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Deep Eutectic Solvent-Based Ultrasound Assisted Extraction of *Piper betle* Leaves Oil and its Antioxidant Activity

Farah Hani Ezzaty Mohd Alias, Wan Nur Aqilah Wan Mohd Samsudin, Siti Ernieyanti Hashim*
Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, Skudai, Malaysia
*Corresponding author: ernieyanti@utm.my

Abstract
Piper betle, commonly known as *betel* leaf, is a tropical plant with rich cultural and medicinal history. Known for its complex composition, the essential oil of *P. betle* leaves is composed of diverse range of chemical constituents. Deep Eutectic Solvents (DES) is gaining popularity as a green alternative to Ionic Liquids. It offers an environmentally friendly approach for pre-treatment by effectively breaking down cell walls and enabling the extraction of a higher yield of chemical constituents during the hydro-distillation process. This study main objective were to investigate the efficacy of DES as a pre-treatment to maximize the yield of P.betle leaves essential oil and evaluate its antioxidant properties. The experimental methodology involved pre-treating the leaves with DES-based UAE prior to the extraction process using hydro-distillation. Analytical techniques employed to identify the specific chemical constituents present in the essential oil were Gas Chromatography (GC) and Gas Chromatography-Mass Spectrum (GC-MS). Major constituents obtained after DES pre-treatment included eugenol (46.23%), acethyl eugenol (14.59%), chavicol (4.56%), α-terpene (3.44%) and germacrene D (2.21%). Furthermore, the antioxidant activity of the extracted essential oil was evaluated using DPPH free radical scavenging method. The results revealed that the DES-based UAE pre-treatment exhibited a significantly lower IC_{50} value of 3.07 µg/mL compared to the UAE pre-treatment with IC_{50} value of 138.80 µg/mL. These findings strongly indicate that DES pre-treatment not only enhances the extraction of chemical constituents but also contributes to the improvement of antioxidant activity of the obtained essential oil.
Keywords: Deep Eutectic Solvent (DES), Ultrasound-assisted extraction, *P. betle*, antioxidant.

1. Introduction

Piper betle is a cosmopolitan plants that belongs to the *Piperaceae* family. Locally, it is referred to as 'sireh' in Malaysia. *P. betle* is one of the medicinal herbs used most commonly by cancer patients as an alternative treatment in traditional Asian medicine (Faruuqui et al., 2016). The plant is well-known in India since it is used in social, cultural, and religious activities as well as other aspects of daily life. Although *betel* leaves are mostly used as a mouthwash, they are also used to treat a variety of contagious and non-contagious illnesses, including stomachaches, rheumatism, and colds. They are also used to treat conditions like pyorrhea, conjunctivitis, wounds, injuries, constipation, boils, leucorrhoea, mastitis, mastoiditis, otorrhoea, swelling of the gums, and abscesses (Suryasnata, 2022).

The majority of the pharmacological, organoleptic, and other beneficial qualities of *betel* leaves are due to essential oil. Essential oil is a combination of several volatile molecules with a complex composition and a distinctive aroma unique to *betel* leaf. It can be used for a variety of medical conditions, including the treatment of pain and the acceleration of recovery (Madhumita, Guha, & Nag, 2019). The oil from *betel* leaves has a unique scent and a pungent spicy smell. The *betel* leaves essential oil has antibacterial, cardiotonic, blood pressure-lowering, cardiac, and respiratory depressive characteristics (Das et al., 2022).

P. betle can be accredited as a potential compound in alternative treatment to prevent or cure certain diseases due to the presence of various bioactive compounds such as eugenol, hydroxychavicol and chavibetol. However, by using conventional extraction method, there is some chemical constituents that is hard to be extracted. In the early 2000s, Deep Eutectic Solvent (DES) were introduced as the alternative ionic liquid (IL). DES can be used to pre-treat the cell-wall of any plants in order to allow more chemical constituents that were hard to obtain (Smith, Abbott, & Ryder, 2014). Thus, this study focused on investigation of the uses of DES as pre-treatment can obtain more *P. betle* leaves oil yield and characterization of chemical constituents of *P. betle* leaves oil. The determination of antioxidants by DPPH free radical scavenging activity can be used to identify the antioxidant properties of *P. betle* leaves oil.

Deep eutectic solvents (DES) are now widely acknowledged as a new class of ionic liquid (IL) analogues because they share many characteristics and properties with ILs. DES can offset the major drawbacks of common ILs, namely highly toxicity, non-biodegradability, complex synthesis requiring purification and high cost of the starting materials (Liu, Ou, Gregersen, & Zuo, 2023). DES can be obtained by combining two safe components which are cheap, renewable and biodegradable which capable of forming a eutectic mixture (Khandelwal, Tailor, & Kumar, 2016; Russo et al., 2022).

In this study, it is really important to have another alternative for pre-treatment using DES that are more convenient and safer. Given that *P. betle* has a long history of usage as a medical herb, this may help to demonstrate that it can be applied for other alternative treatments. Therefore, the outcome of this research is to pre-treat with DES-based UAE to provide additional information on the chemical constituents of *P. betle*.

2. Literature Review

2.1. Traditional Uses of Piper betle

P. betle's fragrant leaves *(piperaceae)*, often called *betel* leaves are used for both medicinal and masticating purposes, prominence in the nations of South East Asia (Gupta, Guha, & Srivastav, 2022). Being both perennial and evergreen, *P. betle* is known as a blessing plant that God created. There are chewing habit practices of *betel* leaves which are believed beneficial for avoiding bad breath, strengthening the gum, preserving teeth, and stimulating the digestive system (Chowdhury &Baruah, 2020; Das et al., 2022; Prabhu, Sudharsan, Ganesh Kumar, Chitra, & Janani, 2022).

It is also found that two months of consuming the combination of *P. betle* and Piper nigrum leaves is recommended in curing obesity. In addition, the leaves can also be applied locally to cure inflammatory swelling such as orchitis, arthritis and mastitis; and also can be used in prevent and treat vaginal ejection, and reduce itching of the vagina. They also showed the applications of *P. betle* roots and fruits to treat malaria and asthma, with the roots also being used in conjunction with *P. nigrum* to induce sterility in women, and the oil derived from *P. betle* being used to treat irritation in throat, larynx, bronchi, gargle and inhalation in diphtheria (Dwivedi & Tripathi, 2014).

2.2. Chemical Constituents of Piper Betle

People around the world starts to be interested in *P. betle* as it is known to have many benefits to human health due to biological active constituents in the essential oil. Some of chemical constituents of *P. betle* oil that have been found are eugenol, hydroxychavicol,chavibethol, chavicol, methyl eugenol, α-terpinene, mycrene and α-humulene (Karak et al., 2018).

Phenylpropanoid are constituents that has a phenyl group which is linked to a 3 carbon propane side chain (Dong & Lin, 2021). Phenylpropanoid constituents found in *P. betle* leaf oil are eugenol, carvacrol, chavicol, methyl eugenol, chavicol acetate, methyl chavicol, acethyl eugenol, phenyl acetaldehyde and estragol, while chavibetol is characteristic of the EOs from the wholeplant (Karak et al., 2018; Xiang et al., 2017). However, in study of the difference of chemical constituents of fresh leaf essential oil (FLEO) and cured leaf essential oil (CLEO). This study found that the chemical constituents of both FLEO and CLEO are the same but with different value of percentage. Phenylpropanoid constituents that found as major constituents are eugenol, estragole, anethol and hydroxychavicol (Madhumita et al., 2019).

Other typical constituents that can be found in *P. betle* leaf oil is monoterpenes such as α-terpinene, p-cymene, eucalyptol (Vu et al., 2021). In a research by using seven *P. betle* leaves from different part of West Bengal discovered 14 monoterpenes in the essential oil; α-thujene, α-pinene, champhene, sabinene, mycrene, α-terpinene, β-phellandrene, eucalyptol, (E)-β-ocimene, γ-terpinene, terpinolene, linalool, teroinen-4-ol and α-terpineol (Karak et al., 2018).

2.3. Biological Activities of Piper betle

P. betle leaves has a wide variety of phytochemicals. These bioactive are phenolic and flavonoid constituents that belong to diverse classes and are particularly well-known for their range of biological activity.

Natural antioxidants are efficient against key elements connected to illnesses associated with ageing. *Betel* leaf oil is a rich source of nutrients like energy, protein, fat, minerals, thiamine, riboflavin, vitamins, and bioactive constituents like hydroxychavicol, eugenol, chavicol, and chavibetol, where estragole is the main bioactive molecule, due to its therapeutic and nutritional characteristics (Arsad, Yunus, Zaini, Rahman, & Idham, 2016). The effect of free radical scavenging on DPPH radicals may be utilized to measure the antioxidant activity of *betel* leaf oil. The antioxidant activity of *betel* leaves oil is measured by DPPH radical scavenging assay. It is found that lower IC_{50} indicates the sample which exhibit higher antioxidant activity and vice versa. This study found a correlation between the chavibetol content and antioxidant activity of the leaf oil. The leaf oil having high chavibetol content showed high antioxidant activity with low IC_{50} value when compared among all the essential oils (Das et al., 2022). These results suggest that *betel* leaf could be used to substitute synthetic antioxidants as a natural antioxidants.

2.4. Deep Eutectic Solvent (DES)

Deep Eutectic Solvent (DES) have gained recognition as cost-effective alternatives to Ionic Liquids(IL). They are referred to as deep eutectic solvents because they form a eutectic points when two constituent parts are combined in the appropriate proportion. Fascinatingly, DES are created by combining organic hallide salts with an organic chemical that act as hydrogen bond donor (HBD), allowing them to establish hydrogen bonds with the hallide ion (Khandelwal et al., 2016). DES commonly classified based on the specific complexing agent employed (Li & Row, 2016; Vanda, Dai, Wilson, Verpoorte, & Choi, 2018).

In addition to their low lattice energy and low melting points, DES possess other favourable chemical and physical properties such as low toxicity, low vapor pressure and non-flammability (Smith et al., 2014). However, DES are characterized by high viscosity, which is considered a drawback. To address this issue, DES can be diluted with water to reduce its viscosity.

2.5 *Ultrasound Assisted Extraction (UAE)*

Ultrasound-assisted extraction (UAE) relies on the phenomenon of acoustic cavitation and mechanical effects for the extraction of constituents from plants sources. Collapse of the cavitation bubbles on the plant matrix's surface causes the cell walls to rupture resulting in higher and faster penetration of the solvent into the plant material. Thus, due to enhanced overall mass transfer, the extraction of the desired constituents are accelerated (Tomšik et al., 2016). UAE benefits include lower time and energy requirements, low-temperature extraction, and extract quality preservation. The physical forces generated during sonic cavitation cause the ultrasound waves to rupture the plant tissue, and they also speed up the mass transfer, allowing the release of extractable components into the solvent in a short amount of time (Albero, Tadeo, & Pérez, 2019).

It is found UAE gave significantly better yield and exhibit greater antioxidant activity compare to extraction by conventional method (Ali, Lim, Chong, Mah, & Chua, 2018) The effectiveness of ultrasound was also noticeable in the general phytochemical screening where additional phytoconstituents were detected in the UAE extracts only.

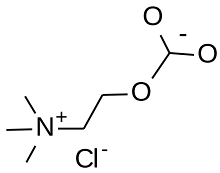
3. Methodology

3.1. Plant Material

Fresh leaves of *P. betle* were brought from Pekan Nenas, Pontian. The leaves is cleaned with water. Once cleaned, the leaves is cut uniformly in order to extract thoroughly.

3.2. Preparation of Deep Eutectic Solvent (DES)

Dark bottle was used in the preparation of DES where mixing choline chloride (ChCl), which act as HBA, and glycerol which act as HBD at ratio 1:2. The mixture is stirred using magnetic stirrer in the dark bottle at 100°C. The bottle is covered with bottle cap and let it heat for about 1 hour until the mixture turn into clear liquid solution



DES structure mixture of ChCl and glycerol

3.3 Extraction and Analysis of Piper betle Leaf Oil

3.3.1. *Pre-treatment of P. betle leaf*

The sample was immersed in DES solution and place in ultrasound-assisted extraction (UAE) by GT SONIC-D series for 30 minutes at 30°C. The second sample immersed in distilled water and also place in UAE for 30 minutes at 30°C. Both beakers are covered with paraffin film.

3.3.2. *Extraction of P. betle leaf Oil*

The pre-treated *P. betle* leaves were filter and transfer into 5L round bottom flask, followed by the addition of distilled water until the sample was fully covered. The Clevenger apparatus were setted up. The sample was heated and the extraction of the essential oil done by hydrodistillation for 8 hours. The oil was collected and extracted with diethyl ether (Et_2O) in separatory funnel and the organic layer is collected. Next, the organic layer was dried with anhydrous magnesium sulphate ($MgSO_4$) until it clumps and filter. The essential oil was kept in a vial, then the percentage yield will be calculated. The essential oil is kept in refrigerator until further use.

3.3.3 *Determination of Chemical Constituents*

The chemical constituents of the essential oil sample were obtained by quantitatively analysing the composition of the essential oil using GC-MS Headspace System brand Perkin Elmer gas chromatography equipped with an HP-5MS capillary column with a dimension of 30 m x 0.25 mm x 0.25 µm. Helium gas (He) was utilised as a carrier gas (mobile phase) and is programmed to control temperature. The analyses were done under the following oven temperature programme: injection at 60°C (5 min), then temperature increase at 4°C/min to 220°C and hold time 10 min. The injection temperature was set at 230°C; the MS transfer line at 280°C; the ion source at 250°C. Helium was used as the carrier gas with flow rate 1 ml / min.

3.4 *Antioxidant Activity*

3.4.1. *DPPH Free Radical Scavenging Assay*

The DPPH stock solution (0.1M) was made by dissolving 0.4 mg of DPPH in 25 mL of methanol, and it was then kept at 20°C until it was needed. An aliquot of 100 µL of this solution was combined with 100 µL of the sample at various concentrations ranging from 3.90 ppm to 1000 ppm. After completely blending, the reaction mixture was left to sit at room temperature in the dark for 30 min. Then, the wavelength be measured at 517 nm. Equation below were used to calculate the DPPH radical scavenging activity. A graph of concentration against % absorbance inhibition and the value of IC_{50} was calculated by using GraphPad prism software.

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_b - A_a}{A_b} \times 100$$

4. Results and discussion

4.1. Determination of Chemical Constituents of P. betle Leaf Oil

After extraction process by hydro-distillation, a faded yellow volatile oil with a strong aroma was obtained from the *P. betle* leaves, yielding 0.65% of essential oil. The GC-MS analysis of the *P. betle* identified various constituents. A total of 39 peaks were detected in the GC analysis, with some major constituents exhibiting high percentage areas. These major constituents include eugenol (46.23%), acethyl eugenol (14.59%), chavicol (4.56%), α-terpnene (3.44%) and germacrene D (2.21%) as shown in Table 4.1. Additionally, there were minor constituents such as terpinolene (0.04%), β-elemene (0.26%), spathulenol (0.08%) and viridiflorol (0.25%). When comparing the result obtained with previous study, Karak et al. (2018), take samples from 7 different region in India and compares their chemical constituents. It is found that in all leave samples, eugenol appear as the major constituents with percentage area over 20%. Other major constituents that found in this study are, germacrene D, and chavicol acetate. These major constituents were also present in the analysis conducted in this research.

Table **Error! No text of specified style in document.**.1: GC-MS Analysis of Chemical Constituents for DES-based UAE Pre-treated Leaf Oil

No	Chemical constituents	RT (min)	Area (%)
1	Eucalyptol	11.05	0.22
2	α-Pinene	11.56	0.04
3	Terpinolene	13.89	0.04
4	α-Terpinol	17.32	0.06
5	Estragole	17.44	0.13
6	Chavicol	19.61	4.56
7	β-Elemene	22.18	0.26
8	α-Terpene	22.62	3.44
9	Chavicol acetate	23.79	0.02
10	Acethyl eugenol	22.96	0.45
11	Eugenol	24.25	46.23
12	Isoeugenol	24.32	0.12
13	Chavibetol	24.50	0.90
14	Methyl eugenol	24.64	0.31
15	Caryophyllene	25.21	0.68
16	Alloaromadendrene	25.47	0.06
17	Germacrene D	25.90	2.21
18	α-Copaene	26.04	0.18
19	Selinene	26.27	0.18

20	Aromadendrene	26.40	0.08
21	α-Gurjenene	26.23	0.37
22	δ-Cadinene	26.74	0.06
23	Muurolene	26.93	0.32
24	α-Elemene	27.22	0.43
25	γ-Cadinene	27.37	0.37
26	α-Cadinene	27.43	0.04
27	α-Muurolene	27.53	0.32
28	β-Bisabolene	27.84	1.25
29	Nerodilol	27.99	5.53
30	Acethyl eugenol	28.76	14.59
31	α-Humulene	28.88	0.18
32	Spathulenol	29.96	0.08
33	α-Selinene	30.45	0.06
34	Globulol	30.78	0.07
35	Viridiflorol	31.22	0.25
36	Cubenol	31.79	5.68
37	Cubebene	31.93	0.21
38	α-Cadinol	32.19	0.98
39	Longifolene	32.26	0.13

RT = Retention Time, Area = Percentage Area

4.2. Antioxidant Activity

4.2.1. DPPH Free Radical Scavenging Assay

In DPPH analysis, numerous studies shown that the free radical scavenging activity of essential oils increases with higher concentrations, indicating strong antioxidant properties. In this study, a serial dilution method was employed to prepare different concentrations of the essential oil (1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 ppm). it was observed that as the concentration of *P. betle* oil increase, the free radical scavenging activity also increased.

The data obtained from the study was used to plot the DPPH assay graph, which represents the percentage inhibition of free radicals against the concentration of the samples. The results are presented as in Figure 4.1 for DES-based UAE pre-treated essential oil and Figure 4.2 for UAE. It is important to note that a lower IC_{50} value indicates higher antioxidant activity. In this study, the IC_{50} values for DES-based UAE were found to be 3.07 μg/mL compared to the UAE only pre-treatment with IC_{50} value of 138.80 μg/mL. The lower value of DES-based UAE pre-treated oil indicates it exhibits higher antioxidant activity compared to the only UAE pre-treated oil.

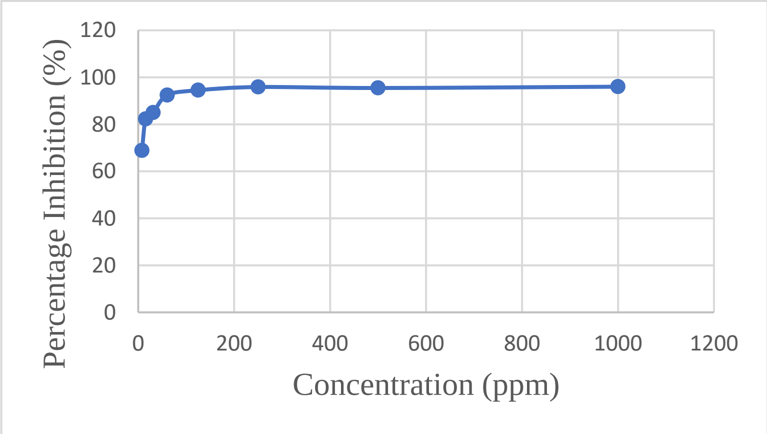


Figure **Error! No text of specified style in document.**.1: DPPH Assay for DES-based UAE Pre-treated Leaf Oil

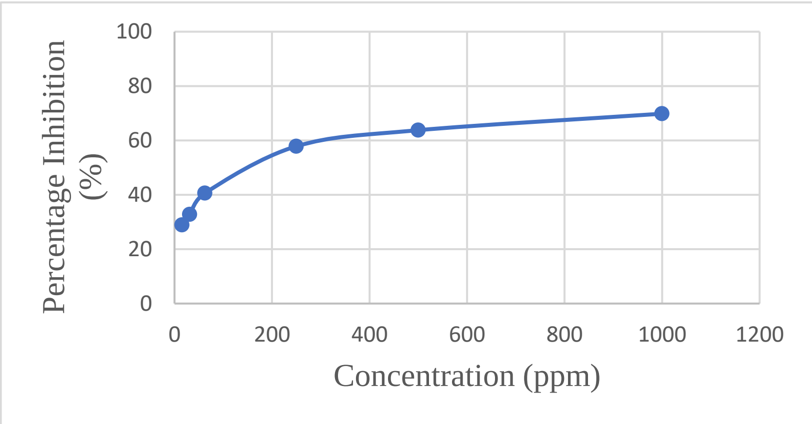


Figure **Error! No text of specified style in document.**.2: DPPH Assay for UAE Pre-treated Leaf Oil

Conclusion

In this project, two approaches were employed for pre-treatment prior to the extraction process. As UAE alone was not adequate enough to extract the complete range of chemical constituents found in the *P. betle* leaf, the other technique sought to increase the production of *P. betle* essential oil in a practical method by employing DES-based UAE. By employing DES, which is renowned for its ecologically renowned for its ecologically benign extraction technique, provided a green alternative method that was less expensive and time-consuming than using harmful and expensive chemicals. The composition of DES was crucial to the effectiveness of essential oil extraction, as it varied properties, including viscosity, surface tension and permeability, influenced the interactions with the extracted chemicals. By effectively dissolving the plant's cell wall, DES facilitated the extraction of constituents in *P. betle* more as compared to the effectiveness of using UAE alone as the pre-treatment. The antioxidant activity seen in the free radical scavenging experiments lends more credence to this idea. This unequivocally demonstrated that the utilization of DES as a pre-treatment not only enhances antioxidant activity but also increases the yield and content of chemical constituents compared to treatments without DES.

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