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Application of deep eutectic solvents in the extraction of polyphenolic antioxidants from New Zealand Manuka leaves (*Leptospermum Scoparium*): optimization and antioxidant activity

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Abstract

Manuka tree (*Leptospermum Scoparium*) is the most widespread native shrub in New Zealand and has an economic impact as a source of honey and essential oils. In the present study, eight types of DESs based on choline chloride (ChCl) and tetrabutylammonium chloride (TBAC) as hydrogen bond acceptors (HBAs) in combination with different polyalcohols and carboxylic acids as hydrogen bond donors (HBDs) were used to assess the extraction of polyphenolic compounds in terms of total phenolic content (TPC) from dried Manuka leaves. ChCl: ethylene glycol DES showed the highest efficiency in the extraction of the polyphenolic compounds (56.87 mg GAE/g DW) followed by ChCl: lactic acid DES (52.51 mg GAE/g DW) and ChCl: 1, 3-propanediol DES (50.67 mg GAE/g DW). Furthermore, the mentioned DESs revealed a better extraction efficiency for polyphenolic compounds than ethanol as a benchmark solvent. Among the successful DESs, ChCl: lactic acid DES was preferred and studied further for optimization using response surface methodology. The highest TPC (59.82 mg GAE/g DW) was extracted at optimum conditions (extraction temperature 50.00 ℃, biomass % 5.07 and extraction time 1.07 h). Besides, the extracted polyphenolic compounds showed good stability in ChCl: lactic acid DES over eight days of storage. Moreover, after four extraction cycles using the DES, 154.65 mg GAE/g DW of TPC advises a possible scaling up of the potential DES. The antioxidant activity of extracts was evaluated using DPPH and FRAP assay. ChCl: ethylene glycol DES extract showed better free radical scavenging activity (12.78 µg TE/ml) while ChCl: lactic acid DES extracts gave higher ferric reducing power (199.78 µg TE/ml) at the same extract concentration with both being higher than the extract obtained by ethanol.

Keywords: Deep eutectic solvent; extraction; total phenolic content; Manuka leaves; antioxidant activity.

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1. Introduction

Manuka tree (*Leptospermum Scoparium*) is the most widespread native shrub in New Zealand and has an economic impact as a source of honey and essential oils. The Maori used the leaves, bark, seeds, and flowers as traditional medicine and for smoking fish [1]. Brooker recorded some traditional uses of the Manuka tree [2]. In a comparison study of the phenolic compounds of New Zealand Manuka honey and from around the world, researchers proved that Manuka honey has the highest amount of phenolic bioactive compounds, and they believe that the antimicrobial activity of Manuka honey showed a strong association with the level of phenolic compounds [3]. Furthermore, the high level of bioactive compounds in Manuka leaves is responsible for the powerful antibacterial and antifungal activities of Manuka essential oil [4]. This can be proven by the healing properties of Manuka essential oil-containing products to treat various skin disorders such as ringworm, acne, eczema, athlete's foot, nail fungus, seborrheic dermatitis, and *Candida Albicans* [4-8]. Given all this, it might be of great interest to investigate the extraction of total phenolic compounds from Manuka leaves.

Phenolic compounds are secondary metabolites in the plant kingdom and play a critical role in protecting the plant from pathogens. The chemical structure of phenolic compounds consists of aromatic rings with one or more hydroxyl groups [9]. They possess multifaceted biological activities such as antimicrobial, antioxidant, anti-inflammatory and anticarcinogenic [10, 11]. Besides, phenolic compounds from plants are considered the primary natural source of antioxidants in food, beverages, pharmaceuticals and cosmetics [12]. Previous studies revealed the localization of polyphenols in the cell wall, vacuoles in leaves of a monocotyledonous and a dicotyledonous plant associated with other macromolecules such as mono and polysaccharides and proteins [13, 14]. Polyphenols comprise a vast family of around 8000 compounds [10, 15]. The first critical step in obtaining these phytochemicals is the extraction [16]. Primarily step in the extraction of polyphenols includes solid-liquid extraction. Solid-liquid extraction such as Soxhlet, boiling, reflux has been employed in numerous industries to recover valuable compounds from plant matrices. Large quantities of toxic solvents used and long processing time are the main drawbacks of these methods. Solvents with a high ability to create hydrogen bonding with phenolic compounds are usually used as extractants such as methanol, acetone, ethanol, propanol, ethyl acetate and their binary mixtures [15, 17-19]. These solvents can not fulfil the trend of both environmental and economic points of view. For this reason, new kinds of solvents are investigated to recover phenolic compounds from various plant matter.

Towards green consumerism, deep eutectic solvents have been replacing the use of toxic solvents. In the past few years, different types of deep eutectic solvents showed tangible proof that these non-toxic, biodegradable solvent could be an acceptable, environmentally friendly media for the recovery of bioactive compounds with many advantages such as easy to prepare and use, cheaper starting material, low vapor pressure, and strong dissolution properties [20-23]. A DES consists of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD). The DESs differ from ionic liquids in two aspects, I) the formation materials used and II) the synthesis [24]. The most popular hydrogen bond acceptor use in the literature is choline chloride which has renowned properties such as non-toxic, inexpensive, biodegradable. On the other hand, the complexing agents are also known to have green usage. Compounds from natural sources such as surges, carboxylic acids, alcohols, amino acids have also been widely used [25]. Over the last few years, DESs have been mostly employed for synthesis reactions [26, 27], metal extraction [28], purification of fuels [29, 30], removal of pollutants [31-33], isolation of target compounds [34-36]. Due to their unique solvation properties [37], DESs became the main interest in the field of pharmaceuticals to recover bioactive compounds from plant material. DESs have shown to enhance the extraction of phenolic compounds in R*uta graveolens* L. [38], coffee beans (*Coffea arabica* L.) [39], olive leaf [40], Greek medicinal plants [41], *Rosmarinus officinalis* L.[42], walnut leaves (*Juglans regia* L.) [43], orange peel waste [44], grape skin [24], onion peel (*Allium cepa* L.) [45], and tea [46].

No previous studies based on DESs have been done for polyphenols extraction from the Manuka tree to the best of our knowledge. Therefore, in this study, for the first time, the extraction of polyphenolic compounds from New Zealand Manuka leaves (*Leptospermum scoparium*) was conducted using different types of deep eutectic solvents DESs as well as determine the free radical scavenging activity (DPPH) and ferric reducing antioxidant activity (FRAP) for the obtained extracts. Moreover, the extraction parameters were optimized based on response surface methodology combined with BBD (Box-Behnken design).

2. Research methodology

2.1. Materials

Samples of fresh Manuka tree grown in the East Cape were kindly provided by Manuka Bioactives Ltd., East Cape, New Zealand. Upon receipt, leaves and branchlets were dried indoor at room temperature for a few weeks and then dried using a convection oven at 35 ℃

for three days. Dried leaves with the moisture content of 5 % were only used for the extraction of total phenolic content. Dried samples were stored at 4 ℃ until it was used. Dried Manuka leaves were milled freshly before each experiment in a high-speed food-grade miller at 28,000 rpm to get a fine powder. The pulverized samples were subjected to sieving using Vibratory Sieve Shaker, AS 200 Germany, to get powder with a particle size of ≤200 µm. The reason for selecting powder with a particle size of 200 µm was to provide a larger surface area and facilitate bioactive diffusion into the DES, which is more viscous than organic solvents. All chemicals used in this study are shown in Table S1.

2.2. Preparation of deep eutectic solvents

Eight DESs were prepared by mixing choline chloride (ChCl) and tetrabuthylammonium chloride (TBAC) as HBAs with different HBDs at different molar ratios shown in Table 1. The eutectic mixtures were heated to 80 °C using heating magnetic with constant stirring until a homogeneous liquid was obtained. The parafilm was used to seal the prepared DESs vails to protect against moisture.

Table 1
Composition of the studied DESs and their abbreviations.

HBA	HBD	Molar ratio	Abbreviation
Choline chloride	Ethylene glycol	1:2	DES1
Choline chloride	1,3-propanediol	1:3	DES2
Choline chloride	Acetic acid	1:2	DES3
Choline chloride	Lactic acid	1:2	DES4
Tetrabuhylammonium chloride	Ethylene glycol	1:2	DES5
Tetrabuhylammonium chloride	1,3-propanediol	1:2	DES6
Tetrabuhylammonium chloride	Acetic acid	1:2	DES7
Tetrabuhylammonium chloride	Lactic acid	1:2	DES8

2.3. Viscosity

The viscosity measurement of the ChCl-based DESs was conducted using AR-G2 magnetic bearing rheometer (TA Instruments Ltd, West Sussex, NB) attached to an aluminium cone geometry. The viscosity measurements were carried out at a temperature of 25 ℃ with shear rates from 1 to 100 s^{-1}.

2.4. Extraction of polyphenolic compounds using DESs and ethanol

Each prepared DES was mixed with grounded Manuka leaves at a solid to liquid ratio of 1:20 (w/v). The extraction was performed at room temperature in a closed vessel for one hour. The stirring speed was set to obtain perfect mixing for all samples. Following extraction, the mixture was centrifuged at 10000 rpm for 10 minutes, and then the resulting extract was filtered by PTFE microfilter (0.45 μm). The filtrate was diluted with ethanol before polyphenolic compounds quantification. Each sample was performed in duplicate. In parallel, ethanol as a benchmark solvent was also examined to extract polyphenolic compounds to compare the extraction efficiency with those obtained by the DESs.

2.5. Optimization and extraction conditions

Design-Expert 7.1 software (Stat-Ease Inc., U.S.A) was used to create different extraction conditions to enhance the recovery of phenolic compounds from Manuka leaves. RSM (response surface methodology) combined with BBD (Box-Behnken design) was chosen to investigate the impact of three influence factors at three levels on the yield of TPC, including extraction time (A), temperature (B), and Biomass percentage (C). Three Manuka biomass percentages (5, 10 and 15%) were used at the temperature within the range of 25-50 ℃ for 1-3 hours. The experimental design resulted in 15 runs with two replicates of the centre points.

Since no previous studies have been conducted on the extraction of phenolic compounds from New Zealand Manuka leaves, a moderate level of variables was used for the optimization. The main goal was to explore the effect of time, temperature and biomass % on the extraction of TPC. Moreover, the extraction of bioactive terpenoids from Manuka leave was successfully conducted using DESs [47]. The extraction parameters were also optimized, including extraction time (1-3 h), biomass% (5-15%) and temperature (25-55 ℃). Therefore, extraction variables were studied at the same level.

The efficiency of the design is decreased with a higher number of variables are involved. However, the Box–Behnken design helps to select a central point from a three-level arrangement and an adequate estimation of the first- and second-order coefficients. In this way, BBD can be considered more efficient and economical than other designs used to optimize a

process [48]. The optimization design using BBD became very popular due to its efficiency and easier to interpret the data than other RSMs in this field [49].

2.6. Determination of total phenolic content (TPC) in the extracts

Phenolic compounds were evaluated in terms of total phenolic content (TPC) by the Folin–Ciocalteu method using a microplate 96-well reader as previously reported by [50, 51]. In a 2 ml Eppendorf centrifuge tube, 20 µL of the sample at appropriate dilution was added, followed by 780 µl of distilled water and 50 µL Folin−Ciocalteu reagent. The tube was mixed thoroughly and, after a few minutes, 150 µL of 20% filtered sodium bicarbonate solution was added. Then samples were filtered using a 0.22 µm PTFE microfilter. Samples were transferred to a 96-well plate and kept in the dark for 60 min at room temperature. The absorbance of the mixture was determined at 750 nm using SpectraMax iD3 Multi-mode Microplate Reader. Gallic acid standard dilutions were prepared to create a calibration curve at concentrations of 10-700 ppm Fig. S1. The results were expressed as µg Gallic acid equivalent (GAE)/g DW [52].

2.7. Determination of antioxidant capacity of the extracts

2.7.1. DPPH radical scavenging assay

DPPH assay estimates the capacity of the antioxidant agent to scavenge free radical DPPH$^{\bullet}$ (2, 2-diphenyl-1-picrylhydrazyl). The antioxidant substances in the extract can donate a hydrogen atom, reducing the free radical to diphenylpicrylhydrazine. This can be monitored by the change in solution colour from violet to yellow as well as spectrophotometrically at 517 nm. The DPPH solution was prepared by dissolving 4 mg of DPPH in 100 ml methanol. Twenty microliters of the sample were added to 200 µl of methanolic DPPH. The plate was kept in the dark at room temperature for 30 min. The absorbance of the mixture was determined at 517 nm using SpectraMax iD3 Multi-mode Microplate Reader. The absorbance of the free radical without sample or standard, i.e., blank, was also measured. All samples were performed in triplicate. Trolox standard solutions were prepared at concentrations of 10-150 ppm Fig. S2. The antioxidant capability of the sample was expressed as µg Trolox equivalent (TE)/ml.

2.7.2. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) is another assay used to confirm the antioxidant capacity of Manuka extracts. This method determines the ability of antioxidant substances to reduce Fe+3-TPTZ (yellow) to Fe+2-TPTZ (navy blue) in FRAP reagent which can be read at 593 nm. TPTZ (100 mM or 0.01M) was prepared by dissolving 78.08 mg in 25 ml of 40 mM HCl. Ferric (III) chloride hexahydrate (20 mM) was prepared by dissolving 135.15 mg in 25 ml of distilled water. The freshly prepared FRAP reagent was prepared by mixing sodium acetate buffer (300 mM), TPTZ and $FeCl_3.6H_2O$ in the proportion of 10:1:1. Ten microliters of the sample were added to 190 µl of FRAP working solution and mixed thoroughly. The 96-well plate was kept in the dark for 60 min. All samples and standards were performed in triplicate. Trolox standard solutions were prepared at concentrations of 10-150 ppm Fig. S3. The antioxidant capability of the sample was expressed as µg Trolox equivalent (TE)/ml [53].

2.8. Stability and reusability study

The stability study of polyphenolic compounds in the DES was carried out for the sample at the optimum extraction condition. The sample was kept at ambient temperature in a dark and dry place in a closed vessel. The TPC quantification was performed on days 1, 2, 3, 4, 5, 6, 7 and 8 by diluting the known amount of sample with ethanol.

A solvent's reusability represents the number of cycles for DESs regeneration to recover more of the interesting compound. Reusability is very desirable from both environmental and economic standpoint to achieve a sustainable extraction solvent, as DESs could be reused up with a limited reduction in its efficiency. The first extraction cycle was carried out for the sample at the optimum extraction condition. After the extraction, the slurry was centrifuged at 10000 rpm for 10 min and filtered through a 0.45-µm PTFE microfilter. Manuka extract was quantified for TPC after diluting with ethanol. The remaining supernatant containing DES was measured and then re-employed for extraction by mixing it with the same biomass %. In this study, the potential DES was reused for four cycles to determine the TPC at the same extraction condition.

3. Results and discussion

3.1. Screening of DESs performance in TPC extraction

With the aim to find the most potent deep eutectic solvent in the extraction of polyphenolic compounds from Manuka leaves, eight types of DESs were synthesized and evaluated. Furthermore, DESs were based on ChCl and TBAC as HBAs combined with two polyalcohols, namely: ethylene glycol and 1, 3-propanediol, and two organic acids, namely: lactic acid and acetic acid.

Since no previous studies on the extraction of TPC from Manuka leaves have been conducted, the selection of the DESs in the extraction of bioactive compounds depends mainly on the properties of the proposed DESs as well as the nature of the constituents. Due to the hydrophilic nature of the phenolic compounds, the quaternary ammonium salts along with the polyalcohols and organic acids increase the polarity of the resulting DESs. Moreover, choline chloride has been widely used in the DESs studies due to its properties such as non-toxic, inexpensive and, biodegradable. Besides, TBAC-based DESs were used to investigate the effect of HBAs on the recovery of TPC from Manuka samples.

In order to compare the DESs efficiency in the extraction of polyphenolic compounds, pure ethanol was used as a traditional solvent at the same extraction condition. A biomass percentage of 5%, at ambient temperature for one hour, was selected as a screening condition to evaluate the performance of DESs and the conventional solvent. Fig. 1 represents the DESs performance in TPC extraction.

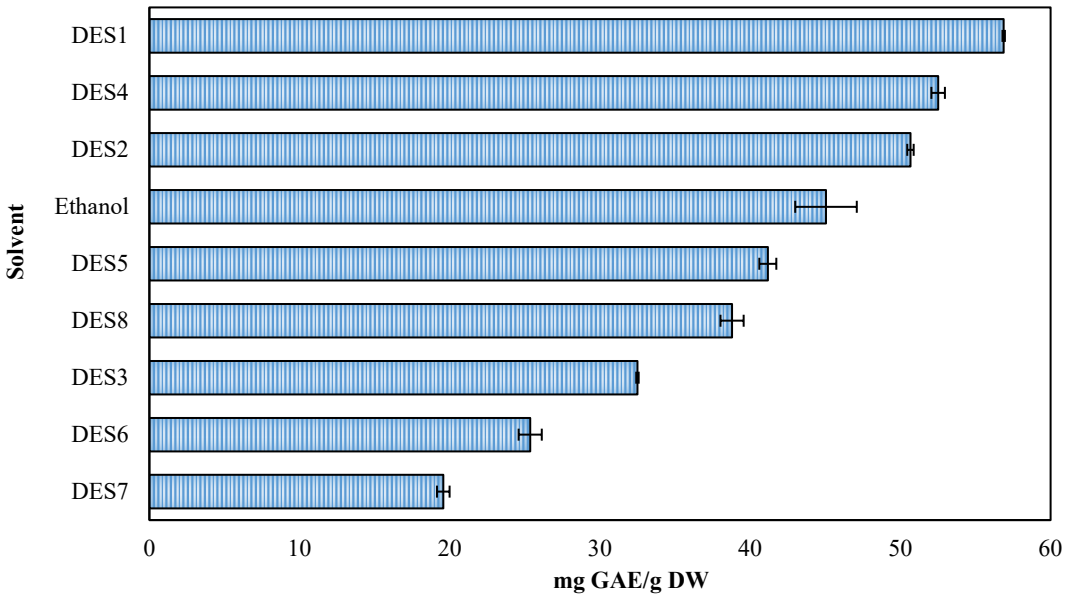


Fig. 1. Deep eutectic solvents performance in the extraction of TPC from Manuka leaves.

The chemical structure of phenolic compounds consists of aromatic rings with one or more hydroxyl groups [9]; thus, it might differ in its polarities. On the other hand, the DESs composition can play a vital role in the extraction efficiency due to the direct effect on its physicochemical properties such as viscosity, polarity and the solubilization of the phenolic compounds [54]. The interaction between the phenolic molecule and the DES is based on the hydrogen bonding availability of OH and/or Cl^- of DES (for the ChCl-based DESs) which facilitates the removal of the target compound. Therefore, DES with a higher ability to interact and to form hydrogen bonding can perform better than other types of DESs. Although DESs with low viscosity are desirable, DESs with higher viscosities can also show high extraction capacity due to the excessive amount of hydrogen bond within the DESs components [25].

In relation to the HBA type, ChCl-based DESs performed better than TBAC. The possible reason could be that ammonium salts like TBAC (with four carbon chain length) based deep eutectic solvents have more hydrophobic behavior, which could contribute to the lower yield of total phenolic content as hydrophilic molecules in nature.

Among the polyalcohols-based DESs, ChCl: ethylene glycol DES1 (56.87 mg GAE/g DW) achieved a higher yield, followed by ChCl: 1, 3-propanediol DES2 (50.67 mg GAE/g DW). This investigation is in close agreement with those results reported by others who found that polyalcohol-based DESs have an excellent ability to extract polyphenols from various plant materials [40, 44, 52, 55-58]. This could be explained by the fact that ethylene glycol and 1, 3-propanediol as HBDs based-DESs display lower viscosity Table 2 and good ability to form hydrogen bonding. Moreover, ethylene glycol ($C_2H_6O_2$) with the ability to form multiple hydrogen bonding with polar molecules such as phenolic compounds and two carbon chain length seems to help in the interaction with the target compounds much easier as compared to the 1,3-propanediol ($C_3H_8O_2$) [59].

Table 2
Viscosity of ChCl-based DESs at 25 ºC.

DES	molar ratio	η @ 25 ºC (mPa.s)
ChCl: ethylene glycol	1:2	32.045
ChCl: 1,3-propanediol	1:3	82.177
ChCl: lactic acid	1:2	301.902
ChCl: acetic acid	1:2	39.000

Carboxylic acids were also used as HBDs such as acetic acid (linear monocarboxylic acids) and lactic acid (hydroxyl monocarboxylic acids) with a molar ratio of 1:2 (ChCl: carboxylic

acid, 1:2) according to the eutectic mixture found by [60]. Among the carboxylic acid used as HBDs, lactic acid was observed to achieve a higher TPC (52.51 mg GAE/g DW) followed by acetic acid (32.49 mg GAE/g DW). Although, ChCl: lactic acid DES4 has a higher viscosity than ChCl: acetic acid DES3 as shown in Table 2, the higher number of –OH present in the lactic acid and the presence of (C=O) group in the acetic acid can explain the higher extraction yield of polyphenolic compounds with ChCl: lactic acid as compared to ChCl: acetic acid. Ethanol as the most commonly used organic solvent to recover phenolic compounds from different natural sources [61] was also employed to extract TPC and resulted in a yield of 45.04 mg GAE/g DW. These results can be supported by [44, 59, 62], who found that DESs enhance TPC extraction as compared to the organic solvents due to favourable hydrogen bonding with the interesting compounds.

Lactic acid is a natural carboxylic acid and found in dairy products and different types of vegetables [63]. The biocompatibility and its low cost as it can be produced quickly by fermenting a carbohydrate source can fulfil all the obligations for the production of natural deep eutectic solvents (NDESs) [64]. On the other hand, the results that have been investigated by [65] indicate that ethylene glycol as a hydrogen bond donor in combination with quaternary ammonium salts is not a safe solvent due to its health and safety issues in pharmaceutical, nutrition and cosmetic applications.

Therefore, ChCl: lactic acid DES4 has been chosen as a potential DES for further study on optimizing of TPC extraction parameters.

3.2. Antioxidant activity results

The antioxidant activity of the extracts obtained by DES1, DES2, DES4 and ethanol was evaluated using the free radical scavenging activity (DPPH) and ferric reducing antioxidant activity (FRAP). The screening extraction conditions (biomass percentage of 5%, ambient temperature and one-hour mixing) were used to obtain the extracts.

It is worth noting that there is no specific method providing universal answers regarding the antioxidant potential of a plant extract. Since there are different mechanisms involved in different antioxidant assays, it is advised to have at least two tests to evaluate the antioxidant capacity. DPPH and FRAP are the most common assays used in the literature. The first method is mainly to study the ability of the extract to scavenge free radicals in the solution, while FRAP is used to measure the ability of the extract to produce a reduction in ferric ions. Besides, there

are some factors that could affect the antioxidant evaluation i.e., pH (the pH of FRAP reagent =3.6). Thus, such an assay is necessitated.

Serial doubling dilution (50, 25, 12.5 mg/ml) was conducted to monitor the antioxidant capacity of the extracts. The antioxidant capacity of Manuka extracts with different solvents is presented in Fig. 2. All extracts showed sample dose-dependent antioxidant capacity, with ethanol being higher at the concentration of 12.5 mg/ml and DES1 at the concentration of 25 mg/ml. Furthermore, ethanolic extracts have quite good antioxidant activity and probably due to the synergistic effects of the extracted molecules. On the other hand, ferric reducing antioxidant power (FRAP) is another assay used to confirm the antioxidant capacity of Manuka extracts. This method determines the ability of antioxidant substances to reduce Fe+3-TPTZ (yellow) to Fe+2-TPTZ (navy blue) in FRAP reagent which can be read at 593 nm. DES4 showed superior antioxidant activity among all tested extracts using FRAP method Fig. 3. However, DES1 extract possesses a higher DPPH value and DES4 has the highest value of FRAP. The relation between the total phenolic compounds extracted and the antioxidant activity of the extracts is still not clear. The mechanisms of action implied in those two different assays could be a possible explanation for the antioxidant results. Nevertheless, different extraction ability of different solvents for various polyphenols mixtures also should be considered.

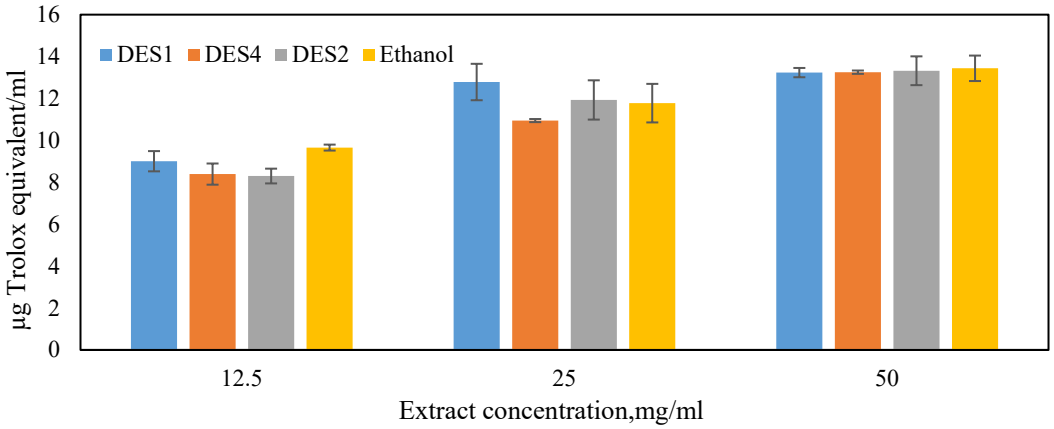


Fig. 2. DPPH values of Manuka leaves extracted by different solvents

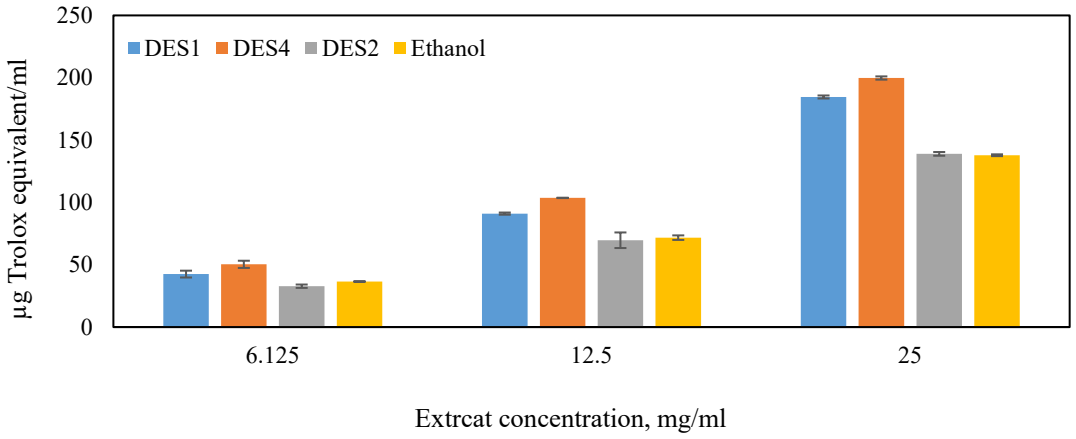


Fig. 3. FRAP values of Manuka leaves extracted by different solvents

3.3. Optimization of experimental variables for TPC extraction using BBD

The key parameters affecting the extraction of polyphenolic compounds from Manuka leaves using DES4 were optimized using Design-Expert software version 7.1 (Stat-Ease Inc., U.S.A.) including extraction time (1-3 hours), extraction temperature (25-50 ℃) and biomass percentage (5-15%). Fifteen runs of Box-Behnken design including three replicates at the center point was performed to optimize the extraction parameters, and the amount of TPC extracted was taken as the response. The independent factors at three levels are depicted in Table 3.

Table 3
Independent variables and levels used for BBD experiments.

Factor	Variable	Level		
		-1	0	1
A	Time (h)	1	2	3
B	Temperature (℃)	25	37.5	50
C	Biomass (%)	5	10	15

The experimental data from BBD were analyzed and the quadratic polynomial equation (Eq 1) was derived to describe the interactive effect between the response (TPC yield) and the extraction variables at different levels.

$$TPC = +50.01 + 1.17A + 3.02B - 4.61C - 1.85AB + 1.54AC - 0.91BC - 1.01A^2 + 0.41B^2 + 0.40C^2 \quad (1)$$

Where TPC presents total phenolic content (mg GAE/g DW), A is extraction time (hours), B is extraction temperature (°C), and C is % w/v biomass. Table 4 represents the experimental BBD matrix of three factors: extraction time, temperature, % biomass, and TPC extracted.

Table 4
Experimental BBD matrix and obtained response values for the extraction of total phenolic content from Manuka leaves.

run	A: Time(h)	B: Temperature (°C)	C: Biomass (%)	Response: mg GAE/g DW
1	1	37.5	5	54.52
2	2	25	5	51.42
3	3	37.5	5	53.92
4	3	37.5	15	47.37
5	2	50	15	48.40
6	1	25	10	43.31
7	2	37.5	10	50.01
8	2	37.5	10	50.11
9	2	25	15	44.44
10	3	25	10	49.21
11	1	50	10	53.31
12	2	50	5	59.02
13	3	50	10	51.81
14	1	37.5	15	41.80
15	2	37.5	10	49.91

The F and p-value were used to testify to the significance of the generated model. In our case, the high F-value (293.39) and the small p-value (less than 0.05) obtained mean the model is significant. Furthermore, the determined coefficient (R-squared) and adjusted (R-squared), as shown in Table 5, were used to validate the model. The correlation coefficient of the model was 0.9981, which confirms that the model can justify 99.81% of the variation. The R-squared (0.9707) predicted by the model agrees with the adjusted R2 (0.9947) and shows that the coefficient of the quadratic polynomial equation adequately expresses the TPC yield. Besides, the "Lack of Fit F-value" implies that the model could fit the response value with a good prediction.

Table 5
Analysis of the Variance for the Fitted Quadratic Polynomial Model of total phenolic content Extraction Using DES4.

Std.dev.	0.33	R^2	0.9981
Mean	49.91	Adjusted R^2	0.9947
C.V.%	0.66	Predicted R^2	0.9707
PRESS	8.38	Adequate precision	64.318

The p-values ($p < 0.05$) presented in Table 6 were used to evaluate the individual variables' significance and explained the interaction between them.

Table 6
ANOVA for Response Surface Quadratic Model of total phenolic content Extraction Using DES4.

Source	Sum of squares	df	Mean square	*F* value	*P*-value prob > *F*	Remarks
Model	285.61	9	31.73	293.39	< 0.0001	Significant
A-Time	10.97	1	10.97	101.39	0.0002	Significant
B-Temperature	73.00	1	73.00	674.92	< 0.0001	Significant
C-Biomass	169.77	1	169.77	1569.53	< 0.0001	Significant
AB	13.69	1	13.69	126.56	< 0.0001	Significant
AC	9.51	1	9.51	87.89	0.0002	Significant
BC	3.30	1	3.30	30.51	0.0027	Significant
A^2	3.75	1	3.75	34.71	0.0020	Significant
B^2	0.62	1	0.62	5.69	0.0627	
C^2	0.60	1	0.60	5.55	0.0650	
Residual	0.54	5	0.11			
Lack of Fit	0.52	3	0.17	17.36	0.0550	Not significant
Pure Error	0.020	2	0.010			
Cor Total	286.15	14				

According to the ANOVA results Table 6, the p-values indicates that (A, B, C), the interaction between time and temperature (AB), time and biomass% (AC) as well as the interaction between temperature and biomass% (BC) were significant. Moreover, the quadratic term coefficients (A^2) seemed to be significant, whereas in the case of (B^2 and C^2) were found to be not significant. To look into the interactions between studied variables on the extraction yield of TPC, three-dimensional plots were depicted, as can be seen in Fig. 4. This figure represents the output of the effect of two independent factors, while the third factor is fixed at the centre point. Fig. 4a demonstrates the interaction between the extraction temperature and the biomass percentage on the yield of TPC. It can be observed that by decreasing the amount of Manuka powder added and increasing the temperature from 25 ℃ to 50 ℃ the TPC was enhanced. The research works reported by other authors showed that increasing the temperature positively affects the extraction efficiency due to the significant impact on the DESs properties such as viscosity, reducing the surface tension, and enhancing the target's solubility compounds [66-69]. It has also been reported that increasing the temperature gradually to up to 120 ℃ can improve the amount of TPC extracted [70]. Fig. 4b represents the effect of both extraction time and temperature on the TPC. The increasing stirring time from 1 to 3 h was less noticeable as compared to the temperature effect. The possible explanation would be that the advantage of

the low viscosity of the DES and the strong ability to create a hydrogen bonding seem to facilitate the extraction within a shorter time. Fig. 4c again confirms that the highest amount of TPC was observed by decreasing the stirring time and the biomass percentage used in the extraction experiments. Consequently, the extraction of TPC from Manuka leaves powder was repeated in triplicate at the optimal conditions to verify the experimental value with the predicted one. A value of (59.82 mg GAE/g DW) was obtained when the extraction temperature, time and biomass percentage were 50.00 ℃, 1.07 h and 5.07% respectively, which is in close agreement with the predicted by the model (60.41 mg GAE/g DW) and fitted within the PIs (prediction intervals) of TPC (59.15-61.68 mg GAE/g DW) with a 0.9862% error.

In conclusion, ChCl: lactic acid DES successfully improved the extraction of phenolic compounds from the leaves of Manuka. However, further investigation will be needed to reduce the extraction time and enhance the yield by employing one of the promising tools such as ultrasound and microwave-assisted extraction.

DESs recyclability remains a crucial step in the extraction of bioactive compounds from various biomaterials. However, there are several approaches have been demonstrated to date to recover the target compound, i.e., such as solid-phase extraction (SPE) [71, 72], macroporous resin adsorption [73-78], anti-solvent precipitation [72, 79, 80], back-extraction [81], and adsorption chromatography [82]. Other strategies that might be used to weaken the intermolecular forces of DESs and, consequently, help in recovering the target compounds are adjusting the temperature and pH. The separation and purification of rutin, quercetin, kaempferol, and daidzein extracted by the NaDESs made with ChCl: glucose: water, 5:2:5 was successfully conducted by Liu et al. [83]. Those molecules were extracted with different organic solvent mixtures, namely, ethyl acetate: butanediol: H2O, 6:4:10, v/v for rutin; hexane: ethyl acetate: MeOH/H2O, 3:7:5:5, v/v for quercetin and daidzein, and hexane/ethyl acetate/MeOH/H2O (4:6:5:5, v/v) for kaempferol. The percentage recovery rutin, quercetin, kaempferol, and daidzein were 95.7, 94.6, 97.0, and 96.7%, respectively.

The recovery of phenolic compounds extracted from virgin olive oil by different types of DESs made up from ChCl associated with sugars, alcohols, organic acids, and urea was successfully applied by Garcıa´ and his co-worker [84]. The phenolic compounds recovery from DES was accomplished by nonpolar resin XAD-16 (styrene-divinylbenzene). First, DES was eluted with water, and then the phenolic compounds was flushed with 100 mL of methanol. Further studies on the recovery of polyphenolic compounds from DESs along with the recovery yield, solvent type and recovery techniques are reported in [85].

Based on rough estimates of ChCl (0.6 USD/Kg) and lactic acid 80% (0.45 USD/Kg), the cost of ChCl: lactic acid DES at 1:2 molar ratio is about 0.9 USD/Kg which is cheaper than ethanol 1.2 USD/Kg. According to the obtained results, ChCl: lactic acid DES is an efficient extraction solvent for the recovery of TPC from Manuka leaves at room temperature (52.51 mg GAE/g DW) as compared to ethanol (45.04 mg GAE/g DW). The optimization of the extraction conditions increased the yield to 59.82 mg GAE/g DW when the extraction temperature increased to 50.00 °C, which will be difficult to demonstrate here with ethanol due to its flammable and volatile nature. Therefore, our results propose ChCl: lactic acid as a good candidate for the large-scale extraction of polyphenolic compounds for possible industrial applications with low cost and environmental impacts.

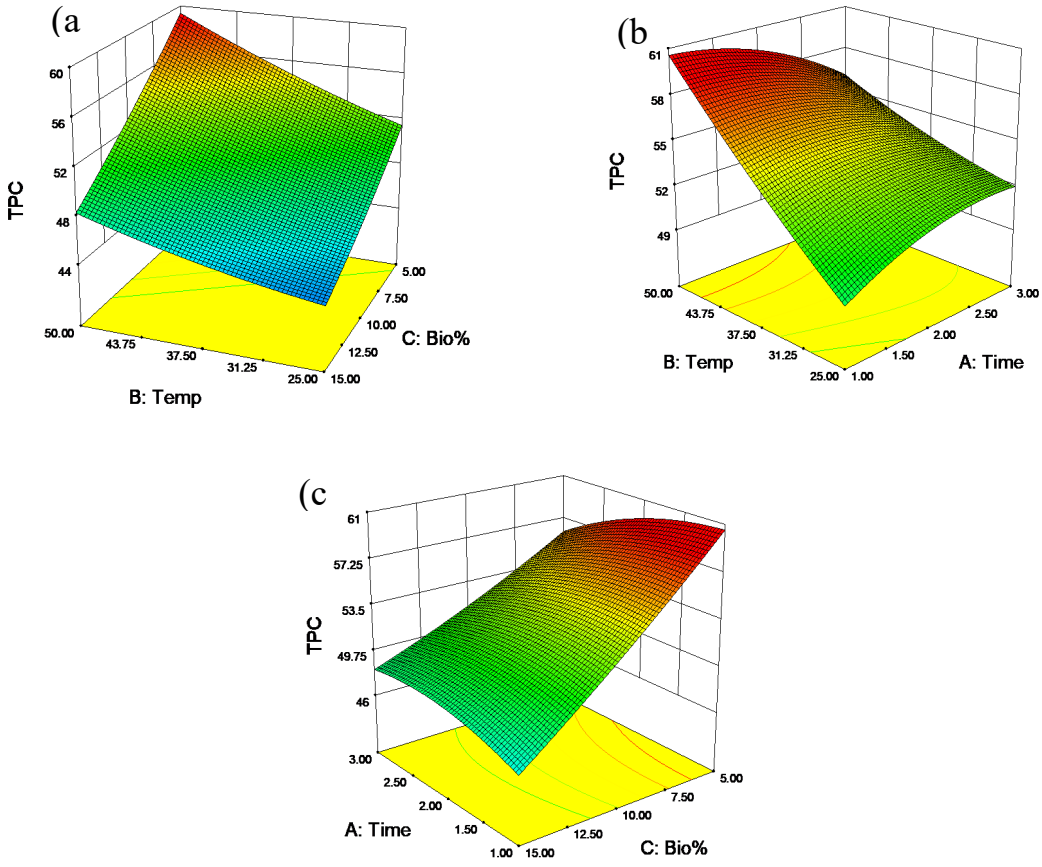


Fig. 4. 3D plots of total phenolic content extraction using deep eutectic solvent based on the interaction between the considered factors: (a) frequency and temperature, (b) frequency and time, and (c) time and temperature

3.4 Reusability and stability results

The reusability of the ChCl: lactic acid DES4 for the extraction of phenolic compounds was conducted at the optimal conditions. DES4 was reused for four cycles to determine the total phenolic content at the same extraction condition. As shown in Fig. 5, the amount of TPC extracted was steadily increased (cycle 1=59.85, cycle 2=95.12, cycle 3=135.05 and cycle 4=154.65 mg GAE/g DW) after four consecutive cycles. However, it can be expected that no further extraction will be observed after a few more cycles. The successful reusability can prompt further studies to be possible scaling up a sustainable process of DESs with different applications.

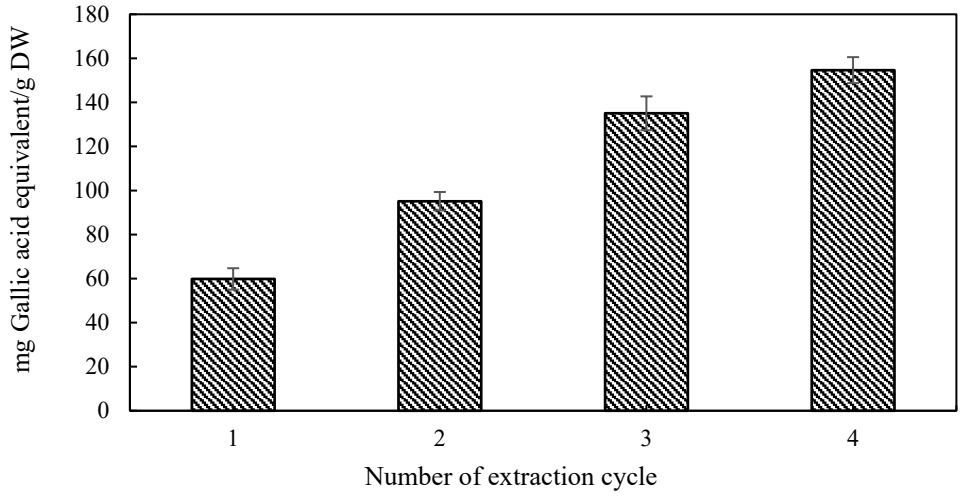


Fig. 5. The amount of extracted TPC in mg GAE/g DW after four extraction cycles

On the other hand, studying the stability of phenolic compounds in the deep eutectic solvent during storage is of great importance. The extracted TPC using DES4 was obtained at the optimal conditions. The DES4 displayed good stability for the target compounds over eight days of storage in a dry and dark place. As shown in Fig. 6, a slight decrease in the TPC was observed at the end of the storage experiment from 64.68 mg GAE/g DW to 63.38 mg GAE/g DW. The negligible volatility of the DESs can add more advantages to the DES-containing bioactive products to fit the application of a wide range of industries such as food, pharmaceuticals and cosmetics.

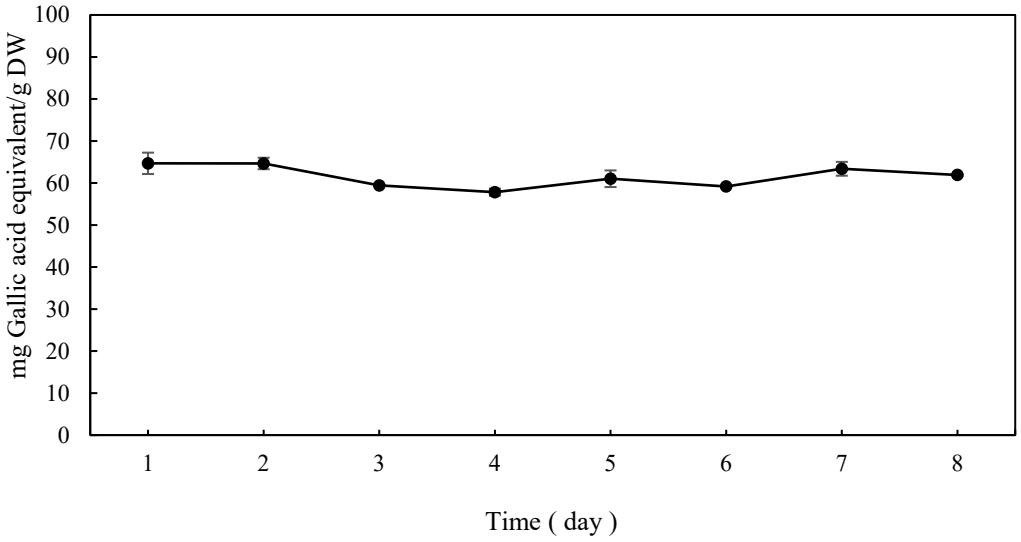


Fig. 6. The amount of TPC in mg GAE/g DW during the storage period.

4. Conclusion

The extraction of phenolic compounds from dried Manuka leaves using DESs-based extraction was conducted for the first time. In the present investigation, choline chloride and TBAC were paired with two polyalcohols (ethylene glycol, 1, 3-propanediol) and two carboxylic acids (lactic acid and acetic acid). The results revealed that ChCl: ethylene glycol DES, ChCl: lactic acid and, ChCl: 1, 3-propanediol DESs have proved to outperform ethanol as a conventional solvent in TPC with 56.87, 52.51, 50.67 and, 45.04 mg GAE/g DW respectively. Among the mentioned DESs, ChCl: lactic acid DES was further optimized and both the stability of TPC and the reusability of the potential DES were also studied. Both the stability of TPC and the potential DES's reusability demonstrate that DES as a green and sustainable solvent can be directly used to recover valuable compounds such as polyphenolic compounds. Furthermore, the antioxidant activity of extracted TPC by the DESs was assayed using DPPH and FRAP methods and compared with the ethanolic extract. Generally, ChCl: ethylene glycol DES extracts showed better free radical scavenging activity (12.78 µg TE/ml) while ChCl: lactic acid DES extracts gave higher ferric reducing power (199.78 µg TE/ml) at the same extract concentration with both being higher than the extract obtained by ethanol. Further studies are needed to recover the TPC from the DES extract by one of the enrichments and separation procedures, such as solid-phase extraction (SPE), macroporous resin adsorption, anti-solvent precipitation, back-extraction, and adsorption chromatography. This study proposes an approach for exploiting native Manuka leaves and developing the recovery of natural products in the pharmaceuticals and food industries.

Declaration of competing interest

The authors declare no conflict of interest.

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