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https://doi.org/10.1016/j.cherd.2020.11.028

Evaluation of deep eutectic solvents in the extraction of β-caryophyllene from New Zealand Manuka leaves (*Leptospermum scoparium)*

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Abstract

Leptospermum scoparium (Manuka) is a small indigenous shrub belonging to the family of Myrtaceae, which is equivalent to a tea tree (*Melaleuca alternifolia*) in Australia. Manuka leaves are rich in bioactive terpenes such as β-caryophyllene, which are scientifically proven to have potential activities in different areas including antimicrobial, anti-inflammatory, antitumour, antioxidant, and anaesthetic. This research considers for the first time the application of deep eutectic solvents (DESs) as green and sustainable solvents for the extraction of β-caryophyllene (β-CAR) from New Zealand Manuka leaves. The extraction was carried out using twenty-six types of hydrophilic and hydrophobic DESs. Menthol: Lactic acid DES was found to have higher extractability of β-caryophyllene than *n*-hexane, methanol, and ethanol as reference solvents. Moreover, Menthol: Lactic acid DES at 1:2 molar ratio was more efficient than conventional steam distillation and Soxhlet extraction. Furthermore, β-caryophyllene showed a good stability in menthol: lactic acid DES over 9 days of storage. The final β-caryophyllene concentration (β-CAR= 8.713605 mg/g Manuka leaf) after 4 cycles of extraction suggests a good reusability of the potential DES. The extraction of β-caryophyllene using this DES was further optimised using RSM (response surface methodology) combined with BBD (Box-Behnken design). The optimum experimental conditions were as follows: temperature 25.07 °C, stirring time 1.09 h, and % w/v biomass of 14.99%, which gave an extraction yield of 10.25 mg of β-caryophyllene/g Manuka leaf.

Keywords: Deep eutectic solvent; extraction; bioactive compound; Manuka leaves; β-caryophyllene.

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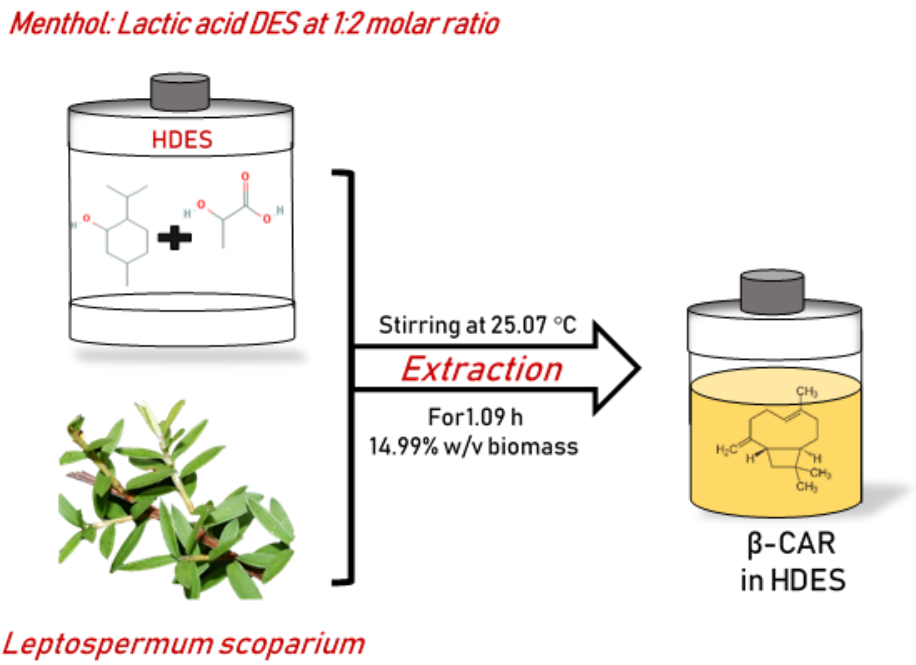

1. Introduction

The native species of New Zealand flora have a significant number of health-promoting components due to geographic isolation and high UVB light level (McKenzie et al., 2006; Wardle, 1985; Zhang and Björn, 2009). *Leptospermum scoparium* (Manuka) is a small indigenous shrub belonging to the Myrtaceae's family, which is equivalent to a tea tree (*Melaleuca alternifolia*) in Australia. Traditionally, Māori and the early 19th century settlers used Manuka for therapeutic purposes. Australian tea tree oil (TTO), is the essential oil extracted mainly from the native plant of Australia by steam distillation. The main application of TTO is to help in cutaneous infections healing and can be widely found over the counter. Besides, the essential oil of kanuka (*Kunzea ericoides*) and Manuka, are referred to as New Zealand TTOs. The early settlers used to make tea from the leaves and foliage of Manuka as they liked the bitter taste of it.

Manuka oil from the East Cape (North Island, New Zealand) has been shown to have an antimicrobial effect against some antibiotic-resistant bacteria such as *Staphylococcus aureus*, enterococci, and tuberculosis (van Klink et al., 2005). In a comparative study, the essential oil from the Manuka tree (*Leptospermum scoparium*) and Kanuka (*Kunzea ericoides*) was shown to have a remarkable inhibition against certain oral pathogen strains compared to Australian tea tree, rosemary, lavender, and eucalyptus essential oil (Takarada et al., 2004). Moreover, the oil is rich in bioactive terpenes, which are scientifically proven to have potential activities in different areas including antifungal (Christoph et al., 2000), anti-inflammatory (Maddocks-

Jennings et al., 2009), anti-acne (Wu, 2011), antiviral (Schnitzler et al., 2008), UVB skin protection (Kwon et al., 2013), herbicidal (Dayan et al., 2011), antioxidant (Lis-Balchin et al., 2000), and anti-ageing (Kwon et al., 2013). For this reason, a wide range of Manuka oil products is commercially available for such applications. Manuka oil that is present in the cellular structure of Manuka leaves is high in bioactive compounds. Ninety-five percent of Manuka oil content was identified as 20-30% triketones, 60-70% sesquiterpene hydrocarbons, and 5% monoterpene hydrocarbons (Christoph et al., 2000). Different factors such as climate, geographic area, season, and the age of the plants affect the composition of Manuka oil (Porter et al., 1998b). It is essential to understand the variation in the Manuka tree oil composition when it comes to therapeutic applications. There are three main chemotypes of *Leptospermum scoparium* available in New Zealand: (i) high in β-triketones (Nazzal et al., 2002) high in monoterpenes and; (iii) high in sesquiterpenes (Porter and Wilkins, 1999; van Klink et al., 1999). Commercially, the essential oils from Manuka leaves and fine branches are produced by steam distillation (Porter and Wilkins, 1999; Southwell, 1973). By prolonging the period of distillation (up to 5 h), the maximum yield of valuable compounds can be achieved. Moreover, the essential oil of Manuka can be extracted by using hexane for 4-7 days, at ambient temperature (Porter et al., 1998a).

Production of Manuka oil is more expensive due to the heavy oil components (sesquiterpenes) compared to Kanuka and Australian tea tree oil which contains light components (monoterpenes) and can be extracted within 20-40 min.

β-caryophyllene, β-CAR is a volatile (lipophilic) active compound and is abundant in the essential oils of many spices and plants extracts such as *Cannabis sativa* (Hendriks et al., 1975), *Origanum vulgare* (Bayramoglu et al., 2008), *Piper nigrum* (Menon et al., 2000), *Eugenia caryophyllata* (Della Porta et al., 1998), *Cananga odorata*, (Gaydou et al., 1986), *Murraya koenigii* (Erkan et al., 2012), *Salvia triloba* (Longaray Delamare et al., 2007) and *Copaifera multijuga* (Cascon and Gilbert, 2000).

From a chemical perspective, caryophyllene belongs to bicyclic sesquiterpene, which consists of a cyclobutane ring and an unsaturated carbon-carbon double bond in a 9-membered ring. Due to its structure, it can readily react or attach to another moiety. The molecular structure of β-caryophyllene was shown in Fig. S1.

Due to its attractive odour, β-caryophyllene has been used in different products such as fragrances, cosmetics, personal care, and bath products like shampoo, toothpaste, detergents,

creams, and lotions since the 1930s. Moreover, it is approved by the Food and Drug Administration (FDA) as an additive in a variety of food and beverage products (Fidyt et al., 2016; Schultes, 1987; Sköld et al., 2006b). β-caryophyllene has exhibited significant effect as an anti-inflammatory activity (Cho et al., 2007; Tung et al., 2008), a local anaesthetic (Ghelardini et al., 2001), antibacterial activity (Haznedaroglu et al., 2001; Koutsoudaki et al., 2005; Magina et al., 2009), antifungal activity (Omidbeygi et al., 2007), antitumor activity (Amiel et al., 2012; da Silva et al., 2007; Kubo et al., 1996) and antioxidant activity (Dahham et al., 2015). This potential pharmacological agent has low solubility in water and is sensitive to oxygen, acids, humidity, and high temperature (Ponce Cevallos et al., 2010; Sköld et al., 2006a; Wang et al., 2011).

According to the literature, the extraction of essential oils high in β-caryophyllene from different biomaterials can be conducted using some conventional methods such as hydro-distillation and solvent extraction (Dahham et al., 2014; Erkan et al., 2012; Gaydou et al., 1986; Jayaprakasha et al., 2003; Longaray Delamare et al., 2007). The main disadvantages of using conventional technologies for extracting valuable compounds are the excessive contact time, high solvent consumption, solvent toxicity, high energy consumption, adverse effect on thermo-sensitive components and poor stability of the extract. To overcome these challenges, novel techniques are emerging to enhance the extraction of valuable compounds and to reduce environmental problems. Supercritical CO_2 is one of the green agents used to extract essential oils containing β-caryophyllene extraction (Wang and Weller, 2006). Despite the advantages of this technique, the high operating cost may restrict its application. Microwave treatment combined with hydrodistillation is another comparable method that has been used to extract essential oils high in β-caryophyllene (Erkan et al., 2012). Although this technique can be very simple and economical, the efficiency of extraction can be limited when the bioactive compounds and the solvent are either non-polar or volatile (Wang and Weller, 2006). Moreover, additional use of toxic organic solvents will be needed for liquid-liquid extraction of the essential oils. Therefore, there is a need to replace the current methods with more feasible ones having environmental and economic benefits.

There is a growing interest in using deep eutectic solvents (Magina et al.; Mišan et al., 2020) for bioactive compounds recovery as a replacement for organic solvents. This kind of solvent shares some properties with another type of solvents widely known as ionic liquids (ILs); it is significantly less toxic, less volatile, relatively cheaper, easier to prepare, biodegradable, sustainable and stable at higher temperatures than other organic solvents (Zhang et al., 2012).

Generally, a DES consists of a hydrogen bond acceptor (HBA) associated with a hydrogen bond donor (HBD). The DESs normally have low volatility because of the hydrogen bonds between HBA and HBD. Choices of mixing HBA and HBD are many, which can offer a large number of solvents with unusual properties. DESs physicochemical properties can be adjusted to extract polar and non-polar compounds via a selection of the starting materials, the molar ratio, and by adding a certain amount of water (Paiva et al., 2014). From an environmental point of view, chemicals from natural sources such as organic acids (Choi et al., 2011), sugars and sweeteners (Carter et al., 2004; Nockemann et al., 2007) and amino acids (Kagimoto et al., 2006), have been considered in DESs composition as starting materials. DESs can be used for many applications including the separation of mixtures (Jiao et al., 2019), desulfurization of fuels (Ahmed Rahma et al., 2017; Li et al., 2019), and extraction of interesting compounds from biological tissues (Benvenutti et al., 2020; Cai et al., 2019; He et al., 2020; Kanberoglu et al., 2019; Ruesgas-Ramón et al., 2020; Saha et al., 2019).

Presently, DESs are gaining growing attention in the extraction of bioactive compounds due to their desirable feature of tunability to become more selective for a particular bioactive compound, their potential for enhancing the thermal stability of bioactive compounds, their low toxicity and lack of adverse effects on the environment. Furthermore, the recyclability of DESs reduces the cost of the extraction process significantly for industrial scale-up. DESs have been utilised to extract the different types of bioactive compounds such as anthocyanin (Dai et al., 2016), terpenoids (Tang et al., 2014), phenolic compounds (Cvjetko Bubalo et al., 2016; Duan et al., 2016; Zhao et al., 2015), polysaccharides (Zhang and Wang, 2017), hydrophobic diterpenoids (Chen et al., 2016) and antifungal compounds (Nadia et al., 2018). For the first time in this study, different hydrophilic and hydrophobic DESs were prepared and utilised for the extraction of β-caryophyllene from Manuka in a single extraction step and their stability and reusability were studied.

2. Research methodology

2.1 Materials

Leaves of the Manuka tree were harvested during February 2019 in controlled plantations at Manuka Bioactives Ltd., located in the East Cape, New Zealand. The materials were left to dry at room temperature for a few weeks and then dried by using a convection oven at 35 ^{o}C for three days. The dried samples were kept at 4 ^{o}C prior to the extraction process. Before each set of experiments, the dried Manuka leaves were blended by a food-grade stainless steel grain miller at 28,000 rpm to make powder. The powder with particle size ≤200 μm was collected

using Vibratory Sieve Shaker AS 200, Germany at 60% power level for 10 minutes. All chemicals used in this study are shown in Table S1. Standard compounds of trans-caryophyllene (99%), α-pinene, β-pinene, linalool, eucalyptol, β-eudesmol were obtained from Sigma–Aldrich, Inc, USA.

2.2 DESs preparation

Twenty-six DESs were prepared by mixing the different HBAs and HBDs at different molar ratios and at 90 °C with constant stirring until a transparent liquid was obtained. Choline chloride (ChCl), tetrabuthylammonium chloride (TBAC), guanidinium chloride (GuHCl), menthol, DL-menthol, chloride (TOAC) and lidocaine were selected as HBA to prepare the hydrophilic and hydrophobic DESs. Different types of HBAs and HBDs were selected to prepare DESs with diverse polarity range. The proposed DESs are presented in Table 1.

Table 1 - List of the proposed DESs.

HBA	HBD	Molar ratio	Abbreviation
Choline chloride	Glycerol	1:2	DES-1
Choline chloride	Ethylene glycol	1:2	DES-2
Choline chloride	Triethylene glycol	1:3	DES-3
Choline chloride	1,2-butanediol	1:3	DES-4
Choline chloride	1,3-propanediol	1:3	DES-5
Choline chloride	Lactic acid	1:2	DES-6
Choline chloride	Acetic acid	1:2	DES-7
Choline chloride	Levulinic acid	1:2	DES-8
Tetrabutylammonium chloride	Polyethylene glycol 300	1:3	DES-9
Tetrabutylammonium chloride	Ethylene glycol	1:2	DES-10
Tetrabutylammonium chloride	Lactic acid	1:2	DES-11
Guanidinium chloride	Polyethylene glycol 300	1:2	DES-12
Guanidinium chloride	Glycerol	1:2	DES-13
Guanidinium chloride	Ethylene glycol	1:2	DES-14
Guanidinium chloride	Lactic acid	1:2	DES-15
Tetrabutylammonium chloride	1-dodecanol	1:2	DES-16
Tetrabutylammonium chloride	Tetradodecanol	1:1	DES-17
Tetrabutylammonium chloride	Capric acid	1:2	DES-18
Menthol	Acetic acid	1:1	DES-19
Menthol	Lactic acid	1:1	DES-20
Menthol	Tetrabutylammonium chloride	2:1	DES-21
Menthol	Levulinic acid	1:1	DES-22
Tetraoctylammonium chloride	Capric acid	1:2	DES-23
DL-menthol	Lactic acid	1:1	DES-24
DL-menthol	Lauric acid	2:1	DES-25
Lidocaine	Capric acid	1:2	DES-26

2.3 Extraction of β-caryophyllene using DESs and conventional solvents

The extraction was performed by mixing the Manuka leaves powder and the prepared deep eutectic solvents at 5% w/v. The extraction was performed at room temperature (~20 ºC) in a screw cap glass vials. The stirring speed was set at 1000 rpm for all solvents. After one hour,

the biomass was centrifuged at 10000 rpm for 10 minutes, the supernatant then filtered by PTFE microfilter (0.45 μm). The final sample was diluted three times with ethanol to reduce its viscosity before HPLC analysis. Each sample was analysed in duplicate. Conventional solvents such as *n*-hexane, ethanol, methanol, and milli-Q water were also examined for extraction of β-caryophyllene at ambient temperature for one hour at a solid to liquid ratio of 1:20 (w/v) ratio. After extraction, Manuka leaves extracts were centrifuged at 10,000 rpm for 10 min. A PTFE microfilter (0.45 μm) was used to filter all sample solutions before HPLC analysis.

2.4 Extraction of β-caryophyllene using conventional techniques

Steam distillations of the Manuka leaves for obtaining the essential oil were performed using a Turbo 500 still. The steam was condensed with a tap water condenser and collected in a glass bottle. The oil was recovered by liquid-liquid extraction in a separating funnel with dichloromethane as a solvent. After extraction, the organic phase (solvent + essential oil) was evaporated using a rotary evaporator (Buchi, Rotavapor R-210/215) under reduced pressure (954 mbar) and at the temperature of (58 ^{o}C). The essential oil yield was calculated based on the dry weight. Finally, the essential oil sample (100 mg) was diluted in 10 ml of ethanol and filtered through a PTFE microfilter (0.45 μm) for GC-MS analysis to determine β-caryophyllene content.

Soxhlet extraction of β-caryophyllene was performed using a Soxhlet apparatus (Buchi, B-811 LSV Extraction System) for three hours, with 200 mL of *n*-hexane. 10 g of the powdered Manuka leaves was placed in cellulose extraction thimbles. After extraction, the solvent was evaporated using a rotary evaporator (Buchi, Rotavapor® R-210/215) under reduced pressure. The resulting dark green powder was diluted in 10 mL ethanol. Finally, a PTFE microfilter (0.45μm) was used to filter the samples for HPLC analysis (Madhava Naidu et al., 2008; Martino et al., 2006).

2.5 Stability and reusability study

Samples for the stability test were prepared by the similar DES extraction method, as described in section (2.3). The DES extracts of β-caryophyllene were stored in glass vials at ambient temperature in a dark and dry place. The β-caryophyllene concentration was measured on days 1, 2, 3, 6, 7, 8, 9, and 10 after the extraction by taking a known amount of the sample and diluting it with ethanol before HPLC analysis. Similarly, the stability of ethanol and *n*-hexane extracts of Manuka leaves was also studied in the same manner. The reusability is defined as

the number of cycles in which the DES solvent can extract additional quantities of the bioactive compound of interest. A good number of repeats is essential from the economic point of view, as this can reduce the amount of DESs needed for β-caryophyllene products formulations.
The first extraction cycle was started by mixing the potential DES with 5% w/v of Manuka leaf powder in glass vials at ambient temperature. The extraction was performed for 1 hour with perfect stirring. After extraction, the mixture was centrifuged and filtered using a 0.45 µm microfilter to separate the solid part. β-caryophyllene containing samples were quantified by diluting 1.0 ml of the extract three times in ethanol. The remaining volume of DES was measured and then reused for Manuka powder extraction with the same biomass percentage for consistency. Four cycles were used for β-caryophyllene extraction with the same extraction conditions for each cycle.

2.6 Analytical methods

The content of β-caryophyllene in the different DESs, conventional solvents and Soxhlet extracts was determined using HPLC Shimadzu (Tokyo, Japan). An Agilent ZorbaxEclipse XDB-C18, 5 µm, 4.6 x 150 mm column was used for chromatographic separation. Acetonitrile 100% was used as a mobile phase. The Photo Diode Array (PDA) detector was used with Deuterium (D2), scan 190 nm to 350 nm, Cell temperature 40 ºC, Slit width 1.2 nm. The presence of β-caryophyllene was verified by a peak between 190 and 350 nm in the HPLC analysis. The column temperature was 25 ºC, and the sample volume for injection was 10 µL. The column was purged for 20 min with the mobile phase before the sample injection at flowrate 0.5 mL/min. The content of β-caryophyllene was determined using a calibration curve established with a regression equation of $Y = 46429X + 105968$ ($r^2 = 0.9998$), where Y and X are the peak area and the concentration (mg/L) of the β-caryophyllene, respectively.

The content of β-caryophyllene in essential oils obtained by steam distillation was determined using GC-MS (Douglas et al., 2004). The samples were analysed by a GC-MS-QP2010 Ultra system (Shimadzu) attached to an AOC-5000 plus autosampler (Shimadzu). HP-5MS (30 m x 0.25 mm, film thickness 0.25 µm x 0.32 mm ID) fused silica capillary column was used. Chromatographic conditions were: injector temperature 280.0 ºC, injection mode: split, split ratio: 50.0. The column temperature program was 40 to 100 °C at a heating rate of 3 °C/min, then 100 ºC to 115 ºC at a heating rate of 1 °C/min for 15 minutes; lastly, from 115 ºC to 200 ºC at a heating rate of 3 °C/min. The carrier gas was helium. One µL of the diluted extract solution was injected. Mass spectrometry conditions were as follows: acquisition of mass

scanning between 50-800, scan time 0.32s, and ionisation voltage of 70 eV. Oil constituents were identified by comparing each component's mass spectra with those values stored in GCMS libraries. A solution of C8-C20 alkanes was also injected using the same column under the same conditions described for GCMS analysis to calculate the retention indices of the obtained components. The obtained peaks are also compared with available standards reference and match them with the MS fragment pattern. For Manuka essential oil produced by steam distillation, the available standard was trans-Caryophyllene (99%), α-Pinene, β-pinene, Linalool, eucalyptol, and β-eudesmol.

2.7 Design of experiment and Statistical Analysis

Design-Expert 7.1 software (Stat-Ease Inc., U.S.A) was used to perform the experimental design and optimisation of the β-caryophyllene extraction process from Manuka leaves. RSM (response surface methodology) combined with BBD (Box-Behnken design) was performed to investigate the impact of three independent variables on the yield of β-caryophyllene, including extraction time (A), temperature (B), and %biomass (C) at three levels. The experiment consisted of 15 runs and included two replicates of the centre points. This potential pharmacological agent is sensitive to the elevated temperature. The values were chosen to avoid thermal degradation of the compound. Optimisation of the extraction process could help in enhancing the yield of the target, reduce the number of experiments needed, investigate the interaction between the studied variables, and minimise cost and time. Among the most reliable analytical technique for optimisation, BBD is very efficient and most frequently used for evaluating the performance of many chemical and physical processes (Ray et al., 2015).

3. Results and discussion

3.1 Screening of DESs performance in β-caryophyllene extraction

Twenty-six different DESs (DES1- DES26) were examined for extraction of β-caryophyllene from Manuka leaves. DESs were classified into two groups, hydrophilic DESs (DES1- DES15) and hydrophobic DESs (DES16–DES26) which varied in their hydrophobicity. The superiority of DESs in β-caryophyllene extraction was also evaluated with conventional solvents (i.e., *n*-hexane, ethanol, methanol, and milli-Q water). The results obtained from DESs extraction and conventional solvents are shown in Fig. 1. The amount of β-caryophyllene varied among the DESs and conventional solvents used. In general, methanol and *n*-hexane yielded a similar

amount of β-caryophyllene (3.24 and 3.21 mg/g dried leaves, respectively), ethanol yielded less with 2.51 mg/g dried leaves, while Milli-Q water did not extract this non-polar compound. The lipophilic nature of β-caryophyllene explains the extraction efficiencies of the organic solvents. Although ethanol and methanol are considered polar solvents because of the existence of the hydroxyl groups, the hydrophobic ends in their structures can help in dissolving β-caryophyllene. However, methanol dissolves significantly more β-caryophyllene due to its lower viscosity. In the solvent extraction, selective solubility "like-dissolve-like" is an important principle to understand the extractability of the organic compounds by polar and non-polar solvents (Smith, 1977).

β-caryophyllene extraction efficiencies by proposed DESs varied greatly depending on the type of DES. However, DESs that show a better affinity for β-caryophyllene are those solvents with hydrophobic characteristics. The hydrophilic DESs (DES1-DES15) used in this study were mainly based on polyalcohol and carboxylic acids. In general, hydrophilic DESs exhibited low to moderate extractability of β-caryophyllene from Manuka powder and DES12 performed best. Furthermore, DESs with TBAC as the HBA extracted a larger amount of β-caryophyllene compared to choline chloride. The possible explanation could be that the long-chain ammonium salts have less solubility in water compared to choline chloride which impacts greater hydrophobicity.

On the contrary, β-caryophyllene showed an excellent solubility in hydrophobic DESs as compared with other solvents. β-caryophyllene is non-soluble in water, so the extraction efficiency will be better in DESs with hydrophobicity like hexane and may be used as alternatives for β-caryophyllene extraction. In this study, the property of the proposed DES was mainly affected by the HBA. Change in the HBA would change the polarity and viscosity of the DESs and thus influence the extraction efficiency of different hydrophobic DESs. This can be supported by the amount of β-caryophyllene in the carboxylic acid-based DESs when the hydrogen bond acceptor is a hydrophobic component.

The DESs containing fatty acids (i.e., Capric acid and lauric acid) as HBDs have a good performance in β-caryophyllene extraction due to the hydrophobic nature of β-caryophyllene. Interestingly, TBAC: capric acid DES (DES14) showed higher extraction of β-caryophyllene compared to TOAC: capric acid DES (DES23) (Fig. 1). The possible explanation for the results could be that TOAC (with eight carbon chain lengths) is considered more viscous, leading to a limit in the diffusivity of the bioactive compound, resulting in a poor yield. Similarly, the hydrophobic deep eutectic solvent (DES16) with 1-dodecanol (solubility in water = 0.004 g/L) as a HBD gave a higher concentration of the β-caryophyllene (β-CAR=2.16 mg/g Manuka leaf)

as compared to tetradodecanol based DES17 (β-CAR=1.17 mg/g Manuka leaf), (immiscible with water). The possible explanation could be the high viscosity of the resultant TBAC: tetradodecanol, which may limit the mass transfer of the target compound.

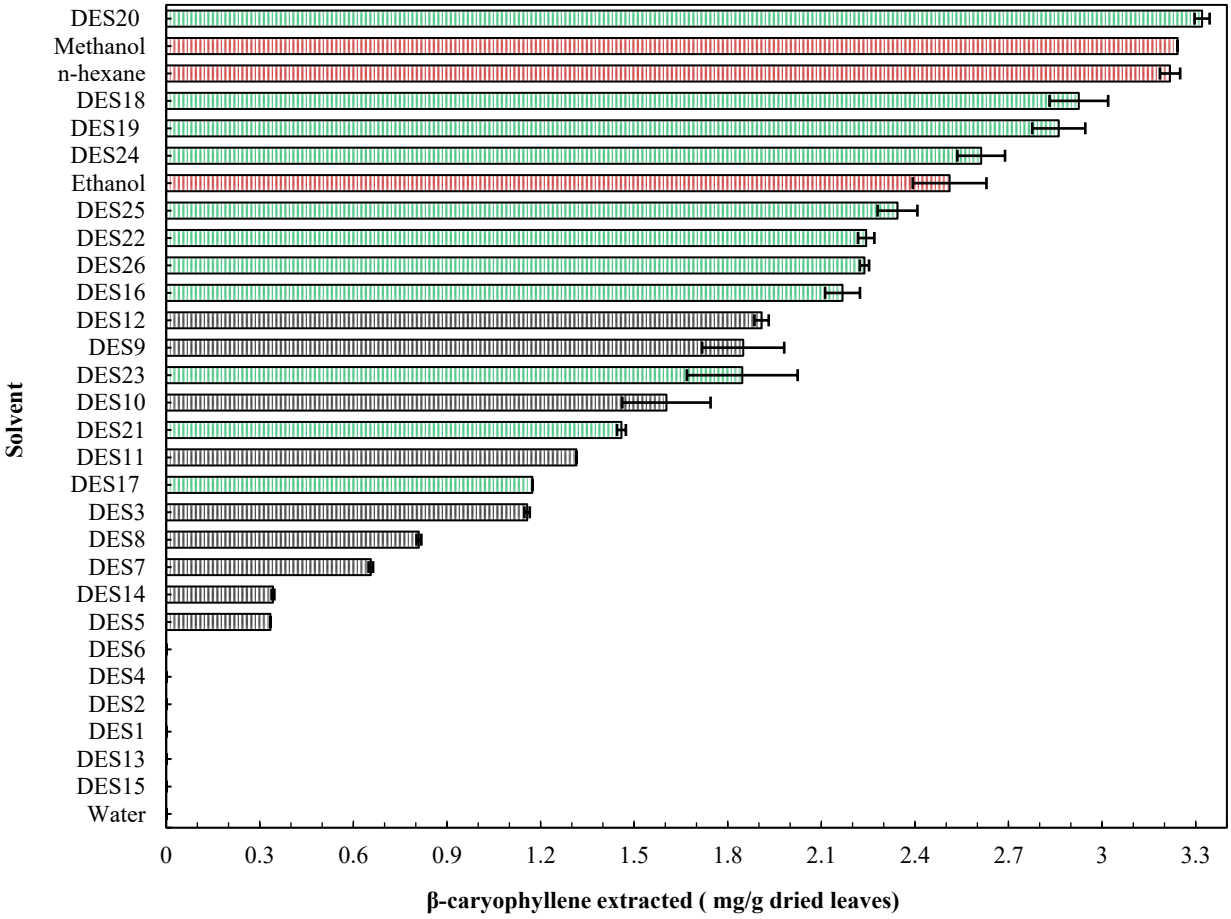

Fig. 1 - The extractability results of β-caryophyllene using different DESs and conventional solvent. (Reference solvents are shown by red colour, hydrophobic DESs are shown in green colour and the hydrophilic DESs are shown is black colour)

Menthol, DL-menthol, and lidocaine as HBAs in DESs showed superior β-caryophyllene extractability, especially the menthol-based DESs. The extraction yields obtained from DES20 were slightly higher than those obtained by *n*-hexane and methanol.

DES26 (lidocaine: capric acid) also gave a good extractability of β-caryophyllene (β-CAR=2.34 mg/g Manuka leaf). This can also be explained by the limited solubility of lidocaine in water (0.41 mg/mL), which gives a better hydrophobic property in combination with capric acid. Based on carefully examining the extraction efficiency of various DESs, it was found that menthol-based DESs have much higher hydrophobic characteristics than those of salt-based DESs due to the poor solubility of menthol in water (Nazzal et al., 2002); this gives them the advantage and enhances the extractability of β-caryophyllene. Therefore, in this study, the

effect of the menthol to the lactic acid ratio on the extractability of β-caryophyllene from Manuka leaves was also studied. β-caryophyllene reusability and stability in the potential DES were also investigated. Lastly, the impact of extraction parameters on the yield of β-caryophyllene, including extraction time (A), temperature (B), and biomass percentage (C) was optimised.

The physiochemical properties can be influenced by DES composition and the molar ratio (Abbott et al., 2011; Kareem et al., 2010; Shahbaz et al., 2011); hence, β-caryophyllene was extracted using different molar ratios of menthol: lactic acid. Menthol: lactic acid DESs at molar ratios of 2:1 to 1:3 were prepared and tested for β-caryophyllene extraction. As indicated in Fig. 2, the extraction yield was influenced by different molar ratios of menthol: lactic acid. β-caryophyllene yield steadily increased when the menthol mole fraction decreased to 0.33. Also, it was observed that a further decrease in the mole fraction of menthol resulted in a decrease in the yield. It is clear that when the menthol mole fraction decreased to 0.33, the amounts of lactic acid increased, and thus the DES viscosity diminished, enhancing the extractability of the target compound (Yusof et al., 2014). When the menthol mole fraction decreased to 0.25, an excessive amount of HBDs led to less menthol moles, which reduced the interactions between the DES components and between the β-caryophyllene and the charges on menthol, thus decreasing the extraction performance (Qi et al., 2015). Based on the results, menthol: lactic acid at a molar ratio of 1:2, gave the best β-caryophyllene extractability, and therefore it was chosen for the subsequent experiments. The lactic acid results as the sole solvent showed no extraction of β-caryophyllene due to its high miscibility with water (Fig. 2).

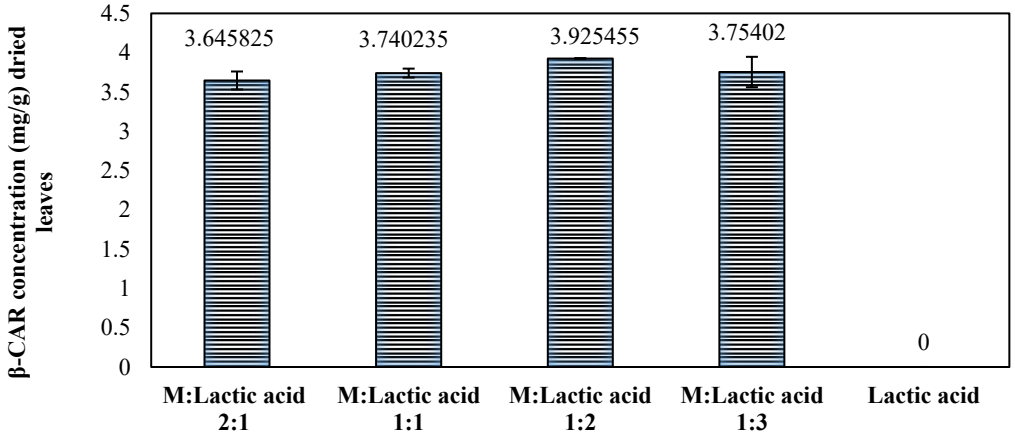

Fig. 2 - Effect of the molar ratio of the menthol: lactic acid DES on β-caryophyllene extractability.

Cao et al. (Cao et al., 2017b) studied the influence of the methyl trioctyl ammonium chloride:1-butanol molar ratio on the recovery of artemisinin from the leaf of *Artemisia annua*. In their

investigation, they found that the reduction in the methyl trioctyl ammonium chloride mole fraction from 0.33 to 0.2 resulted in an improvement in the yield of artemisinin. Further reduction in the mole fraction to 0.16, decreased extraction efficiency. The high concentration of 1-butanol could limit the interaction between the hydrogen bond acceptor and the hydrogen bond donor and the artemisinin with chloride anion, hence reducing the recovery of the target compound. Similar findings were observed by Cao et al. (Cao et al., 2017a). They found a significant influence on the extraction of polyprenyl acetates from *Ginkgo biloba* using a different molar ratio of hydrophobic DESs.

3.2 Reusability and stability results

The reusability of the potential DES for extracting β-caryophyllene from Manuka was studied using the same screening condition as for the single-step extraction. The extraction was performed for 1 hour with stirring. After extraction, the remaining volume of the potential DES was measured and then reused with the same biomass percentage of Manuka powder. Fig. 3 represents the amount of β-caryophyllene after four consecutive cycles of extraction using menthol: lactic acid DES at 1:2 molar ratio as a solvent. The concentration of β-caryophyllene steadily increased after three cycles (β-CAR= 3.32, 6.07, and 8.77 mg/g dried Manuka leaves, respectively). However, the extraction efficiency decreased to zero and the β-caryophyllene concentration remained constant after the third cycle with the final concentration of β-caryophyllene in menthol: lactic acid DES (β-CAR= 8.71 mg/g Manuka leaf). Good reusability of DESs is important from the economic perspective, as this reduces the amount of DESs needed for β-caryophyllene-rich products. Due to the nontoxicity, biodegradability, and low volatility of menthol, it is considered as a good and sustainable candidate for preparing hydrophobic DESs.

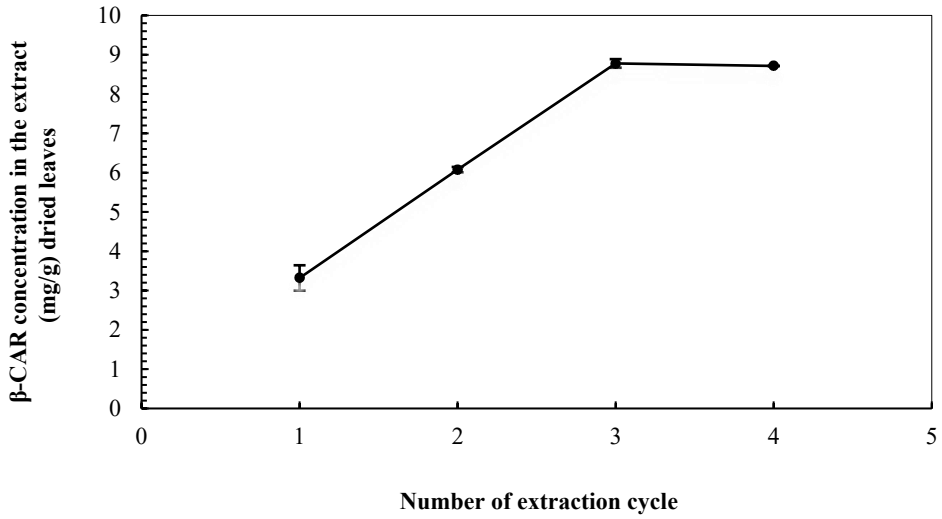

Fig. 3 - β-caryophyllene concentration in the menthol: lactic acid DES after four cycles of extraction.

It is essential to study the stability of the extracted bioactive compounds in the deep eutectic solvent during storage. Samples for the stability test were prepared by the similar DES extraction method, as described in section (2.3). β-caryophyllene showed a good stability in menthol: lactic acid DES over 9 days of storage with a slight decrease of β-CAR concentration from 10.82 to 10.26 mg/g dried leaves (Fig. S2). It can be concluded from the stability results that, due to the non-volatile nature of DESs, DES-containing β-caryophyllene would be more stable.

Menthol-based DESs can also be used as a formulation medium. Moreover, menthol possesses a cooling effect along with biological activity, and for that reason, menthol is used in a wide variety of health-care products. Therefore, DES can be incorporated into cosmeceuticals, pharmaceuticals, and industrial applications.

3.3 Comparison of β-caryophyllene extraction with different extraction techniques

In order to assess the superiority of DESs as a green extraction solvent, different extraction methods were used. The β-caryophyllene was extracted using menthol: lactic acid DES, steam distillation and Soxhlet techniques, and the β-caryophyllene yield obtained from these techniques are shown in Table 2. The amount of β-caryophyllene obtained by Soxhlet extraction was 2.11 times higher than that of steam distillation. However, the highest β-caryophyllene concentration was found using menthol: lactic acid DES (1:2 molar ratio) with 1.39, and 2.93 times higher than Soxhlet extraction and steam distillation, respectively.

Table 2 - Comparison of the β-caryophyllene yields from *Manuka* leaves obtained by menthol: lactic acid DES, steam distillation, and Soxhlet extraction techniques.

Extraction methods	β-caryophyllene, mg/g dried leaves
Steam distillation	1.13±0.06
Soxhlet extraction (*n*-hexane)	2.38±0.0019
Menthol: lactic acid DES extraction	3.32±0.024

The extraction using Soxhlet extraction resulted in higher extraction impurities due to the larger amount of solvent (*n*-hexane) being used, thus, greater solvent /Manuka powder ratio. Furthermore, the extraction using Soxhlet apparatus took a longer time (3 hrs) and a higher temperature (*n*-hexane boiling point = 68 ºC), which led to greater extraction of the impurities but less amount of β-caryophyllene as compared to the extraction using a deep eutectic solvent. On the other hand, after the extraction of Manuka leaves using conventional steam distillation, sixty compounds of Manuka essential oil were detected by gas chromatography-mass spectrometry (GCMS) analysis and thirty-eight of them could be identified. The GC-MS results indicate that β-caryophyllene (15.35%), α-pinene (11.41%), linalool (11.31%), eucalyptol (7.81%), α-selinene (4.53%), β-selinene (4.19%), α-eudesmol (4.21%), β-pinene (3.65%), and α-terpineol (3.11%) are the major components of Manuka essential oil. However, the elevated temperature used with the steam distillation could be the reason behind the poor β-caryophyllene yield which is considered as a sensitive compound in nature.

According to the literature, the extraction of essential oils high in β-caryophyllene from different biomaterials can be conducted using some conventional methods such as hydro-distillation from *Cinnamomum zeylanicum* fruit stalks (β-CAR=22.3% total peak area) (Jayaprakasha et al., 2003), supercritical CO_2 from Clove (*Eugenia caryophyllata* Thun) bud (β-CAR=11.10%) (Della Porta et al., 1998), and 80% ethanol extraction from *Aquilaria crassna* (β-CAR=10.5%) (Dahham et al., 2014). For the production of essential oils from various medicinal plants, conventional steam distillation and Soxhlet extraction are the most used (Burt, 2004; Lahlou, 2004). However, both methods have disadvantages such as thermal degradation of sensitive compounds, time consumption, high energy consumption, use of a flammable and harmful solvent which contributes to environmental problems, high amount of impurities in the extract, loss of volatiles, and cost of solvent recovery (Ali et al., 2019a; Burt, 2004; Gao et al., 2017; Gavahian et al., 2015; Upadhyay et al., 2015). The main advantage of using DESs as a solvent is the capacity of their components to accept and donate protons and electrons thereby making hydrogen bonds between molecules (Paiva et al., 2014; Zhang et al., 2012). In this case, DESs have a higher affinity to the target compound. It has also been

reported that DESs have excellent solubility for diverse types of compounds including pharmaceuticals, metal oxides, and carbon dioxide (Abbott et al., 2004; Aroso et al., 2016; Leron and Li, 2013). Based on the comparison results of the β-caryophyllene extracted by the aforementioned methods, the deep eutectic solvent could be the viable option for the extraction of compounds like β-caryophyllene which are unstable at an elevated temperature.

3.4 Optimization of β-caryophyllene extraction using BBD

To obtain the optimal conditions for β-caryophyllene extraction using DESs, RSM with Design-Expert software version 7.1 (Stat-Ease Inc., U.S.A.) was employed. A 15 run of three levels of the major independent extraction variables, including temperature (25 ºC, 40 ºC, and 55 ºC), % w/v biomass (5, 10 and 15%), and extraction time (1, 2, and 3 hours) were performed using a BBD design. Response surface methodology (RSM) is a popular technique used to determine the processing conditions that achieve the best yield of the target compound. The independent variables and levels are shown 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 (ºC)	25	40	55
C	% W/V biomass	5	10	15

The experimental data were analysed using multiple regressions to develop a quadratic polynomial equation and to describe the interaction between the extraction yield of β-caryophyllene and extraction variables. As a result, the following regression model was established:

$$\beta\text{-CAR} = 6.13 - 0.11A - 0.54B + 2.82C - 6.089^{*}10^{-3}AB - 0.072AC - 0.32BC - 0.043A^2 + 0.18B^2 - 0.46C^2 \quad (1)$$

where β-CAR is the amount of β-caryophyllene (mg/g dried Manuka leaves), A is stirring time (hours), B is extraction temperature (°C), and C is % w/v biomass.

Table 4 shows the experimental data of three factors (at three levels) and experimental response values of the target with different levels of temperature, time, and % w/v biomass.

The validity of the model was testified in terms of the determination coefficient (R^2) and adjusted (R-squared), as presented in Table 5. The model has a correlation coefficient of 0.9975, indicating that the model could justify 99.75% of the variation. The predicted R-squared (0.9651) is closed to adjusted R^2 (0.9930), meaning the model can adequately represent the data and also can be reliable to predict responses for new experiments. Besides, the lack-of-fit at the 95% confidence level is also considered as an important factor to evaluate the

agreement between the experimental and the predicted data. In our case, the lack-of-fit has a p-value (probability of error value) of 0.2099, which again supports the accuracy of the model to represent the experimental data.

Table 4 - Experimental BBD matrix and obtained response values for the extraction of β-caryophyllene from Manuka leaves.

run	*B*: Time (h)	*A*: Temperature (°C)	*C*: biomass (%)	Response: extraction yield (mg β-CAR/g dried leaves)
1	2	55	5	2.73
2	3	25	10	6.72
3	2	25	15	9.61
4	3	40	5	2.92
5	1	40	5	2.94
6	2	40	10	6.25
7	2	40	10	6.04
8	1	25	10	6.99
9	1	55	10	5.83
10	3	55	10	5.53
11	3	40	15	8.16
12	2	25	5	3.09
13	1	40	15	8.47
14	2	55	15	7.97
15	2	40	10	6.09

Table 5 - Analysis of the variance for the fitted quadratic polynomial model of β-caryophyllene extraction using menthol: lactic acid DES (1:2 molar ratio).

Std.dev.	0.18	R^2	0.9975
Mean	5.96	Adjusted R^2	0.9930
C.V.%	3.08	Predicted R^2	0.9651
PRESS	2.35	Adequate precision	45.169

The p-values of ANOVA demonstrated the significance of the corresponding coefficient and also explained the interaction between the variables. The higher the F-value and the lower the p-value were, the more significant the model was. Based on the ANOVA results from Table 6, the p-values of (*B*, *C*), quadratic term coefficients (C^2), and the interaction between temperature and % biomass (BC) appeared to be significant (p-value < 0.05) whereas, in the case of (*A*, A^2, B^2, *AB*, and *AC*) were found to be not significant. The quadratic effect of A^2 (stirring time) and B^2 (temperature) as independent variables did not significantly influence the extraction yield of β-caryophyllene. The possible explanation is that the solvent was very efficient in extracting almost all the β-caryophyllene in the sample within 1 hour. Meanwhile, the quadratic effect of the C^2 (% biomass) has the greatest influence on the extraction yield of β-caryophyllene.

Table 6 - ANOVA for response surface quadratic model of β-caryophyllene extraction using menthol: lactic acid DES (1:2 molar ratio).

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*P*-value prob > *F*	Remarks
Model	67.31	9	7.48	222.46	< 0.0001	Significant
A-Time	0.10	1	0.10	3.05	0.1410	
B-Temp	2.37	1	2.37	70.43	0.0004	Significant
C-Bio%	63.45	1	63.45	1887.24	< 0.0001	Significant
AB	1.483E-004	1	1.483E-004	4.411E-003	0.9496	
AC	0.021	1	0.021	0.62	0.4658	
BC	0.41	1	0.41	12.15	0.0176	Significant
A^2	6.786E-003	1	6.786E-003	0.20	0.6720	
B^2	0.12	1	0.12	3.65	0.1141	
C^2	0.78	1	0.78	23.35	0.0047	Significant
Residual	0.17	5	0.034			
Lack of Fit	0.14	3	0.048	3.92	0.2099	Not significant
Pure Error	0.024	2	0.012			
Cor Total	67.48	14				

The 3D plots were used to visualise the effect of temperature, stirring time, and % w/v biomass on the yield of β-caryophyllene in terms of mg/g dried Manuka leaves Fig. 4. It was observed from Fig. 4a that the extraction yield of β-caryophyllene increased significantly when the % w/v biomass increased from 5 to 15%, and the increase in the extraction temperature did not enhance the yield. However, some researchers have well described the viscosity-temperature relationship. They showed that the reduction in the viscosities trend can be observed as the temperature increased. (Abbott et al., 2006; AlOmar et al., 2016; D'Agostino et al., 2011; Kareem et al., 2010). However, that was not the case in this study, as the low viscosity of the potential DES has a positive effect on the extractability of β-caryophyllene. Fig. 4b and 4c indicate that the increase in the stirring time from 1 to 3 hours did not improve the extraction yield. Menthol-based DESs are known to have lower viscosities compared to other types of DESs (Ribeiro et al., 2015), and that gives the advantage to facilitate the mass transfer and complete the extraction within a shorter time. In summary, the highest β-caryophyllene yield of 9.64 mg/g dried Manuka leaves predicted by the model can be achieved when the % w/v biomass, stirring time, and extraction temperature were 14.55, 1.09 and 25.44 ^{o}C, respectively.

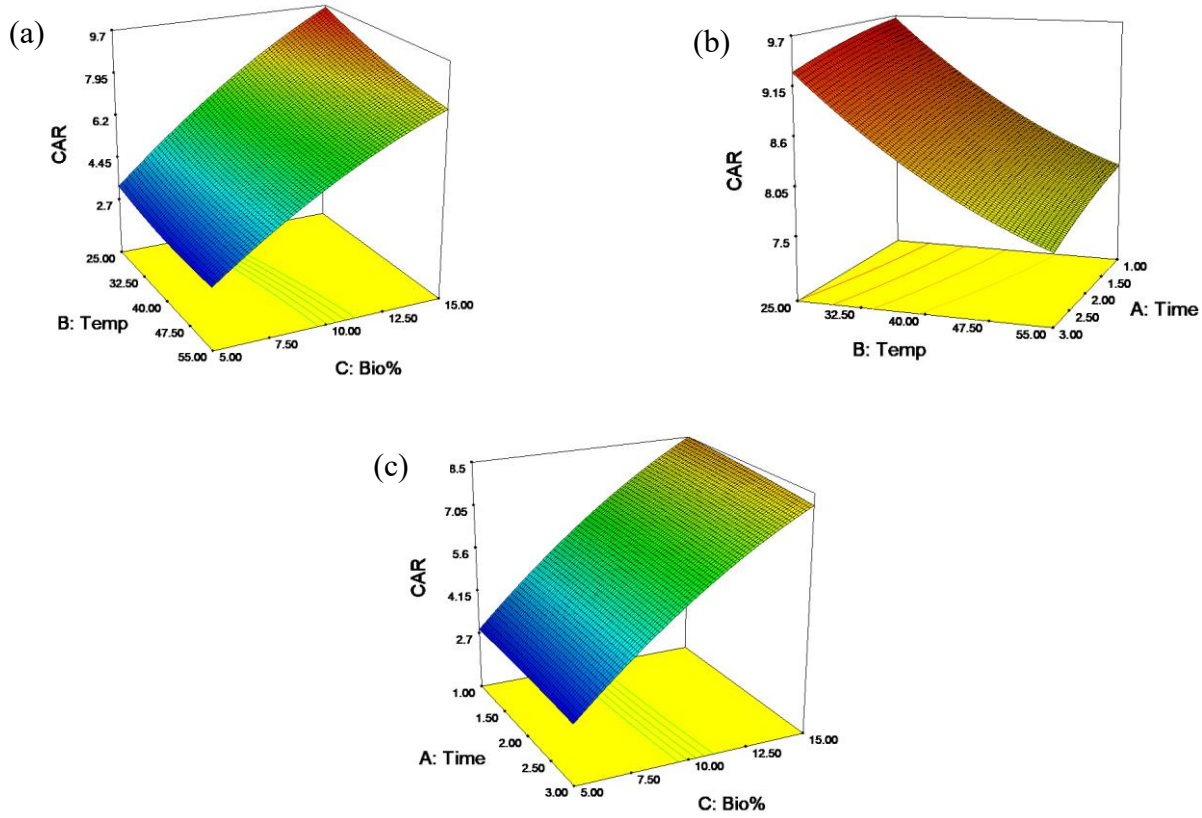

Fig. 4 - 3D plots of β-caryophyllene extraction using menthol: lactic acid DES based on the interaction between the considered factors: (a) temperature and % w/v biomass, (b) temperature and time, and (c) time and % w/v biomass.

Finally, the extraction under the optimum condition was determined to verify the yield with the value predicted by model Table 7. The experimental yield of β-caryophyllene was found to be 10.25 mg/g dried Manuka leaves which was close to the predicted value and fitted within the PIs (prediction intervals) of β-caryophyllene (8.94-10.35 mg/g Manuka leaf) with 0.95% error. In conclusion, hydrophobic DESs hold promise for improved extraction of β-caryophyllene from Manuka leaves. However, this study suggests using ultrasound-assisted extraction and microwave-assisted extraction as an intensification technique to significantly decrease the extraction time and increase the extraction yield(Ali et al., 2019b).

Table 7 - Experimental and predicted results of β-caryophyllene extraction using M: LA 1:2 at optimum condition.

Temperature (°C)	Time (h)	% W/V biomass	β-caryophyllene yield (mg/g dried leaves)		
			Predicted	Measured	Error (%)
25.07	1.09	14.99	9.64	10.25	0.95

4. Conclusion

The extraction of β-caryophyllene as a bioactive sesquiterpene was evaluated for the first time using deep eutectic solvents. In this study, twenty-six types of DESs were adapted for β-caryophyllene extraction from Manuka leaves. DESs were classified into two groups, hydrophilic DESs (DES1- DES18) and hydrophobic DESs (DES19 – DES26). The results revealed that the hydrophobic deep eutectic solvents showed higher extractability of β-caryophyllene than conventional solvents such as *n*-hexane, methanol, ethanol, and water. Moreover, menthol: lactic acid DES at 1:2 molar ratio was more efficient than conventional steam distillation and Soxhlet extraction. The extraction of β-caryophyllene using menthol: lactic acid DES was further optimised using RSM (response surface methodology) combined with BBD (Box-Behnken design). The optimum experimental conditions were as follows: temperature 25.07 °C, stirring time 1.09 h, and % biomass of 14.55wt%, which gave an extraction yield of 10.25 mg of β-caryophyllene/g dried Manuka leaves. Furthermore, β-caryophyllene showed a good stability in menthol: lactic acid DES over 9 days of storage with a slight decrease of β-CAR concentration from 10.83 to 10.27 mg/g dried leaves. The final β-caryophyllene concentration (β-CAR= 8.71 mg/g manuka leaf) after 4 cycles of extraction suggests a good reusability of the potential DES. In conclusion, menthol-based DES can be a potential, green, and sustainable replacement for organic solvents and conventional extraction methods in the extraction of β-caryophyllene from Manuka leaves and could also be extended for extracting bioactive terpenoids from another chemotype leaf in a single extraction step.

Acknowledgement

This research was carried out as part of the Food Industry Enabling Technologies (FIET) program funded by the New Zealand Ministry of Business, Innovation, and Employment (contract MAUX1402). The authors would also like to acknowledge Manuka Bioactives Ltd., located in the East Cape, New Zealand, to provide Manuka fresh leaves.

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