Optimization of NADESs-UAE extraction of phytochemicals from African nutmeg (Monodora myristica (Gaertn.) Dunal) Peels: antioxidant and antiradical scavenging activity by Rotatable Center Composite Design-Response Surface Methodology (RCCD-RSM)

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Abstract

A RCCD-RSM was used to optimize the extraction of bioactive compounds from African nutmeg peels based on UAE-NADESs. Four independent factors determined in previous studies that significantly affect bioactive molecule extraction were optimized to elucidate the best conditions that produce the maximum TPC and TFC extracts, with high antiradical scavenging activity through CUPRAC, FRAP, and DPPH inhibition. The optimized conditions with maximum desirability for TPC, TFC, CUPRAC, FRAP, and DPPH were temperature, time, volume, and concentration of 45°c, 5 min, 25 ml, & 90%; 30°c, 5min, 25 ml, & 90%; 30°c, 5 min, 25 ml, % 90%; 45°c, 25 min, 25 ml, & 50%; and 50°c, 15 min, 15 ml, & 70%, respectively. CaFr11 performs highest for TPC and TFC extraction as well as in CUPRAC antiradical scavenging activity at the optimized conditions than the rest of the NADESs studied. CaSu11 had the highest FRAP power, while CaXy12 gave the highest DPPH inhibition at optimized conditions. Besides, all the studied NADESs were better than water in TPC, TFC extraction, and in DPPH inhibition. Our results are similar to results obtained by other researchers who performed optimization of extraction based on UAE-NADESs using other plant materials.

1.0 Introduction

Today's aging but health-conscious population is pushing science and industry to finding safer, more environmentally friendly manufacturing processes and products as alternative to the conventional approaches. The need and applications of plant-derived bioactive compounds and extracts have been rising over the past several years mainly as nutraceuticals and functional food ingredients. Among several plant-derived bioactive compounds, polyphenols, and in particular flavonoids represent the main secondary metabolites that have been recognized for their multi-functional and multi-industrial applications for promoting human health [1].

Extraction is an important process in many household and industrial derivatization of necessary components. It is a crucial step in pharmaceutical and food processing operations in terms of extracting desired bioactive compounds in their optimum yield without affecting their functionality. Ultrasonic-assisted extraction (UAE) is one of the advanced forms of innovative extraction techniques and uses ultrasonic waves to generate cavitation bubbles which on collision generate a hot spot/micro-jet with higher temperature and pressure, thus

resulting in faster and efficient extraction of components from the plant matrix [2]. The quality and yield of the extracts are significantly affected by various factors including but not limited to the UAE such as ultrasonic power/intensity, reaction vessel type, extraction temperature and time, solvent pH, concentration and volume [3]. Application of UAE for extraction of bioactive compounds from plants abound in the literature for example, anthocyanins [4], chlorogenic acid [5], antioxidant [6], polysaccharide [7], and so on. Therefore, it is very important in order to achieve considerable UAE application to extraction process, determination of significant process factors and optimization of their levels is key to getting superior quality of extract.

Phenolic compounds are micronutrient endowed with many biological effects including antinflammatory and antioxidant properties [8]. Research highlights that a diet abundant in fruits and vegetables, enriched with spices, herbs, and seaweeds, correlates with a decreased risk of chronic degenerative diseases affecting cardiovascular, neurological, metabolic, and oncological health, suggesting the protective role of polyphenols in preventing various disorders and abnormalities [9 10 11 12 13 14 15 16 17 18 19 20 21]. Several foods and food-derived substances rich in phenolic compounds (phenolic acids, flavonoids, stilbenes, & tannins) have been proven to exert cardioprotective and antioxidant properties [22 23 24]. Many of these substances have been extensively researched for their potential as nutraceuticals in clinical applications, before drugs beyond the foods, highlighting the importance of discovering new extraction techniques that enable the obtaining of phyto-complexes and isolated compounds with minimal environmental impact and in alignment with the principles of the circular economy [25 26 27 28 29 30 31 32 33]. African nutmeg is a perennial edible berry plant that grows well in African evergreen forests, and is widely used for medicinal and culinary purposes. Phytochemical screening of hydroalcoholic extracts of the seeds indicated they contain high amounts of polyphenols, saponins, tannins, carbohydrates, and other valuable substances [34 35 36 37 38 39 40]. All parts of the plant, including the bark, roots, seeds, and leaves, are very useful for treating various ailments in African traditional medicine. The root is munched to prevent toothaches and arthritis and is also utilized in the management of anaemia, haemorrhoids and sexual weakness[41]. The stem bark of *M. myristica* is used to treat haemorrhoids, stomachache, fever, pain, and eye diseases[39]. Studies have reported the antioxidant properties of *M. myristica* seeds[35][36][41]. The minty smelling seed has also been shown to possess cholesterol-lowering[39], anti-sickling[42], antimicrobial[40], and anthelmintic activities[43].

The solvents commonly used to extract bioactive compounds and nutraceuticals in the pharmaceutical, food, and cosmetic industries are mostly organic polar solvents such as ethanol, methanol, and water. These organic solvents have high dissolution ability and extraction power but are of non-renewable origin except water. These solvents accordingly have desirable applications for extracting bioactive compounds, however, they also possess high adverse effects on human health and the environment, and therefore, not the best option in terms of extraction of bioactive compounds for human use [44 45 46]. Green solvents can offer much fewer adverse effects, reduced extraction energy consumption, and higher safety for extracting bioactive compounds [47 48 49 50 51 52 32 53 54 55]. One of the emerging solvents that have recently been considered for extracting bioactive compounds and nutraceuticals in the food and pharmaceutical industries is deep eutectic solvents (DES). These are solvents made by mixing two or more compounds in certain ratios primarily by hydrogen bonding to produce a lower melting point mixture than those of each of the reacting species alone. Two fundamental principle of DES formation are the presence of hydrogen bond donor (HBD), and hydrogen bond acceptor (HBA) among the reacting species [56 57 58 59]. If the DES is based on natural compounds such as choline chloride, carbohydrates, amino acids, and organic acids, these green solvents are natural deep eutectic solvents (NADESs) [60]. The main factor for the formation of NADES is the use of natural compounds as the source of hydrogen bond that occurs between the HBD and HBA species. The effects NADESs have

on solubility, polarity, electrical conductivity, density, and viscosity, may affect extraction efficiency, therefore, combining NADESs with modern extraction methods such as ultrasonication can significantly improve the extraction efficiency of bioactive compounds [61 62 63 64 65 66 67].

In recent years, the use of ultrasound technology as a new method with advantages such as high efficiency, short extraction time, ease of use, and low maintenance costs, has found an essential place in the food and pharmaceutical industries [68]. High power ultrasound is used as a tool to change the food properties, such as homogenization, cleaning, sterilization, heating, emulsification, inhibition of enzymes and microbial activities, cell disintegration, intensification of oxidation reactions, meat modification, crystallization correction, and so on [69 70 71 72 73 74 75 76 77]. Extraction of bioactive compounds from organic sources is one of the other practical applications of ultrasonic waves that have attracted the attention of many researchers for over two decades because the process increases mass transfer, reduce extraction time and increase extraction efficiency [64 78 79 80 81 82 83]. In ultrasound-assisted extraction (UAE), ultrasonic waves change the physical and chemical characteristics after interacting with the target sample matrix through the cavitation phenomenon leading to the collapse of the cell wall, and accompanied by increase in mass transfer the bioactive compounds from the sample matrix into the solvent. Ultrasound extraction technique has been tagged a clean and green method because it requires less amounts of solvent and is accomplished at a shorter process time. Different sample matrices from plants, microbes, or inorganic compounds containing aromatics and flavoring substances, pigments, antioxidants, and other high value ingredients have been extracted by ultrasound techniques, and the efficiency of the process evaluated[84 85].

The aim of the present study is to optimize an UAE-NADES extraction of bioactive compounds from African nutmeg peels and evaluate the antiradical scavenging potentials of the extracts using in vitro methods. To the best of our knowledge, there is no published studies on the optimized ultrasonic assisted extraction of bioactive compounds in African nutmeg peels in the literature. By optimizing a combined ultrasound and NADESs extraction of bioactive compounds from African nutmeg peels, the present study is the first attempt to comprehensively evaluate the extraction of bioactive compounds from *M. Myristica*. After extracting the compounds, the total phenolic content (TPC), the total flavonoid content (TFC) was analyzed by spectrophotometric methods, and the antiradical scavenging activity evaluated by various in vitro techniques (CUPRAC, FRAP, and DPPH tests).

2.0 Results

2.1 Results

Different factors affecting the efficiency of ultrasound-assisted extraction include the temperature of extraction, time of extraction, volume of solvent, and concentration of solvent. In order to optimize the extraction conditions, a comprehensive evaluation involving a four-factor, five-level, central composite design within the framework of response surface methodology (RSM) was employed, as detailed in **Supplementary Table 1 (ST 1)**. The experiment's independent variables included the extraction

temperature (UT), ranging from 30 to 70 ºC, the extraction time (ET), spanning 5 and 25 min, the solvent volume (SV), from 5 to 25 ml, and the solvent concentration (SC) which ranged from 50 to 90%. Experimental data were used to compute the coefficient of the polynomial equation that predicts response values. Regression equations for TPC, TFC, CUPRAC, FRAP, and DPPH calculated by response surface methodology for various responses are presented in **Supplementary Table 2 (ST 2)**. Analysis of variance showed that some of the experimental results could be well fitted with a full quadratic polynomial model, some with linear plus squares model, and others with linear plus interactions model determined by F-value and the significance of P-value and presented in **Supplementary Table 3 (ST 3)**. Coefficients of determination (R^2) for TPC, TFC, CUPRAC, FRAP, and DPPH varied from 0.9977 to 1.000, 0.9985 to 1.000, 0.7004 to 0.9994, 0.7639 to 0.9999 and 0.9981 to 0.9999, respectively and are presented in **Supplementary Table 4 (ST 4)** with other statistical parameters calculated from ANOVA. The quadratic, the linear plus squares, as well as the linear plus interactions models were found to be statistically significant with a non-significant lack of fit ($p < 0.05$) with respect to pure error among the parameters measured which showed that the models are statistically accurate for the data. The R^2 values closer to unity are an indication of better fitted model to experimental data, and contrary if the values of R^2 are lower, which shows that the independent variables are not predicting the values of responses accurately [86].

2.1. Effect of Factors on Total Phenolic Content (TPC)

The total phenolic content of extract was measured using Follin–Ciocalteau reagent, which assesses phenolic oxidation and the subsequent interaction with phosphomolybdic-phosphotungstic acid complexes. The total phenolic content of the extracts is presented in **Table 1**; expressed as equivalents of gallic acid (GAE), with values spanning from 29.902 to 1382.35 µgGAE/g. The highest TPC extract was obtained from CaFr11 as shown in run 22, which have operating conditions of ultrasound temperature, extraction time, solvent volume, and solvent concentration set at 50_°C, 15 min, 25 ml, and 70%, respectively. The lowest TPC was obtained found CaXy21 found in run 14 with conditions of ultrasound temperature, extraction time, solvent volume, and solvent concentration set at 40°c, 20 min, 20 ml, and 80% for extraction, respectively. The analysis of variance pareto chart shows that squared solvent volume (i.e. SV*SV) has the maximum significance on TPC extraction using CaFr11, followed by solvent volume (SV) and squared solvent concentration (i.e. SC*SC), while other variables' contributions were not significant (see **Supplementary Figure 1a (SF 1a**). Three-dimensional surface plots of the effects of factors on the total phenolic contents for CaFr11 and CaXy21 are presented in **Figure 1**. **Figure 1A** shows the effect of temperature and time of extraction on the total phenolic content of extract while keeping the volume and concentration of solvent constant. **Figure 1B** displays the effect of temperature and solvent volume on TPC extraction while keeping the time and concentration constant. **Figure 1C** presents the effect of temperature and solvent concentration on phenolic extraction when time and volume are kept constant. In **figure 1D**, the effect of time and volume on TPC extraction while keeping temperature and concentration constant, is presented, whereas in **Figure 1E,** the effect of time and concentration on TPC when temperature and volume is kept constant is presented. Lastly **Figure 1F** displays the effect of

volume and concentration of solvent on phenolic content while temperature and time are kept constant. From the plots, it could be clearly observed that the highest TPC extract was obtained when solvent volume was above 24ml and solvent concentration was above 75% and other factors kept constant when CaFr11 is the extracting solvent (Figure 1F), while the lowest TPC was gotten from CaXy21 when extraction time was above 24 min, solvent concentration above 75%, while other factors remain unchanged (Figure 1E).

2.2. Effect of Factors on Total Flavonoid Content (TFC)

The total flavonoid content was measured as quercetin equivalent per gram of samples in dry weight basis. The maximum and minimum flavonoid contents were 1722.02 µgQE/g and 56.451 µg QE/g respectively and were obtained from the same solvent (CaGc11) at different operating conditions. The maximum extract was found in run number 18 with operational conditions of temperature, time, volume, and concentration set as 40°c, 10 min, 20 ml, and 60%, respectively, while the minimum TFC was in run number 30 with temperature, time, volume, and concentration of 50°c, 15 min, 5 ml, 70% respectively as shown in **Table 1**. Analysis of variance table shows that the quadratic model was statistically significant for the total flavonoid content of extracts from the studied NADESs except in CaSu11 and CaFr12 where linear plus interaction model was significant with a non-significant lack of fit with respect to pure error (see **SF 1b**). Surface plots illustrating the interactions between various extraction parameters and their influence on flavonoid yields from CaGc11 are shown in Figure 2. Figures 2A, 2B, and 2C show how variations in temperature and time, with a constant volume and concentration, temperature alongside volume with fixed time and concentration, and temperature coupled with concentration under unaltered time and volume settings, uniquely impact TFC extraction, respectively. Figures 2D, and 2E display the effect of time and volume when temperature and concentration are kept constant, and the effect of time and concentration while keeping temperature and volume constant, and finally Figure 2F presents the effect of volume and concentration on TFC extraction when temperature and time are kept constant.

2.4. Effects of Factors on Antiradical scavenging activity

The antiradical scavenging activity of the extracts using different levels of independent variables was measured based on CUPRAC, FRAP, and DPPH inhibition methods. The results obtained for CUPRAC and DPPH were reported in micro mole of Trolox equivalent per gram (µMTE/g) of sample in dry weight, while DPPH was reported as percentage of inhibition. The results for the three methods are presented in **Table 1**. The highest CUPRAC activity was obtained as 26.2785µMTE/g, found in run 22 using CaFr11 as solvent under extraction temperature, extraction time, solvent volume, and solvent concentration of 50°c, 15 min, 25 ml, and 70% respectively. Analysis of variance table shows that linear plus interaction's (LI) model predominates among the solvents except in CaFr12, CaXy21, CaGr11, CaGr12, and CaGr21 where full quadratic (FQ) model presents the best fit (see **ST 3**). Pareto plot (**SF 1c**) shows that solvent volume, squared solvent volume, squared extraction time, and extraction time are significant while other factors are not for CaFr11 which produced the highest CUPRAC capacity. Overall, LI and FQ models gave significant effects for CUPRAC capacity among the NADESs studied. The FRAP power ranged from

0.3672 to 23.688 µMTE/g. The highest FRAP power (23.688µMTE/g) was found in run 20 from CaGr21 at extraction temperature, extraction time, solvent volume, and solvent concentration of 40°c, 20 min, 20 ml, and 80% respectively, while the smallest FRAP power was observed in CaFr11 under temperature, time, volume, and concentration of 40°c, 20 min, 10 ml, and 60% respectively. The analysis of variance table (**ST 3**) shows that FQ model presented the best fit for all the NADESs except for the glucose-based (CaGl11 and CaGl21) NADESs which follows a linear plus squares (LS) model. The percentage inhibition measured by DPPH gave the maximum percentage inhibition of 92 from CaFr11 in run number 2 under extraction temperature, extraction time, solvent volume, and solvent concentration of 40°c, 20 min, 10 ml, and 60% respectively, while the minimum inhibition of 1.13 was observed from CaXy21 under two different sets of conditions, specifically, runs 27 and 28 with extraction temperature, extraction time, solvent volume, and percentage concentration of solvent of 30°c, 15 min, 15ml, and 70%, and 50°c, 5 min, 15ml, and 70%, respectively. Significant factors include extraction temperature, squared extraction temperature, extraction time, and squared extraction time, respectively and shown in the pareto chat (See **SF 1e).** The analysis of variance table (**ST 3**) shows FQ was the best model for many of the solvents except for CaXy12, CaGr11, and CaGl11 which follows a LI model fitting, and CaGr21 which has a squared plus interaction (SI) model effect. It should be noted that the effects of the interactions between time and volume, time and concentration, as well as volume and concentration, could not be fitted in the DPPH modelling and were removed.

2.5. Optimization and validation of model

Using Minitab software, numeric optimizations of responses were carried out by the desirability function. Optimization was carried out with the goal set to maximize the total phenolic content, total flavonoid content, and maximum antiradical scavenging activity using the independent variables of temperature, time, volume, and concentration of solvent in the ranges that were used in the experiment. Five different solutions were calculated by Minitab software with different levels of independent variables with good desirability. The solution with maximum desirability (0.993634) had a predicted temperature of 44.9495°c **(45°c)**, time of 5 min, volume of 25 ml, and 90% concentration of solvent for TPC extraction. For TFC, CUPRAC, FRAP, and DPPH, the maximum desirability, and predicted conditions of temperature, time, volume, and concentration are (0.900334), 30°c, 5 min, 25 ml, 85.5556 % **(90%)**; (1.000), 30°c, 5 min, 25 ml, 90%; (0.960543), 42.9293°c **(45°c)**, 25 min, 25 ml, 50%; and (0.931252), 50.6061 °C **(50°c)**, 16.3131 min **(15 min)**, 13.8889 ml **(15 ml)**, 69.3939 % **(70%)**, respectively. The predicted conditions were applied to the samples, however, due to limitations of instrument, the values in bold were used in place of the actual predicted values. The Fitted responses with standard errors of fits, the experimental responses with standard deviations obtained under the predicted conditions are presented in **Table 2**. Water as the greenest solvent was used to compare the performance of the NADESs in this study and the results at the optimized conditions presented in **Figure 3** for TPC and TFC, and **Figure 4** for CUPRAC, FRAP, and DPPH, respectively.

3.0 Discussions

It has already been established that the practical application of extraction of bioactive compounds with NADES is more suitable than organic solvents for use in food and pharmaceutical products [87 51 88]. Therefore, citric acid-based NADESs, were investigated for efficient extraction of phenolic compounds using a multifactorial modelling in our previous study [89]. The NADESs were found to be suitable solvents for extracting bioactive compounds from African nutmeg peels and were selected for the present optimization study using rotatable central composite response surface methodology. The behavior of phenolic compounds with more tendency towards acidic NADESs was seen as the basis for increase in the extraction efficiency of phenolic and antioxidant compounds [90]. Extraction with NADESs coupled with ultrasonication techniques have been reported by different authors [91 92 93 94 95]. Bertolo et al. prepared NADESs with choline chloride as HBA and glucose, sucrose, glycerol, lactic acid, and citric acid as HBDs and reported that choline chloride-lactic acid (ChCl-LA) as the best solvent for extracting bioactive, and phenolic compounds from pomegranate peel waste using NADES coupled with ultrasonic bath [95]. They used various NADES solvents based on choline chloride containing glucose, sucrose, glycerol, citric acid, and lactic acid. Based on the results obtained by these researchers, it was found that the phenolic content of compounds extracted by ChCl-LA was significantly higher than other NADES and ethanolic solutions. They attributed this to a more acidic pH of lactic acid than other solvents. Also, Obluchinskaya et al., Olfat et al., and Koraqi et al. studied the extraction of bioactive compounds from arctic *Fucus vesiculosus* (brown seaweed), *Hypnea flagelliformis,*and *Rosa damascene* Mill. by NADESs coupled with ultrasonication, respectively. They reported that NADESs based on lactic acid coupled with ultrasonic bath will lead to better extraction of phenolic and bioactive compounds [94 68 92]. In our study, we found similarity of efficiency between citric acid-fructose based NADESs to the ChCl-LA based NADES in TPC extraction. Therefore, it may be appropriate to assert that the acidic condition plays a crucial role in the extraction of phenolic compounds using NADESs coupled to ultrasonic technique.

Based on the rotatable central composite response surface methodology (RCCRSM) design to optimize the bioactive compounds extraction from African nutmeg peels, the results of TPC, TFC, CUPRAC, FRAP, and DPPH extraction efficiency using NADESs coupled to ultrasound bath are shown in **Table 1**.
Evaluation by RCCRSM showed variability among the studied NADESs with a second-order polynomial (quadratic) model, linear plus squares, linear plus interaction models with appropriate statistical parameters selected for data analysis. ANOVA for the model responses is shown in Fisher's statistical tests (*F*-test) and probability (*p*-value) in **ST 3**. Accordingly, as shown in **ST 3**, $p < 0.05$ and indicated in bold show that the model is significant, and the data fit well with the proposed model. High R^2 (0.97), non-significant Lack of Fit ($p \geq 0.14$), and $p \leq 0.04$ indicate a good fit of the model for reasonable prediction of data in the experimental range. Therefore, as shown in **ST 3**, linear terms, interaction terms, and quadratic terms have a significant effect ($p < 0.05$) on the response variables. In the presence of a solvent, the cavitation phenomenon occurs by exposing the African nutmeg peelsto ultrasound waves. Cavitation is microbubbles that are formed by the application of ultrasonic waves. Ultrasound waves, due to the cycle of compression and rarefaction, cause the formation, growth, and implosion of bubbles. As the bubbles explode, the tissue matrix of the peelcollapses. The cycle of compression and rarefaction is directly related to the solvent volume, so that at higher volumes, the rate of cavitation will be higher.

Therefore, as a result of increasing the solvent volume, the ultrasound cavitation increases leading to increased tissue disruption and ultimate increase in TPC extraction [96 97 98]. The two main steps on which the UAE process is based are the washing and the slow extraction mechanisms. In the washing stage, the soluble compounds on the surface or close to the surface are washed and extracted by solvent, which is accompanied by a rapid increase in the extraction slope, while during the slow extraction stage, soluble materials are slowly extracted from the sample matrix into the solvent by both diffusion and osmotic phenomena. At the slow extraction stage, temperature plays a role as it helps in diffusion and osmosis through increased molecular mobility. At this mechanism, sufficient time to extract bioactive compounds from the target matrix is required [99]. Therefore, providing sufficient time will cause the most bioactive compounds to be extracted and thus increase the extraction efficiency. However, prolonging the time may lead to polyphenol degradation. The concentration of the solvent increases by reducing the amount of water added to the NADES. Increasing the solvent concentration causes the mass transfer more rapidly and the phenolic compounds in the African nutmeg peels to diffuse into the solvent [77]. This may be because higher solvent concentration means availability of more hydrogen bond species for interaction with the bioactive molecules leaving the sample matrix into the solvent. higher concentration of NADESs will increase the content of hydrophilic compounds, because of the acidic nature of solvents.

Comparison of TPC and TFC compounds extracted by the UAE-NADESs method at optimized conditions with the compounds extracted by the greenest solvent (water) at the same conditions is shown in **Table 2 and Figure 3**. The total phenol content of the bioactive compound extracted by the UAE optimal method with eutectic solvents showed a better performance than water among all the formed NADESs. The combination of ultrasonic process and eutectic solvent, probably due to its role in improving the mass transfer process of bioactive compounds from the African nutmeg peels, will cause more bioactive and phenolic compounds to be extracted and will increase the total phenol content [100]. The UAE-NADESs, due to their role in the stability of solutes, prevents the degradation of these compounds during the extraction process and thus increases the extraction efficiency. In this extraction process, due to the formation of strong hydrogen bonds between solutes and NADES molecules, the stability of the target compounds may increase against adverse environmental conditions (oxidative degradation) [101]. Also, during the UAE process, the high solubility of NADES may confer a significant advantage for the extraction and stability of bioactive compounds (polyphenols, flavonoids, anthocyanins, isoflavones, catechins, sesamol, *etc.*), at such shorter extraction time than conventional extraction processes [102]. Macchioni et al. reported similar results for extracting phenolic compounds from *Humulus lupulus* L. using NADES and UAE [103]. They stated that the extraction efficiency of phenolic compounds by lactic acid-based eutectic solvents is higher than the conventional extraction method using ethanol 80 %. They attributed this behavior to the improvement of the mass transfer process by the UAE method and the increase in the stability of phenolic compounds induced by eutectic solvents [103].

The antiradical scavenging activity (CUPRAC, FRAP, and DPPH) of bioactive compounds extracted from African nutmeg peels using the UAE-NADESs method was compare with UAE-water at the optimized

conditions is presented in **Table 2 and Figure 4**. Based on the results obtained from the analysis of antiradical activity data by Fisher's least significant difference (LSD) test, it was found that in all scavenging tests by DPPH inhibitions performed significantly better in all UAE-NADESs than in UAE-water ($p < 0.05$). Test by CUPRAC shows that all UAE-NADESs were significantly higher than UAE-water except in UAE-CaGc11 where there is no significant difference with that from UAE-water ($p < 0.05$). However, UAE-water performed significantly higher than UAE-CaGl11, UAE-CaGl21, and UAE-CaGc21 using FRAP test, but significantly lower in all other UAE-NADESs studied ($p < 0.05$) (see Figure 4). The free radical scavenging activity of DPPH, CUPRAC and FRAP of UAE coupled with NADES may probably be connected to the content of higher phenolic compounds extracted by the UAE method using eutectic solvents. These bioactive (nutraceuticals) have various antioxidant and biological activities in food and biological systems due to their structural properties [104 105]. The hydrogen donating capability and reduction ability of phenolic compounds cause the oxidation chain reaction to be broken, and these compounds are referred to as potent antioxidants [68]. These findings are consistent with the results of other researchers. Mansinhos et al. studied the extraction of phenolic compounds from *Lavandula pedunculata* subsp. Franco *lusitanica* (Chaytor) with UAE and NADES, and found that the combination of UAE and NADES resulted in higher antioxidant activity compared to conventional methods [106]. They stated that scavenging of DPPH free radicals by UAE-NADES methods was greater than that of extracts obtained by conventional methods, which attributed this higher antioxidant activity to the content of higher phenolic compounds in the UAE method [106].

4.0 Materials and Methods

4.1 Materials

4.1.1 Chemicals

All the chemicals and solvents used were of analytical grade and standard grade. Citric acid monohydrate (≤ 100% purity), sucrose (99.8% purity), glucose monohydrate (99.0% purity), fructose (98.5-101.2% purity), xylitol (99% purity), glycerol (99-101% purity), sodium carbonate (99% purity), sodium acetate (99% purity), potassium acetate (99% purity), iron (iii) chloride hexahydrate (99% purity), and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (99% purity) were supplied by (Sigma–Aldrich, Germany), Glycine (99% purity), and potassium persulfate (≥ 99% purity) were supplied by (Fluka Analytica, Germany), methanol (HPLC grade) (≥ 99.9% purity) was supplied by (Sigma–Aldrich, France), ethanol (96% purity) was purchased from (Fillab, Bulgaria), ammonium acetate (≥ 98% purity) was supplied by (Sigma–Aldrich, Netherlands), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (≥ 99% purity), and Folin-Ciocalteu's phenol reagent were supplied by (Sigma–Aldrich Switzerland), copper (ii) chloride (99% purity) was bought from (Sigma–Aldrich United Kingdom), aluminum (iii) nitrate nanohydrate (98.5% purity) was purchased from (Chem-Lab NV Belgium), Neocuprine (99.9% purity) was supplied by (Sigma–Aldrich Austria), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (≥ 99% purity), 3,4,5-trihydroxybenzoic acid (Gallic acid) (99% purity) were purchased from (Sigma–Aldrich Milan, Italy). Water was purified using a Milli-Q Plus 185 system from Millipore (Milford, MA, USA).

4.1.2 Plant Material

Dried seeds of African Nutmeg (*Monodora myristica* (Gaertn.) Dahl fruits from the 2022 harvest were purchased from the Zuba Fruit Market, Abuja, Nigeria. The seeds were cleaned, packed under vacuum following WTO guidelines on sanitary and phytosanitary measures adopted in Nigeria as provided by the Standards Organisation of Nigeria through (https://epingalert.org/), and brought to the Biotechnology Laboratory at the University of Food Technologies, Plovdiv, Bulgaria. In the laboratory, the seeds were peeled, and the peels were collected and ground with an electric coffee grinder. The ground peels were vacuum sealed and stored in a desiccator until further use [89].

4.2. Methodology

4.2.1 Preparation of Natural Deep Eutectic Solvents (NADESs)

Natural deep eutectic solvents (NADESs) were formulated by mixing hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) components in specified molar ratios. These mixtures were then sonicated using an ultrasonic bath at 35 kHz and 240 W (USTS 5.7-150 Siel, Gabrovo, Bulgaria) to achieve homogeneity. For detailed preparation methods, refer to our preceding publication [89].

4.2.2 Ultrasound-assisted extraction (UAE) experimental design

In this study, a rotatable central composite response surface design (RCCRSD) with four factors at five levels was used**.** The independent variables chosen are temperature (UT), time (ET), volume (SV), and concentration (SC), while the dependent variables are total phenolic content (TPC), total flavonoid content (TFC), cupric ion reducing antioxidant capacity (CUPRAC), ferric reducing antioxidant power (FRAP) and 2,2-Diphenyl-2-picrylhydrazyl (DPPH) inhibition. A total of ninety experimental runs with forty-eight cube points, eighteen center points (twelve on cube, and six on axial), and twenty-four axial points were performed based on rotatable central composite design of thirty base experiments replicated three times. The RCCRSD was chosen because orthogonality was absent among the models from our prior investigations, leading us to select the RCCRSD for its efficacy in optimization experiments under such conditions, as described by Montgomery [86].

Ground African nutmeg (Monodora myristica) peels (1.0 g) were weighed into synthesis bottles, and an appropriate volume of solvent (NADES) at the right concentration added, and irradiated at the designed temperature and time using an ultrasonic bath operated at a frequency of 35 kHz with a maximum input power of 240 W (USTS 5.7-150 Siel, Gabrovo, Bulgaria) with automatic temperature control. At the end of each extraction, the bottles were removed from the bath, the contents transferred to centrifuge tubes and centrifuged at 4500 rpm for 10 minutes (centrifuge MPW-260R Labtech, Germany). After 10 minutes of centrifugation, the supernatants were filtered through solvent bottles using Whatman No. 1 filter paper. The bottles were sealed and kept in a refrigerator at -4°C until further analysis.

4.2.3 Antioxidant Power

4.2.3.1 Total Phenol Content (TPC)

The TPC assay was performed spectrophotometrically modifying the method of Ianni et al., [107]. The Folin–Ciocalteu's (F-C) reagent method determined the TPC by measuring the absorbance at 765 nm. In a reaction tube, 0.1 mL of extract samples were mixed with 0.5 ml of F-C reagent. After approximately 1 min, 0.4 mL of 7.5% Na2CO3 was added, the mixture was incubated at 50°C for 5 min, and the absorbance was measured in a microplate reader (SPECTROstar Nano Microplate Reader, BMG LABTECH, Ortenberg, Germany) against the appropriate blank sample. In this study, the TPC was evaluated using a gallic acid standard curve and reported in mgGAE/g dry weight of sample using **Equation 1**

TPC = ((Y-0.04)/0.0204*D*C)/B*1000) mgGAE/g, DW) …… (1)

Where:

Y = Area of standard curve measured at 765 nm

B = Weight of sample used in the extraction

C = Volume of solvent used in the extraction

D = Dilution factor (where applicable)

DW= Dry weight of sample

4.2.3.2 Total Flavonoid Content (TFC)

The TFC was evaluated according to the method described in Kivrak et al., with minor adjustments [108]. In Eppendorf tubes (2 mL), aliquots of 0.25 mL of the extracts were added to 0.025 mL of 10% Al-$(NO_2)^3$, 0.025 mL of 1 M CH_3COOK, and 0.95 mL of ethanol. The mixture was incubated at room temperature (RT) for 40 min, after which the absorbance was read at 415 nm in a microplate reader (SPECTROstar Nano Microplate Reader, BMG LABTECH, Ortenberg, Germany) against the designated blank control. The TFC calibration curve was determined by relying on the quercetin equivalence (QE) and was used as a standard to calibrate and standardize the spectrophotometric measurements. **Equation 2** was used to determine and express the TFC in microgram equivalent of quercetin per gram of dry sample (µgQE/g, DW).

TFC = (((Y-0.0075)/0.0128) *D*C)/B) µgQE/g…… (2)

Where:

Y = Area of the standard curve measured at 415 nm

B = Weight of sample used in the extraction

C = Volume of solvent used in the extraction

D = Dilution factor (where applicable)

4.2.4 Antiradical scavenging activities

2.2.4.1 Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was carried out according to the procedure of Benzie and Strain, as slightly modified in subsequent studies [109 110]. The FRAP reagent was prepared fresh each day and warmed up to 37°C before use. The FRAP reagent consists (1) 300 mM acetate buffer (pH 3.6), (2) TPTZ (10 mM in 40 mM HCl), and (3) ($FeCl_3.6H_2O$, 20 mM). Solutions (1), (2), and (3) were mixed at a ratio of 10:1:1 to form the FRAP reagent. In a 15 mL tube, 2.85 mL of freshly prepared and warmed FRAP reagent was blended with 0.15 mL of sample and incubated at 37°C for 4 min, after which the absorbance was measured at 593 nm in a microplate reader (SPECTROstar Nano Microplate Reader, BMG LABTECH, Ortenberg, Germany) against the appropriate blank. The result is expressed as µMTE/g dry sample derived from **Equation 3**.

FRAP = (((Y+0.0017)/0.0013) *D*C)/B) µMTE/g, DW…… (37)

Where:

Y = Area of standard curve measurement at 593 nm

B = Weight of sample used in the extraction

C = Volume of solvent used in the extraction

D = Dilution factor where applicable

2.2.4.2 Cupric Ion Reducing Antioxidant Capacity (CUPRAC) Assay

The CUPRAC assay was performed according to the procedure of Apak et al., as described in Akyuz et al. [111 112] with slight modifications. Briefly, 0.25 mL of 1.0×10^{-2} M copper (II) chloride solution, 7.5×10^{-3} M Neocuprine (Nc), 1 M CH_2COONH_4 buffer (pH 7.0) solution, and distilled water were mixed in a 2 mL Eppendorf tube, after which 0.025 mL of sample was added and mixed. The resultant mixtures were allowed to stand at RT for 30 min to incubate. At the end of the incubation period, the absorbance was measured at 450 nm against the blank using a microplate reader (SPECTROstar Nano Microplate Reader, BMG LABTECH, Ortenberg, Germany). Trolox was used to prepare a standard curve, and the total antioxidant capacities of the samples were calculated following **Equations 4** and are expressed as µMTE/g dry weight (DW).

CUPRAC = (((Y+0.0372)/0.4128) *C*D)/B) µMTE/g, DW…… (4)

Where:

Y = Area of the standard curve measured at 450 nm

B = Weight of sample used in the extraction

C = Volume of solvent used in the extraction

D = Dilution factor where applicable

2.2.4.3. 2,2-Diphenyl-2-picrylhydrazyl (DPPH) Assay

The antioxidant assay of the African nutmeg peels extracts was performed by using the radical 2,2-diphenyl-2-picrylhydrazyl (DPPH) based on the method of Brands-Williams et al. [113] with adjustments for specificity to our samples. To prepare the DPPH stock solution we dissolved 24 mg of DPPH in 100 mL of methanol. This solution was then diluted to achieve the absorbance of 1± 0.02 using the spectrophotometer (SPECTROstar Nano Microplate Reader, BMG LABTECH, Ortenberg, Germany). For the assay, we combined forty microliters of extract with 3 mL of the prepared DPPH working solution and kept in the dark for 30 min at room temperature. Subsequently, we measured the absorbance at 515 nm using a spectrophotometer (SPECTROstar Nano Microplate Reader, BMG LABTECH, Ortenberg, Germany), to assess the reduction in DPPH concentration, indicative of the antiradical activity utilizing the formula:

$$ARA\ (\%) = \left(\frac{AC - AS}{AC}\right) X100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where:

ARA (%) = Antiradical activity

AC = absorbance of control

AS = absorbance of sample

2.4. Experimental Design and Statistical Analysis

To investigate the effect of independent variables related to temperature (UT), time (ET), volume (SV), and concentration (SC) on total phenolic content, total flavonoid content, and antiradical scavenging activity by CUPRAC, FRAP, and DPPH, a rotatable central composite response surface methodology design with five levels in and four factors was used. The coded and uncoded levels of independent variables that were used in the design are presented in S**T 1**. Actual values of independent variables were calculated by following **Equation 6**. Central points used for temperature (°C), time (min), volume (ml), and concentration (%) were 50, 15, 15, and 70, respectively. The step change for temperature and concentration was 10, while that for time and volume was 5, respectively. The full design matrix is shown in **ST 1**.

$$X = \frac{X0 - XC}{\Delta X} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots. (6)$$

Where:

X = coded values of independent variables

X0 = actual values of independent variables

ΔX = change in step, and

XC = the values at central points.

The analysis of variance technique was used to calculate the RCCRSD statistical parameters. For building the model, calculating the predicted values and optimal desirability for each of the solvents, plotting three-dimensional surface graphs to show the effects of independent variables on the response factors, calculating regression equations for the best-fitted model having non-significant lack of fit with effect to pure error, the response optimizer, and every other data analysis, Minitab statistical software 21.4.0 (Minitab LLC, Penn, USA) was used. The Fisher least significant difference (LSD) test value (F-value), coefficient of determination R^2, and lack of fit were used to determine the quality and adequacy of the model at ($p > 0.05$).

5.0 Conclusion

In this study, we optimized the extraction of bioactive compounds from African nutmeg (*M. myristica)* peels using NADESs (as green, novel, and potential solvents) coupled with ultrasonication. We had in our previous investigation [89] determined that all our synthesized solvents were very effective at extracting TPC and TFC from African nutmeg peels. In the present study, we optimized the independent variables with the aim to obtain maximum TPC and TFC extracts, as well as maximum antiradical scavenging activity. Optimal extraction parameters, yielding the highest measures of TPC were determined to be temperature (UT), time (ET), volume (SV), and concentration (SC), specified as follows: 45ºc, 5 min, 25 ml, 90%, and similarly optimal conditions were identified for other parameters. These conditions resulted in maximal desirability indices and activity. Among the NADESs, CaFr11 emerged as superior demonstrating the highest efficiency in extracting TPC, TFC extracts, along with CUPRAC scavenging capacity at the optimized conditions. However, CaFr11 (26.15±0.11 μMTE/g), and (73.55±0.24%) follows CaSu11 (26.17±0.17 μMTE/g), and CaXy12 (97.42±0.40%) in FRAP and DPPH potential respectively. The antiradical scavenging activity (using the CUPRAC, FRAP, and DPPH methods) of the extracts obtained by UAE-NADESs was similar to results obtained by other researchers from plant materials using NADES coupled to UAE [114 48 115 116]. The bioactive compounds extracted from *M. myristica* by UAE-NADESs exhibited volume and concentration dependency. This result is consistent with our expectation considering that higher volumes provide wider area for cavitation activity of ultrasonic waves which

leads to biomolecule release from sample matrix. Shorter time and medium temperature with a high solvent volume is also ideal for selective extraction of the polyphenols. However, a high temperature may be necessary for viscous solvents to lower the viscosity of the solvent in order to increase the surface area available for interaction.

The comparative analysis under varying conditions revealed a significant correlation with industrial scalability and environmental impact. Specifically, the application of NADESs, characterized by their biodegradability and low toxicity, underscores a pivotal advancement towards sustainable and eco-friendly extraction technologies. This highlights NADES practical applicability in enhancing the yield and quality of bioactive extractions, thereby promoting a greener approach in the phytochemical industry.

Declarations

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Conflict of interest

All the authors declare no conflicts of interest.

Data availability

The datasets generated and analyzed in the current study are available from the corresponding author at reasonable request.

Author Contributions:

U.J.O- Conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, and writing original draft.

M.M – Supervision, writing review and editing.

D.M- Supervision, resources, writing review and editing.

A.C- Supervision, editing, Funding acquisition

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Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures

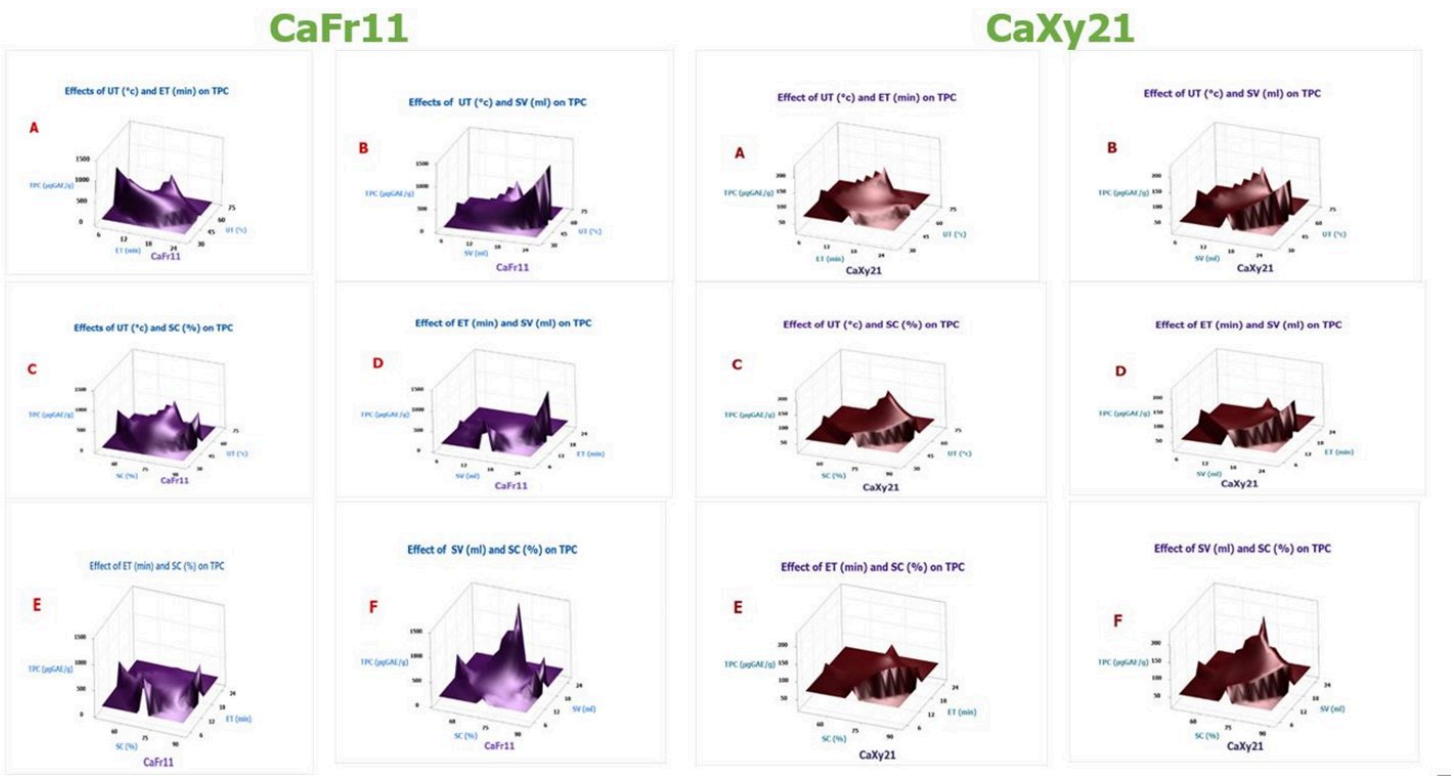

Figure 1

Effects of Factors on Total Phenolic Content Extraction with CaFr11 and CaXy21

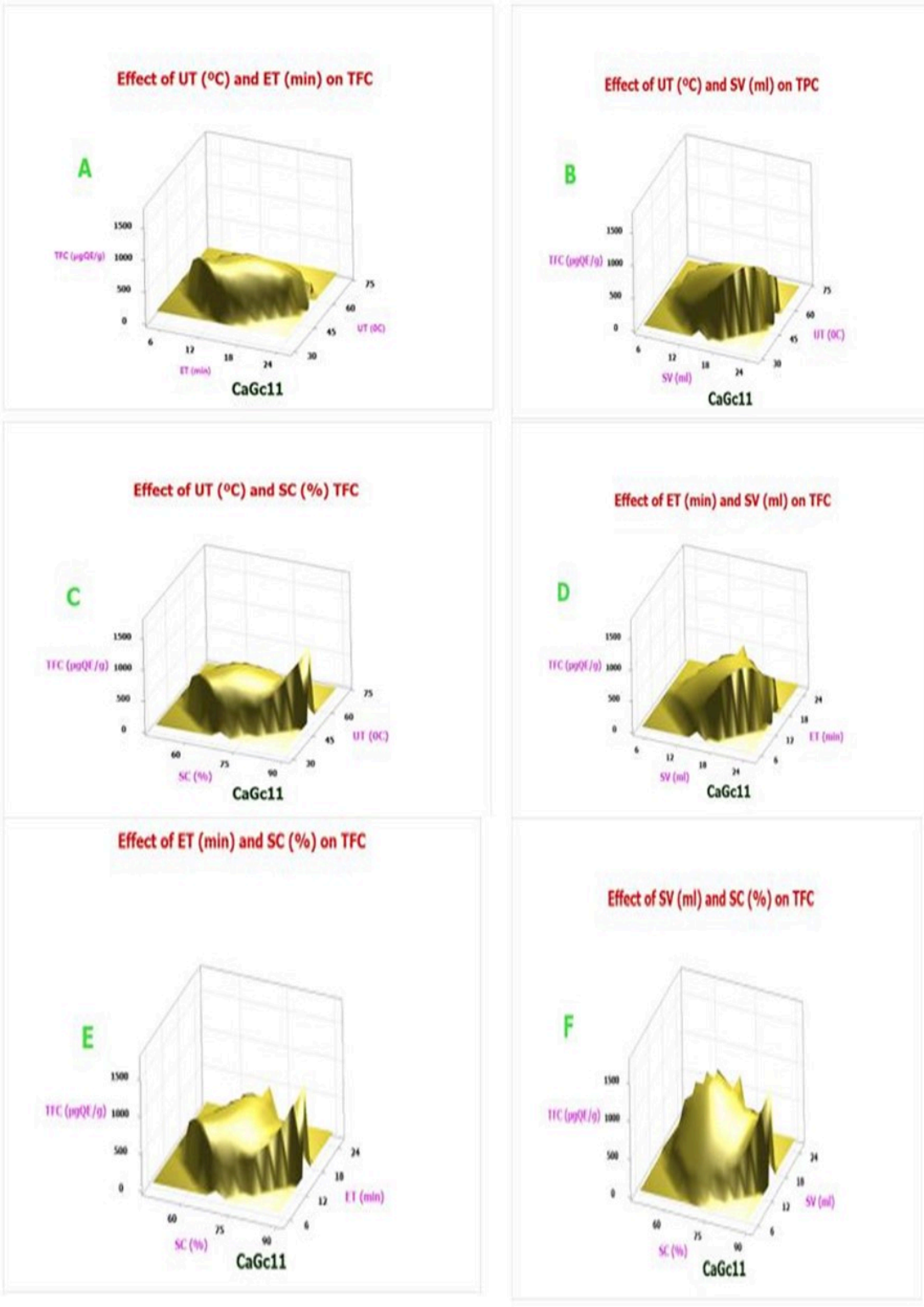

Figure 2

Effects of factors on total flavonoids contents (TFC) using CaGc11

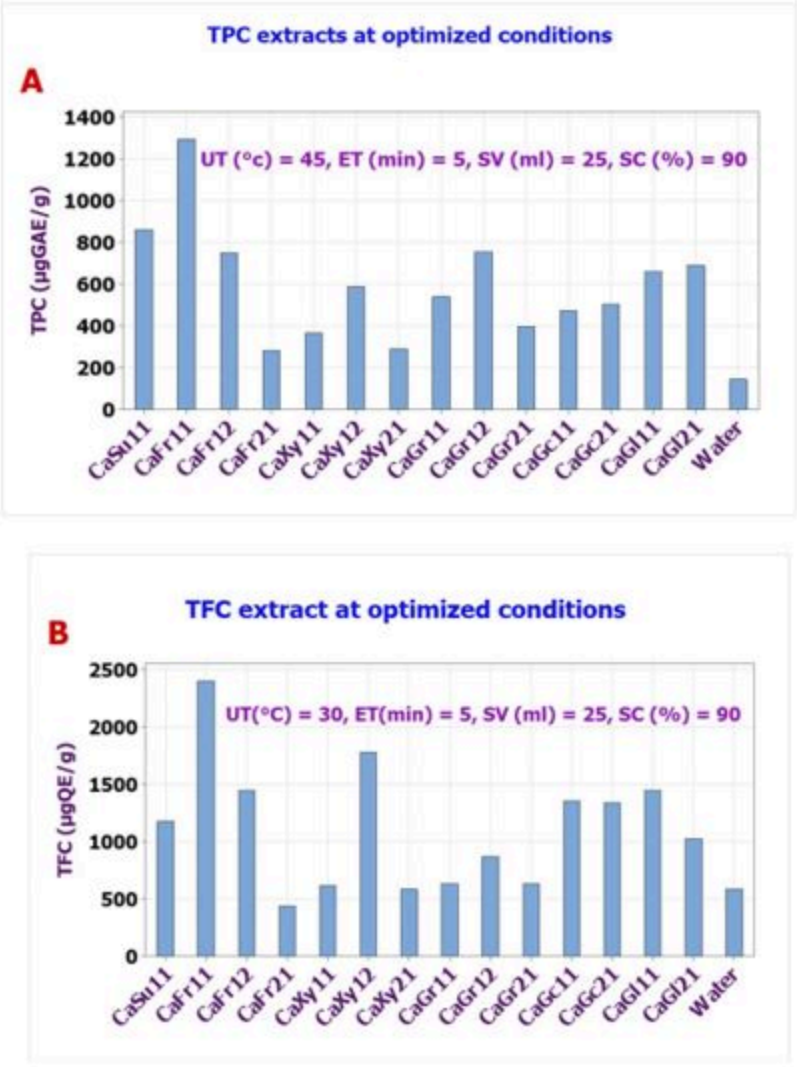

Figure 3

Comparison of antioxidants (A) TPC, and (B) TFC extracted from African nutmeg peels using NADESs and Water at optimized response conditions

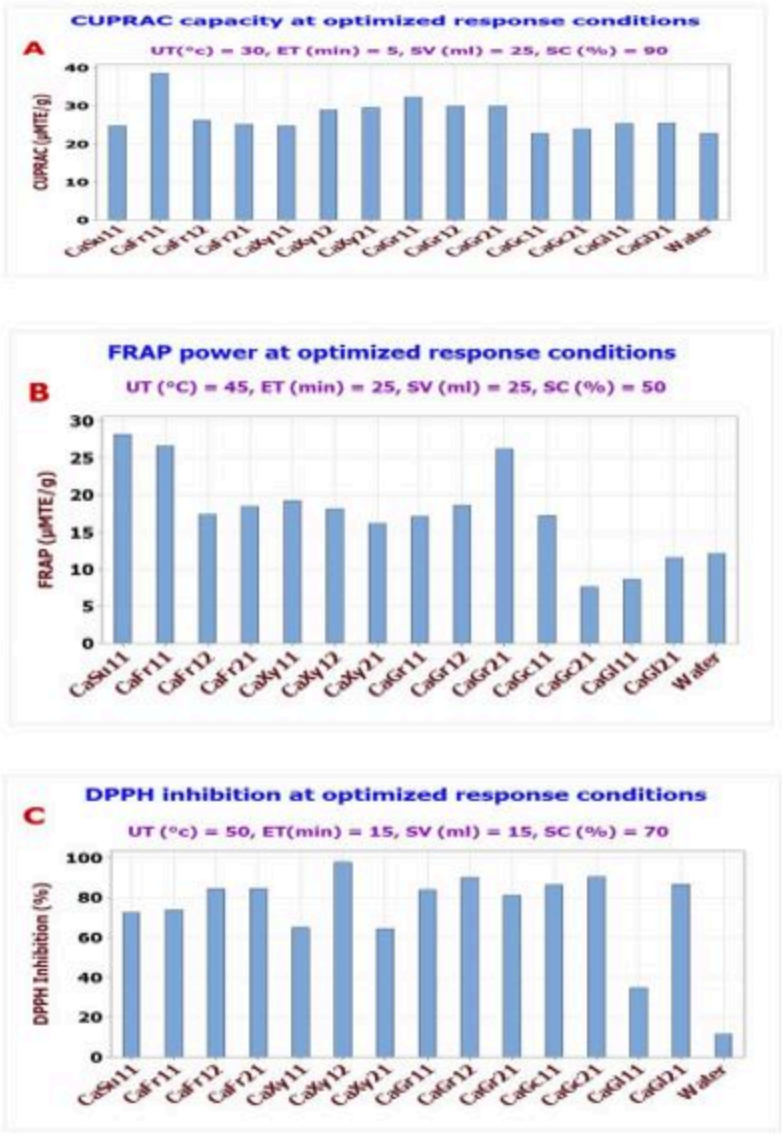

Figure 4

Comparison of antiradical scavenging activity (A) CUPRAC, (B) FRAP, and (C) DPPH of extracts from African nutmeg peels using NADESs and Water at optimized response conditions

Supplementary Files

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