Experimental design

The present work used a completely randomised design (CRD) with three factors, *i.e*., solvent type (ethanol and NADES-LAS), particle size of 40- and 60-mesh, as well as extraction time of 10, 20, and 30 min (Table 1). Each combination was conducted in duplicate, thereby producing 24 experimental units.

Table 1. Experimental design of kola nut extraction.

Maceration			Ultrasound-assisted extraction		
Solvent	**Particle size**	**Extraction time (d)**	**Solvent**	**Particle size**	**Extraction time (min)**
Ethanol (70%, v/v)	40 mesh	2	Ethanol (70%, v/v)	40 mesh	10 20 30
	60 mesh			60 mesh	
			NADES-LAS	40 mesh	10 20 30
				60 mesh	

Sample preparation

Kola nut samples were sorted, washed, cut into small pieces, and oven-dried at 40°C for 18 h. The dried pieces were crushed with a disk mill, and separated using 40- and 60-mesh sieves. The resulting powder was stored in airtight containers until further use.

Proximate analysis

The proximate composition of kola nut was performed following the methods described by the Association of Analytical Chemists (AOAC, 2005). Meanwhile, the calculation of the carbohydrate content was carried out using the difference method.

Solvent preparation

Two solvents were used, *i.e*., 70% (v/v) ethanol and NADES. The preparation of NADES solution was conducted by mixing lactic acid and sucrose (NADES-LAS) in a 3:1 (w/w) ratio. The mixture was stirred using a magnetic stirrer in a glass beaker at 50 - 80°C, then water was added at a 1:1 (w/w) ratio, and shaken until a clear solution formed.

Extraction by maceration

The maceration method was carried out as described by Yuniarti *et al*. (2019), with slight modifications. Kola nut powder was dispersed in 70% ethanol at a 1:50 (w/v) ratio, specifically 2 g of powder in 100 mL ethanol. The dispersion was stirred for 30 min, and incubated in the dark for 6 h before use. Subsequently, the mixture was stirred and incubated for another 18 h. The suspension was separated through filter paper (Whatman no. 3), and the filtrate was collected. The residue was macerated using the same volume of ethanol (100 mL) for 24 h, and filtered. The filtrate from the second maceration was collected and mixed with that from the first maceration. This mixture was concentrated using a rotary vacuum evaporator to obtain a dry extract, which was stored at 4°C for further analysis. The extraction was carried out in duplicate. Maceration was not feasible for application with NADES-LAS because it has a significantly higher viscosity than ethanol. Therefore, NADES-LAS was excluded from the experiments using the maceration method.

Extraction by UAE

Kola nut powder was extracted using UAE by fixing the solid-to-solvent ratio at 1:10 g/mL using 70% ethanol and NADES-LAS. The UAE was set at an amplitude of 60% with a frequency of 20 kHz and power of 200 W. The extraction was performed at a solvent temperature of 40°C for 10, 20, and 30 min. The dispersion obtained after conducting UAE was separated using filter paper (Whatman no. 3). The filtrate was collected and concentrated using a rotary vacuum evaporator to obtain a dry or thick extract, which was stored at 4°C for further analysis.

Extraction yield

The yield of extraction by maceration or UAE was calculated based on the ratio of the extract weight per gram of kola nut powder, as shown in Eq. 1:

$$Yield\ (\%) = \frac{ME}{MS} \times 100\% \qquad \text{(Eq.1)}$$

where, *MS* = mass of the kola nut powder (g) and *ME* = extract mass (g).

Qualitative analysis of caffeine and polyphenols

The caffeine presence in the kola nut extract was determined using the Parry and Murexide tests. The Parry test was performed by adding Parry reagent and a few drops of 5% ammonia to 1 mL of extract positioned in a tube. The extract colour changed to intense blue or green when the tested sample contained caffeine (Kusbandari and Safitri, 2022). Meanwhile, the Murexide test was performed by adding 1.5 mL of 3% H_2O_2 and two drops of 2% HCl to 1 mL of extract kept on a porcelain dish, then evaporated to dry. A few drops of 5% ammonia were added to the resulting residue, and the colour turned intense purple when the tested sample contained caffeine (Drommond *et al*., 1952). The presence of polyphenols in the kola nut extract was determined using a 1% $FeCl_3$ solution. Three drops of 1% $FeCl_3$ were added to 1 mL of kola nut extract, followed by homogenisation and observation of the mixture. The examined sample was considered to contain polyphenols if a blackish-green or blackish-brown colour formed (Padamani *et al*., 2020).

Quantification of caffeine content

The caffeine content was determined using a UV-Vis spectrophotometer according to Fatmawati *et al*. (2018), with slight modifications. A series of caffeine standard concentrations, *i.e*., 0, 10, 20, 30, 40, and 50 mg/L, were prepared to build a calibration curve. A stock solution of the samples was prepared by placing 10 mg of the extract in a 100-mL volumetric flask. Aqua PA was added to the flask up to the 100 mL-tare line, and homogenised. The test samples measured using a spectrophotometer were prepared by diluting 1 mL of the stock solution in aqua PA to a total volume of 10 mL. The caffeine content was determined by measuring the absorbance of the samples at 287 nm.

Quantification of polyphenol content

The total polyphenol content (TPC) was determined using a UV-Vis spectrophotometer according to Shui and Leong (2006), with slight modifications. A stock solution of the test samples was prepared by diluting 10 mg of the extract in aqua PA to a total volume of 100 mL. A volume of 1.8 mL Folin-Ciocalteu reagent (1/10, v/v) was added to 1 mL of the sample solution. This mixture was held for

5 min, and then 1.2 mL of sodium carbonate (7.5%) was added to the mixture. The mixture was incubated for 1 h in the dark at room temperature. The absorbance was measured at 765 nm using distilled water as a blank. A calibration curve was constructed by diluting 10 mg of gallic acid in aqua PA to a total volume of 100 mL. A series of gallic acid concentrations, *i.e*., 0, 0.2, 0.4, 0.8, and 1.2 mg/mL, were prepared from the stock solution standard. Each standard concentration was subjected to the same treatment as the test samples. Analyses were performed in duplicate, and the TPC of the extract was expressed as milligrams of gallic acid equivalents (GAE) per gram of kola nut extract.

Statistical analysis

The data collected were analysed through analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) at the 5% level. DMRT was conducted when the initial results showed statistically different effects. All analysis was performed using IBM SPSS 25 software.

Result and discussion

Proximate composition of kola nut powder

Kola nut powder, on wet basis, contained 17.23% moisture, 7.57% protein, 0.82% fat, 3.67% ash, and 70.72% carbohydrate. The oven drying applied led to a relatively high moisture content of the powder. The moisture content of the kola powder varied between 8.05 - 12.46% (Lowor, 2008; Dah-Nouvlessounon *et al*., 2015) and 20.62 - 22.50% (Ajai *et al*., 2012), which was affected by the drying conditions. A moisture content greater than 10% would facilitate microbial growth capable of damaging the powder. Therefore, the kola nut powder was stored in an air-tight container, and extracted rapidly in a short time. The dried kola nut powder majorly contained carbohydrates, which agreed with other studies (Ajai *et al*., 2012; Benjamin *et al*., 2022).

Extraction yield

Kola nut powder was prepared in two sizes, *i.e*., 40- and 60-mesh, where the 40-mesh had coarser particles. Smaller particle sizes were expected to generate a higher yield of the extract because of a more rapid solvent diffusion (Zhang *et al*., 2018). However, the yield of kola extract was not affected by the particle size when the maceration method or UAE was applied (Table 2). An extraction time longer than 20 min did not necessarily increase the yield. The extraction time was reduced from the two days required in maceration to 20 min using UAE to obtain a kola extract yield similar to the type resulting from the use of ethanol as the solvent. A study reported that UAE effectively reduced extraction time of phycocyanin from *Spirula plantesis*, specifically from the two days required in freeze thawing method to 10 min using UAE (Hardiningtyas *et al.*, 2022).

Table 2. Effect of solvent type, particle size, and extraction time on yield of kola nut extract (%).

Solvent	Particle size (mesh)	Maceration	Ultrasound-assisted extraction time (min)		
			10	20	30
Ethanol	40	7.83 ± 0.25	7.73 ± 0.18	8.03 ± 1.03	8.08 ± 0.81
	60	9.10 ± 1.77	7.83 ± 0.60	8.13 ± 0.53	8.28 ± 0.88
NADES-LAS	40	-	48.16 ± 10.08	51.02 ± 13.64	56.09 ± 8.98
	60	-	53.34 ± 1.95	52.51 ± 7.08	57.02 ± 3.43

Within each solvent group, statistical analysis revealed no significant effect of particle size or extraction time on extract yield.

UAE with NADES-LAS produced a significantly higher extract yield than UAE with ethanol (Table 2), but a direct comparison could not be carried out using the data in Table 2. The extract resulting from NADES-LAS was jelly-like and sticky, while a powder form was obtained with ethanol. The jelly-like and sticky product was heavier than the powder. When this weight was normalised to the initial weight of the sample before extraction, the jelly-like and sticky product had a higher ratio than the powder.

Additional calculations were performed to estimate the yield of the extract obtained using UAE-NADES-LAS when the final product after evaporation was in powder form. Using a moisture content of 17.23% based on proximate analysis, the extract yields obtained with UAE-NADES-LAS for 40-mesh particle sizes was 39.9, 42.2, and 46.4% for

10-, 20-, and 30-min of extraction, respectively. Meanwhile, the yield for 60-mesh particle sizes was 44.1, 43.5, and 47.2% for 10-, 20-, and 30-min of extraction, respectively. These calculated yield values were higher than the values obtained using UAE-ethanol. However, the extract obtained with NADES-LAS contained dried kola extract as well as components of lactic acid and sucrose. Although there are no reports on the yield of kola extract obtained using NADES, most studies showed that NADES produced a higher polyphenol or caffeine content in the extract than conventional solvents, such as ethanol and methanol (Panić *et al*., 2019; Syakfanaya *et al*., 2019; Yuniarti *et al*., 2019; Ahmad *et al*., 2020).

Qualitative analysis of caffeine and polyphenols

Table 3 shows the presence of caffeine and polyphenols in the kola nut extract. The colour produced by the reactions was more intense in the extract obtained using UAE-ethanol than in the extract from UAE-NADES-LAS. Caffeine and polyphenol contents of the extract appeared more soluble in ethanol than in NADES-LAS.

Table 3. Qualitative determination of caffeine and polyphenols in kola nut extract.

Solvent	Compound	Reagent	Colour	Quality
Ethanol	Caffeine	Parry + Ammonia 5%	Green	++
		Murexide (H_2O_2 3% + HCl 2M + Ammonia 5%)	Violet	++
	Polyphenols	$FeCl_3$ 1%	Dark brown	++
NADES-LAS	Caffeine	Parry + Ammonia 5%	Yellowish green	+
		Murexide (H_2O_2 3% + HCl 2M + Ammonia 5%)	Nc	-
	Polyphenols	$FeCl_3$ 1%	Yellow	+

(++): moderately positive (solid colour); (+): mildly positive; (-): absence; and Nc: no colour was developed.

Caffeine content

Caffeine can be analysed spectrophotometrically at 274 nm (Nyamien *et al*., 2013) or 272 nm (Navarra *et al*., 2017). Other studies used different wavelengths ranging from 250 to 300 nm (Hagos *et al*., 2018). Therefore, the maximum wavelength was determined by scanning the caffeine standard solution in the range of 250 to 300 nm. The maximum wavelength of the caffeine standard in the present work was found to be 287 nm. Furthermore, a caffeine calibration curve was constructed at 287 nm, and used to quantify the caffeine content in the kola nut extract.

Caffeine content of the kola extract varied depending on the solvent used for extraction, particle size, and extraction time (Table 4). The particle size did not statistically affect the caffeine content of the extract when the maceration method was applied. The size slightly affected the caffeine content when UAE was performed using ethanol. The 40-mesh particle size led to a higher caffeine content of the extract when the extraction time was prolonged from 10 to 20 min. However, a significant increase in the content was not observed during the extension of the extraction time from 20 to 30 min. The smaller particle size eliminated the effect of extraction time on the caffeine content. A significant effect of particle size on caffeine content was observed with a smaller particle size (60-mesh) when UAE was performed using NADES-LAS. The caffeine content in the extract significantly decreased (~40% reduction) by prolonging the extraction time from 10 to 20 min, but no further decrease was observed by extending the extraction time from 20 to 30 min. Previous investigations reported that time was among the parameters affecting the performance of NADES

Table 4. Effect of solvent, particle size, and extraction time on caffeine content (mg/g) in kola nut extract.

Solvent	Particle size (mesh)	Maceration	Ultrasound-assisted extraction time (min)		
			10	20	30
Ethanol	40	2.77 ± 0.49	1.40 ± 0.01^{a}	2.40 ± 0.41^{b}	3.17 ± 0.17^{b}
	60	2.83 ± 0.44	2.83 ± 1.26^{a}	2.91 ± 1.02^{a}	2.97 ± 0.28^{a}
NADES	40	-	0.99 ± 0.18^{a}	0.75 ± 0.27^{a}	0.88 ± 0.13^{a}
	60	-	1.02 ± 0.13^{a}	0.57 ± 0.09^{b}	0.57 ± 0.13^{b}

Within similar row, means followed different lowercase superscripts are significantly different.

during the extraction of copper from sediment, or manganese from vegetables (Bağda *et al*., 2017a; 2017b). Other parameters such as NADES composition, sample/NADES ratio, and extraction temperature also played important roles in determining the extract yield.

The results showed that the solvent type used with UAE governed the effects of particle size and extraction time on caffeine content of the kola nut extract. UAE performed with ethanol produced a higher caffeine content in the kola nut extract than using NADES-LAS. This result differed from other studies that showed a higher caffeine content in kola nut extract when extraction was performed using NADES compared to conventional solvents (Syakfanaya *et al*., 2019; Yuniarti *et al*., 2019).

NADES solvents are composed of various primary metabolites, such as sugars, sugar alcohols, organic acids, amino acids, amines, and water, in certain molar ratios (Duan *et al*., 2016). This has properties, such as polarity, solubilisation power, and extraction ability, which can be adjusted by changing the composition and ratio. Therefore, NADES type, composition ratio, liquid-to-solid ratio, and extraction time are parameters that determine the caffeine yield. Extraction using NADES produced a lower caffeine content than using ethanol when these four parameters were not optimised (Syakfanaya *et al*., 2019; Yuniarti *et al*., 2019).

Polyphenol content

The total polyphenol content (TPC) of the kola nut extract was determined spectrophotometrically. Gallic acid solution was used as a standard, and it was scanned at wavelengths between 400 and 800 nm. The maximum wavelength was 765 nm, consistent with other reports (Shui and Leong, 2006; Martinović *et al*., 2022). A calibration curve was constructed using a series of gallic acid standard concentrations measured at the maximum wavelength. The calibration curve was used to quantify the TPC of the kola nut extract, with Table 5 presenting the results.

Table 5. Effect of solvent type, particle size, and extraction time on the TPC (mg/g) in kola nut extract.

Solvent	Particle size (mesh)	Maceration	Ultrasound-assisted extraction time (min)		
			10	20	30
Ethanol	40	3.56 ± 1.15	10.90 ± 3.43^{a}	6.51 ± 0.38^{ab}	3.91 ± 0.76^{b}
	60	5.12 ± 1.48	12.80 ± 1.48^{a}	10.82 ± 2.74^{a}	3.84 ± 0.11^{b}
NADES	40	-	1.18 ± 0.05^{a}	1.08 ± 0.03^{a}	1.19 ± 0.06^{a}
	60	-	1.22 ± 0.05^{a}	1.10 ± 0.02^{a}	1.06 ± 0.11^{a}

Within similar row, means followed different lowercase superscripts are significantly different.

Particle sizes of kola nut powder did not significantly affect the TPC when the maceration method was applied. No significant differences were observed when UAE-ethanol or UAE-NADES-LAS was applied at the same extraction time. However, the extraction time significantly affected the TPC of the kola nut extract. Prolonging the extraction time decreased the TPC when UAE-ethanol was applied. A significant decrease was observed during the extension of the extraction time from 20 to 30 min. A significantly higher TPC was obtained with UAE-ethanol than using maceration. The extraction time of 10 min with UAE-ethanol was extremely shorter than the two days spent in maceration method. This may be explained by the physical and mechanical effects of cavitation that enhance the damage to wall structures, and facilitate the release of phenolic compounds (Coelho *et al*., 2021; González-Silva *et al*., 2022). Prolonging the extraction time with UAE (> 10 min) negatively affected polyphenols (Anaya-Esparza *et al*., 2018). The reason for the effects is the increasing temperature, which tends to cause degradation of heat-sensitive compounds of polyphenols. Similar trends of TPC with extraction time were observed for UAE-ethanol and UAE-NADES-LAS.

The TPC obtained using UAE-NADES-LAS was lower than the content obtained using UAE-ethanol. Dabetić *et al*. (2020) reported that grape extraction with NADES comprising choline chloride-citric acid led to a lower TPC (mg GAE/g) than extraction using acidified aqueous ethanol. The same study also reported that the significance of these differences depended on the grape variety. The efficacy of NADES in extracting phenolic compounds appeared to depend on the NADES composition and the type of plant extracted. Ethanol consistently produced a higher TPC than NADES

when used to extract bilberry leaves through sonication method (Martinović *et al*., 2022). In contrast, NADES composed of betaine and urea produced a higher TPC than ethanol when used to extract green tea leaves. The NADES-LAS used in the present work appeared unsuitable for the extraction of phenolic compounds from kola nut. NADES-LAS was diluted with water that increased polarity of NADES as the solvent (Savi *et al*., 2019). This type of solvent might be more appropriate for extracting polar phenolic compounds from kola nut, such as tannic acid and catechin (Jayeola *et al*., 2018). The dilution of NADES-LAS with water at a 1:1 ratio tended to weaken the intensive H-bonding interactions between lactic acid and sucrose (Dai *et al*., 2015), producing a mixture that merely consisted of dissociated NADES compounds (Savi *et al*., 2019). Further study is required to optimise the NADES-LAS composition.

Conclusion

The present work demonstrated that UAE significantly reduced extraction time compared to maceration, using ethanol as the solvent in both methods. UAE produced comparable kola nut extract (7.73 - 8.28%) with maceration (7.83 - 9.10%), but in 20 min rather than two days. UAE-ethanol had a better performance than maceration, generating kola nut extract with significantly higher caffeine and polyphenol contents. Replacing ethanol with NADES-LAS during UAE did not lead to higher extract yield. The caffeine and TPC of the kola extract obtained with UAE-NADES-LAS were significantly lower than the values obtained using UAE-ethanol or maceration method. Further studies should be conducted to optimise the application of UAE-NADES-LAS for kola nut extraction. This should also validate the effectiveness of NADES-LAS over ethanol in extracting caffeine and TPC from the kola nut.

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