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Research Paper

Evaluation of Bioactive Compounds, Antioxidant, and Anti-Diabetic Activities in Hexane and Supercritical Carbon Dioxide Extracts of Sweet Potato (*Ipomoea batatas* L.) Leaves

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Abstract

Sweet potato leaves are a rich source of bioactive compounds with potential health benefits. Advanced methods are being explored to harness these beneficial compounds efficiently. Applying the supercritical carbon dioxide (SCO_2) technique, as an environmentally friendly extraction technique, offers many advantages over traditional solvent extraction. This study, therefore, aimed to investigate the effect of SCO_2 extraction on the bioactive properties of sweet potato leaves, focusing on antioxidant and anti-diabetic activities. Then, the SCO_2 extracts were compared with the hexane extract. A completely random design was used, performing 3 pressures (2000, 3000, and 4000 Psi) and 3 temperatures (40, 50, and 60°C) for the SCO_2 extraction. In addition, a maceration using hexane solvent was performed in a shaker for 24 hours. Total polyphenol and flavonoid concentrations were quantified, and gas chromatography-mass spectrometry (GCMS) analyses were used to identify the extracted bioactive compounds. Antioxidant and anti-diabetic activities were also assessed. This yield of SCO_2 extraction ranged from 0.49% to 0.89%, which was significantly lower in yield, polyphenol, flavonoid concentration, and antioxidant activity compared to hexane extract (P<0.05). Despite the lower yield, this study observed a higher concentration of bioactive terpenoids such as phytol, caryophyllene oxide, and squalene. In terms of anti-diabetic activity, the extracts at SCO_2-specific conditions (4000 Psi 40°C, 3000 Psi 60°C, 3000 Psi 40°C, and 60°C, 2000 Psi 60°C) exhibited potent alpha-glucosidase inhibition, with IC_{50} values comparable to acarbose, and hexane extract. Dipeptidyl peptidase 4 (DPP4) inhibition was the highest in the hexane extract ($p < 0.05$), followed by SCO_2 extracts. Thus, these findings highlight new possibilities for developing anti-diabetic agents derived from sweet potato leaves using the green SCO_2 extraction technique.

Keywords

Non-Polar Compounds, DPPH Inhibition Activity, Anti-α-Glucosidase Activity, Anti-Dipeptidyl Peptidase 4 (DPP4)

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1. INTRODUCTION

Sweet potato (*Ipomoea batatas* L.) leaves (SPL) are often overlooked as agricultural byproducts, yet they possess several health benefits because they are rich in health-promoting bioactive compounds. These leaves are particularly abundant in bioactive compounds, such as polyphenols, flavonoids, and essential oils (Arisanti et al., 2023; Tajik et al., 2017), which have been linked to various health benefits such as antioxidant, antimicrobial, and anti-diabetes properties (Bovell-Benjamin, 2007; Laveriano-Santos et al., 2022), making SPL a promising resource for health intervention. Despite their health properties, SPL remains primarily overlooked, particularly in Indonesia, where the prevalence of diabetes mellitus had doubled from 1.5% in 2013 to 3% in 2018 (Azam et al., 2022). This alarming rise in diabetes cases underscores the urgent need to explore the SPL's potential in combating this health crisis, mainly through sustainable and efficient methods to harness its bioactive constituents.

Traditional solvent extraction has been commonly performed to extract bioactive compounds from SPL. However, conventional methods that utilize organic solvents such as

ethanol, methanol, or hexane present significant drawbacks, including environmental and health risks due to their toxicity and flammability (Soquetta et al., 2018). In addition, the prolonged extraction times can lead to bioactive compounds' degradation, resulting in lower yields and reduced efficacy (Azmir et al., 2013). The increasing demand for cleaner, more sustainable extraction has encouraged the development of greener techniques, with supercritical fluid extraction (SFE) emerging as a powerful method.

SFE is a green technology for obtaining active compounds from natural products, offering several advantages over traditional extractions (Khaw et al., 2017). Compared to conventional solvent extraction, SFE provides enhanced selectivity, shorter extraction durations, reduced solvent usage, and solvent-free extracts (Arumugham et al., 2021). Carbon dioxide (CO_2), a naturally abundant, non-toxic, cost-effective, non-flammable, and environmentally friendly gas, is commonly used as the supercritical fluid in SFE (Herrero et al., 2010). Its unique characteristics to transform into a supercritical state, displaying characteristics of both liquid and gas, improves its dissolution ability and facilitates mass transfer, allowing for the efficient extraction of bioactive compounds (Arumugham et al., 2021).

Despite the promising potential of SPL as a source of bioactive compounds, exploring non-polar constituents through greener extraction techniques, such as supercritical carbon dioxide (SCO_2) extraction, remains limited. Most studies have focused on the polar concentrations of SPL (Laveriano-Santos et al., 2022), leaving a gap in understanding the non-polar constituents of the leaves. Non-polar contents, such as lipids and essential oils, were dominantly found when using the SCO_2 extraction due to its polarity that is comparable to that of liquid hexane (Capuzzo et al., 2013). Previous studies on the non-polar extraction, mainly through distillation, identified sesquiterpene as the predominant bioactive compound in SPL, with a notable concentration of β-caryophyllene, β-copaene, γ-elemene, and β-elemene, ranging from 1.08% to 28.63% across various cultivars of SPL (Marques et al., 2022). Similarly, a study in the Chinese Xiangshu-17 leaves revealed caryophyllene (28.73%), γ-muurolene (13.07%), and β-caryophyllene epoxide (9.04%) as the major components (Wang et al., 2010). These compounds, particularly terpenoid groups such as β-caryophyllene epoxide and β-elemene, have been found to possess anti-diabetic properties (Mahnashi et al., 2022).

Thus, this study aimed to extract non-polar compounds of SPL using hexane solvent, a conventional extraction, and SCO_2 extraction that provide a highly effective, sustainable, and adaptable substitute for conventional solvent-based extraction techniques. Then, it examined the bioactive compounds of SPL extracts, including polyphenol, flavonoid concentrations, and non-polar bioactive compounds, and evaluated their biological activities, focusing on antioxidant and anti-diabetic properties. An investigation was also conducted to compare the characteristics and bioactivities between hexane extract and SCO_2 extracts.

2. EXPERIMENTAL SECTION

2.1 Materials

Sweet potato leaves (*Ipomoea batatas* L.) were harvested from local farmers in Sumedang, Jawa Barat, Indonesia. It was then washed and dried in the oven air-drying, applying 400°C for 32 hours, which resulted in a water content of 8%. Chemical reagents were supplied by Merck (Germany) such as hexane, methanol, gallic acid, carbonate acid, Follin Concialteu, aluminium chloride, potassium acetate, quercetin, 2,2-diphenyl-1-picryl-hydrazil-hydrate (DPPH), p-nitrophenyl- α-D-Gluco side, and dipeptidyl peptidase 4 (DPP4) inhibition assay (MAK 203). In addition, α-glucosidase was bought from Wako, Japan. Lastly, the CO_2 was supplied by PT Samator Indo Gas Tbk (Indonesia).

2.2 Methods

2.2.1 Supercritical Carbon dioxide (SCO_2) and Hexane Extraction

A completely randomized design (CRD) with two replicates for each extraction condition was applied in this study, performing 3 pressures (2000, 3000, and 4000 Psi) and 3 temperatures (40, 50, and 60°C) at a flow rate of 20 mL/minute as extraction conditions in a supercritical extraction apparatus (Supercritical Fluid Technologies, Inc., USA) (Tyskiewicz et al., 2018). The extraction of 20 g of dried SPL was performed for 2.5 hours. In addition to SCO_2 extraction, maceration using hexane for 24 hours at room temperature in a shaker (Heidolph TitraMax 1000 Vibrating Platform Shaker, Germany) and at a ratio of 1:10 (w/v) was done to study the conventional nonpolar extraction method (Gori et al., 2021). Lastly, the yield of the extracts was calculated using the formula (Equation 1):

$$\%yield = \frac{\text{Weight dried extract (g)}}{\text{Weight plant sample (g)}} \times 100\% \tag{1}$$

2.2.2 Total Flavonoid Assay

The quantification of total flavonoids is conducted using aluminium chloride (Johnson et al., 2022). A standard stock solution containing quercetin was prepared within a 15-120 ppm concentration range. Subsequently, 50 μL of either blank (methanol), sample, or standard were introduced into each well of a 96-well plate. Next, 10 μL of 1 M potassium acetate and 10 μL of aluminium chloride 10% were incorporated and incubated for 30 minutes. The absorbance was then measured at a wavelength of 425 nm using an ELISA reader (BioTek Synergy HTX, Agilent Technologies, USA). The results were then graphed on the standard curve of quercetin and presented in milligrams equivalent to quercetin per gram of extract dry matter (mgQE/g extract DM).

2.2.3 Total Polyphenol Assay

The total polyphenol content was assessed utilizing the Folin-Ciocalteu reagent (Permatasari et al., 2023). Gallic acid standards were prepared within a concentration range of 15 to

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120 ppm. First, the sample extract, blank, or standard solution (20 μL) was inserted into individual wells within a 96-well plate. Subsequently, 100 μL of the Folin-Ciocalteu reagent was added to each well and incubated for 10 minutes in darkness. Afterward, 100 μL of a 7.5% $NaCO_3$ solution was inserted, and another incubation for 90 minutes in darkness was conducted. The resulting mixture absorbance was then calculated at a wavelength using an ELISA reader at 750 nm (BioTek Synergy HTX, Agilent Technologies, USA). The results were reported in milligrams equivalent to gallic acid per gram of extract dry matter (mgGAE/g extract DM).

2.2.4 GCMS Analysis

All the extracts were analyzed using a gas chromatography-mass spectrophotometry (GCMS) with an Agilent Technologies 7890A instrument equipped with a 113-2032 Carbowax column with dimensions of 30 m × 320 μm × 0.25 μm. The temperature was set from 60°C to 220°C, with an initial temperature of 40°C. The holding time was 2 minutes, and the post-run temperature was 220°C. A sample volume of 1 μL was injected for analysis. Subsequently, the spectra were compared with the NIST 20 database.

2.2.5 Antioxidant Activity

A 2,2-diphenyl-1-picrylhydrazil (DPPH) assay was used to analyze the antioxidant activity (Barzan et al., 2024). Samples were prepared at a 2500 ppm concentration in methanol, with ascorbic acid serving as the positive control and methanol as the blank. Subsequently, 100 μL of the sample, positive control, or blank was dispensed into individual wells of a 96-well plate. It was followed by 100 μL of 0.2 mM DPPH solution added to each well. As sample color absorbance, 100 μL of the sample was added with 100 μL methanol. The plate was then incubated for 30 minutes and measured at a wavelength of 517 nm. DPPH inhibition was determined by applying the formula below (Equation 2).

$$\text{Percent Inhibition (\%)} = \frac{\text{Blank Absorbance} - (\text{Test Absorbance} - \text{Sample Color Absorbance})}{\text{Blank Absorbance}} \quad (2)$$

The results of percent inhibition were then graphed on the standard vitamin C curve and presented in mg vitamin C equivalents per g of plant extract (mgVCE/g extract DM).

2.2.6 Anti-Diabetic Activity

Two techniques were performed to study the potency of the SPL extract, namely alpha-glucosidase and dpp4 inhibitor.

- **α-Glucosidase Inhibitory Activity**
  The α-glucosidase enzyme inhibition assay was performed as described by Najmah et al. (2021) with some modifications. In a 96-well microplate, a mixture containing 60 μl of the sample dissolved in DMSO and 0.1M PBS pH 7 and 25 μL of α-glucosidase solution (0.04 U/mL) dissolved in 0.1M PBS (pH 7) was prepared. Subsequently, 25 μL of a 20mM p-nitrophenyl α-D-glucopyranoside reagent was introduced into the mixture and allowed to incubate at 37°C for 30 minutes. Subsequently, the mixture in the microplate was read using a microplate reader (BioTek Synergy HTX, Agilent Technologies, USA) at 410 nm, performing the reading precisely at minutes of 0 and 30. The protocol testing was repeated for acarbose and quercetin (positive control). The inhibition percentage was determined using the specified formula (Equation 3):

$$\text{Percent Inhibition (\%)} = \frac{(B-A)-(C-D)}{(B-A)} \quad (3)$$

Where:
A: Absorbance of the blank prior to incubation
B: Absorbance of the blank following incubation
C: Absorbance of the sample/standard prior to incubation
D: Absorbance of the sample/standard following incubation
The IC_{50} value was obtained by plotting a graph of the concentration (ppm) on the x-axis and the inhibition (%) on the y-axis.

- **DPP4 Inhibition Assay**
  The DPP4 assay was performed using an ELISA kit provided by the manufacturer. The underlying principle of this assay involves the cleavage of the substrate (H-Gly-Pro-AMC) by DPP4, resulting in the release of the quenched fluorescent group, AMC (7-amino-4methylcoumarin), at an excitation/emission wavelength of 360/460 nm. Sitagliptin, at a concentration specified by the manufacturer, was employed as a positive control inhibitor, with an inhibitory activity of 50%. A concentration of 2500 ppm SCO_2 and hexane extracts was used as a sample test. Initially, 50 μL of the enzyme dissolved in a buffer solution was incubated for 10 minutes at 37°C. Subsequently, 23 μL of the test sample, blank, or positive control was added. Lastly, 2 μL of the substrate was introduced into the microplate wells and then measured in a microplate reader (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific, USA) for 30 minutes, with data collection occurring every 1 minute. To calculate the inhibition, first, compute the slope in ΔFLU per minute by choosing the two-time point of fluorescence (flu) in the linear plot and, subsequently, subtract the slope of the sample blank from the slope of each sample (Equation 4).

$$\text{Slope } (\Delta\text{FLU per minute}) = \frac{(Flu2 - Flu1)}{(Time2 - Time1)}$$

$$\text{Inhibition (\%)} = \frac{(\text{Slope}EC - \text{Slope}EM)}{\text{Slope}EC} \times 100\% \quad (4)$$

Where: Flu 1 and time 1 = fluorescence absorbance 1 of each sample/blank at time 1, Flu 2 and time 2 = fluo-

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rescence absorbance 2 of each sample/blank at time 2, Slope SM = The sample inhibitor slop, Slope EC = The enzyme control slope

2.2.7 Statistical Analysis

The differences in yield, total phenolic, and flavonoid concentration among the SCO_2 extracts and hexane extract were analyzed using an ANOVA test, followed by Tukey's post hoc test to identify specific group differences. In biological activities, the difference was also examined, including the standard, such as vitamin C for antioxidant activity and acarbose and quercetin for anti-α glucosidase activity. Statistical significance was determined at an alpha level of less than 0.05 (Wiguna et al., 2023). All analyses were performed using the software package PAST.

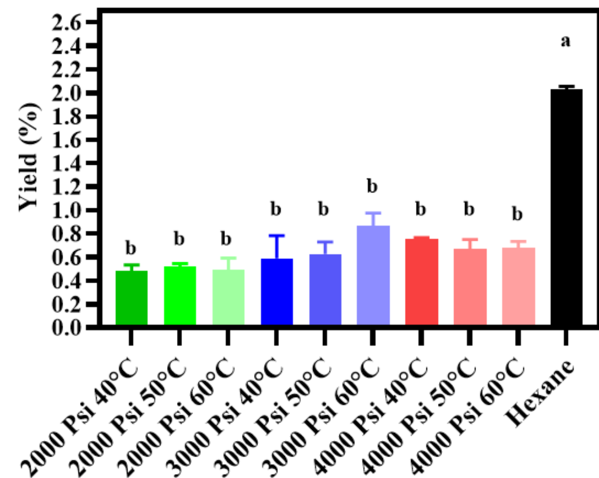
3. RESULT AND DISCUSSION

3.1 Yield, Total Flavonoid and Polyphenol Concentration

The application of SCO_2 extraction on SPL, mainly to extract the non-polar components, has yet to be fully explored. SCO_2 offers green technology to extract generally non-polar bioactive compounds from natural or food products. Similarly, hexane as a solvent with a lower polarity (0.1) also exhibits a liquid to extract non-polar compounds (Capuzzo et al., 2013). Both approaches are suitable for extracting considerably non-polar compounds, with some capacity to extract slightly polar compounds.

In this study, the highest yield was obtained from hexane extraction ($p < 0.05$), which resulted in 2% yield. All SCO_2 treatments showed a similar yield ($p > 0.05$), ranging from 0.49% to 0.89% (Figure 1). Other studies have also found that SCO_2 extraction yields are generally inferior to conventional hexane extraction (Feng and Meier, 2016; Saito et al., 2021; Yeddes et al., 2012). For instance, in green and red propolis extraction, SCO_2 yielded only 50% less than hexane extract, yielding 9.8% vs. 20.6% and 16.3% vs. 31.8%, respectively (Saito et al., 2021). Similarly, hexane extraction from seeds of Tunisian prickly pear (*Opuntia ficus* Indica) was also found to be the highest extraction than the SCO_2 technique, resulting in 10% vs 3.4%, respectively (Yeddes et al., 2012). These results are aligned with the current study, where the yield of the SCO_2 extraction was lesser than that of hexane extraction, showing a third yield of that obtained from hexane extraction. On the one hand, SCO_2 is a non-polar solvent that effectively mainly extracts fatty acids and terpenoids (Herrero et al., 2006). On the other hand, hexane is a broader solvent that might yield a wider variety of compounds, including polar ones (Yeddes et al., 2012). Thus, it can be shown that hexane solvent resulted in a higher yield than the SCO_2 technique.

In addition, this study examined total flavonoid and polyphenol concentrations. The results showed that applying 4000 Psi pressure on SCO_2 at 40 and 50°C yielded the highest polyphenol concentration ($p < 0.05$) among SCO_2 treatments. In contrast, 2000 Psi resulted in the lowest polyphenol and flavonoid concentration ($p < 0.05$) (Figure 2A). Several SCO_2



Distinct letters signify notable differences among the extraction treatments at $p < 0.05$.

Figure 1. The Yield Percentages of Hexane Extract and SCO_2 Extracts with Different Extraction Pressures (2000, 3000, and 4000 Psi) and Temperatures (40, 50, and 60°C)

were observed to obtain similar results for flavonoid concentration, such as a 4000 Psi pressure on SCO_2 at 40, 50, and 60°C and a 3000 Psi pressure on SCO_2 at 40 and 60°C (Figure 2B). However, all SCO_2 groups were lower than that of hexane, which performed as the best-standing extraction for yield, polyphenol, and flavonoid concentrations ($p < 0.05$) (Figure 1). Lower values of flavonoid and polyphenol in SCO_2 extraction were observed in this study, with concentrations ranging from 1.68 to 5.48 mg GAE/g and from 11.73 to 19.96 mg QE/g, respectively. The best SCO_2 extractions for extracting polyphenol and flavonoid concentration were at 4000 Psi with either 40 or 50°C. SCO_2 extraction is one approach to extracting polyphenols and flavonoids from various plants (Tyskiewicz et al., 2018). A review study also revealed that applying SCO_2 parameters at a pressure ranging from 1500 to 4500 Psi and a temperature of 50°C yielded the best level of polyphenol content of chocolate (*Theobroma cacao*), mango (*Magnifera indica*), pomegranate (*Punica granatum*) leaves (Tyskiewicz et al., 2018). Studies also revealed that performing higher pressure (Wu et al., 2014) and higher temperature (Rombaut et al., 2014) SCO_2 increased the polyphenol yield but decreased the extraction efficiency, resulting in low yield.

The result also showed that hexane extract had the highest total polyphenol and flavonoid concentration compared to SCO_2 extracts. Specifically regarding the polyphenol level, a study found that the hexane extraction of slow pyrolysis liquid of beech wood resulted in 1.5 times greater polyphenol than SCO_2, showing 2% and 3.5% yields, respectively (Feng and Meier, 2016). In addition, SCO_2 extraction from two Salvia species (*Salvia chrysophylla* and *Salvia microstegia*) found that SCO_2 extracts were inferior to the standard extraction using hexane in terms of total polyphenol and flavonoid concentra-

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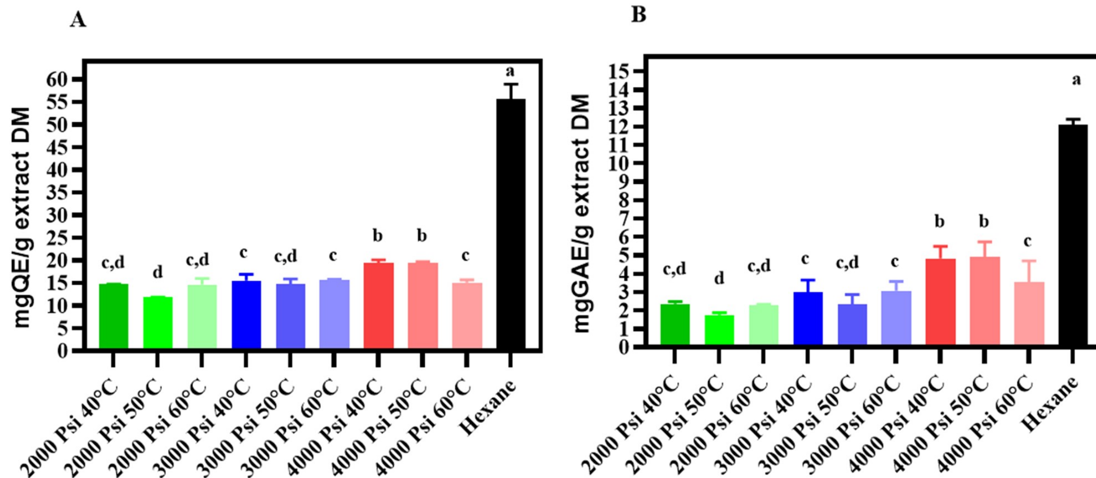


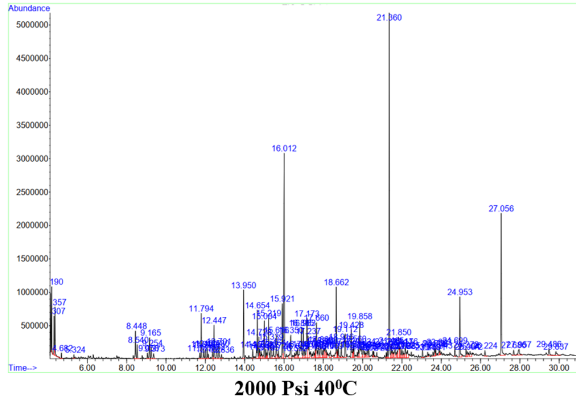
Distinct letters signify notable differences among the extraction treatments at $p < 0.05$.

Figure 2. The Total Flavonoid (A) Expressed as mg Quercetin Equivalents Per Gram of Extract (mgQE/g extract DM) and Polyphenol Content (B) Expressed as mg Gallic Acid Per gram of extract (mgGAE/g extract DM) of Hexane Extract and SCO_2 Extracts with Different Extraction Pressures (2000, 3000, and 4000 Psi) and Temperatures (40, 50, and 60°C)

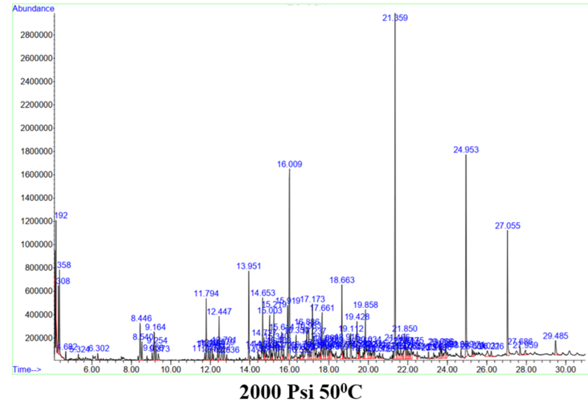
Table 1. The Top 10 most Abundant Predicted compounds in Each Hexane Extract and SCO_2 Extract with Different Pressures (2000, 3000, and 4000 Psi) and Temperatures (40, 50, and 60°C)

Groups	Compound Name	RT	2000 Psi 400°C	2000 Psi 500°C	2000 Psi 600°C	3000 Psi 400°C	3000 Psi 500°C	3000 Psi 600°C	4000 Psi 400°C	4000 Psi 500°C	4000 Psi 600°C	Hexane
Alkenes	2-Bromo dodecane	17.173	#	#	2.02^{9}	#	2.4^{7}	2.48^{6}	#	#	#	#
	Dodecane	18.662	#	2.16^{8}	2.11^{7}	#	2.61^{5}	2.11^{9}	#	2.04^{5}	2.40^{7}	#
	Heneicosane	14.654	2.02^{8}	#	#	2.02^{9}	#	2.42^{7}	2.08^{6}	1.90^{6}	2.23^{8}	3.12^{6}
	Heptacosane	19.427	#	#	#	#	1.59^{10}	#	2.13^{5}	#	#	#
	Heptadecane	15.219	#	#	#	#	#	#	#	2.09^{4}	#	3.10^{8}
	Hexadecane	15.219	1.97^{9}	#	#	#	#	#	#	#	#	#
	Neophytadiene	18.662	2.57^{4}	2.18^{7}	2.08^{8}	2.05^{7}	2.5^{6}	#	2.05^{7}	#	1.55^{10}	#
	Pentacosane	11.794	#	#	2.12^{6}	#	#	#	1.52^{8}	#	#	2.69^{9}
	Pentacosane	15.219	#	#	#	2.10^{6}	#	#	#	#	2.47^{5}	4.20^{3}
	Pentadecane	11.795	#	#	#	#	#	#	#	1.49^{9}	1.72^{9}	#
	Tridecane, 3-methyl-	17.66	1.96^{10}	2.11^{9}		#	2.03^{8}	#	#	#	#	3.37^{4}
	Tridecane, 5-propyl-	17.174	#	#	#	2.03^{8}	#	#	#	#	#	#
Alkohols	1H-Cycloprop[e] azulen-7-ol, decahydro-1,1,7	15.921	2.35^{7}	2.02^{10}	1.94^{10}	1.76^{10}	1.84^{9}	1.43^{10}	1.4^{9}	1.39^{10}	#	#
Fatty Acids	9,12,15-Octadeca trienoic acid, methyl ester, (Z,Z,Z)-	21.264	#	#	#	#	#	#	#	#	#	3.14^{5}
	Eicosonae	19.858	#	2.36^{6}	#	#	#	#	#	#	#	#
	Hexadecanoic acid, methyl ester	19.562	#	#	#	#	#	#	#	#	#	3.11^{7}
Phthalates	Bis(2-ethyl hexyl) phthalate	24.953	2.43^{5}	6.32^{3}	2.32^{5}	2.28^{4}	7.95^{2}	7.77^{2}	2.38^{4}	1.67^{8}	4.80^{2}	#
Terpenoids	Caryophyllene oxide	16.012	8.44^{2}	7.37^{2}	6.81^{2}	6.25^{2}	6.85^{3}	4.77^{3}	4.11^{3}	4.38^{3}	4.71^{3}	2.39^{10}
	Caryophyllene	13.95	2.39^{6}	2.68^{5}	2.45^{4}	2.26^{5}	#	3.96^{5}	1.34^{10}	1.68^{7}	2.44^{6}	#
	Germacrene D	14.729	#	#	#	#	#	2.17^{8}	#	#	#	#
	Phytol	21.36	11.17^{1}	9.47^{1}	9.34^{1}	9.26^{1}	9.72^{1}	8.79^{1}	10.38^{1}	9.09^{1}	8.10^{1}	8.66^{1}
	Squalene	27.05^{6}	5.59^{3}	4.19^{4}	5.18^{3}	4.84^{3}	4.21^{4}	4.48^{4}	5.54^{2}	4.752	3.97^{4}	4.44^{2}

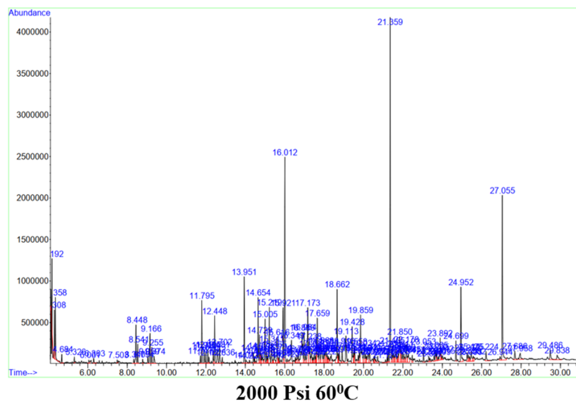
1-10 indicates the rank of the compounds for each extraction.
# indicates the compound was not ranked among the top 10 most abundant compounds for that particular extraction.



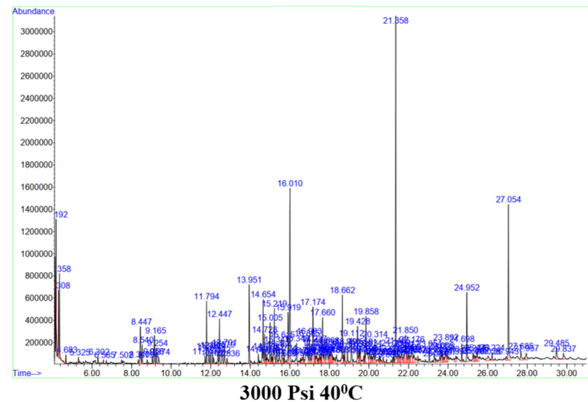
2000 Psi 40^{0}C



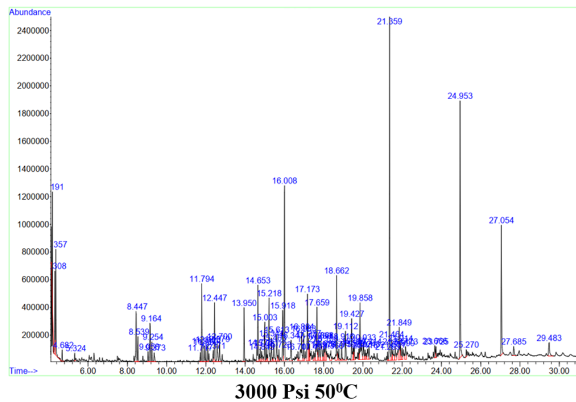
2000 Psi 50^{0}C



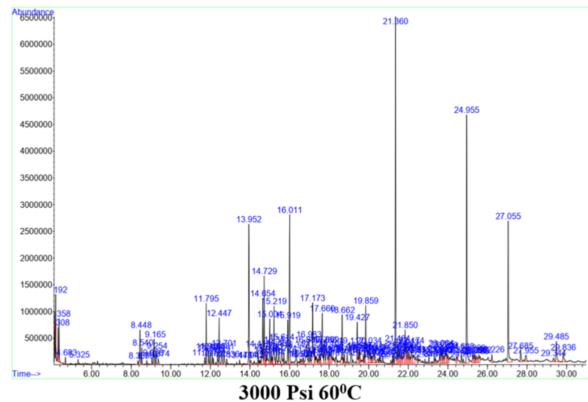
2000 Psi 60^{0}C



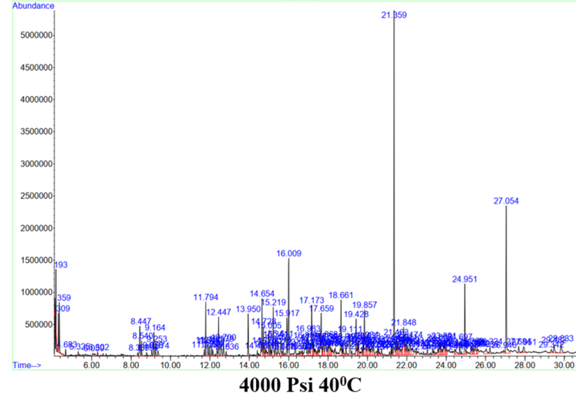
3000 Psi 40^{0}C



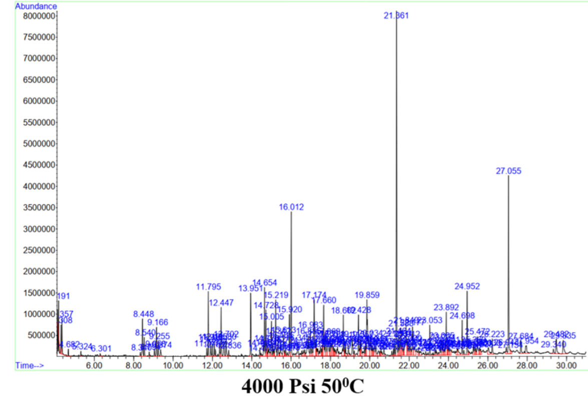
3000 Psi 50^{0}C



3000 Psi 60^{0}C



4000 Psi 40^{0}C



4000 Psi 50^{0}C

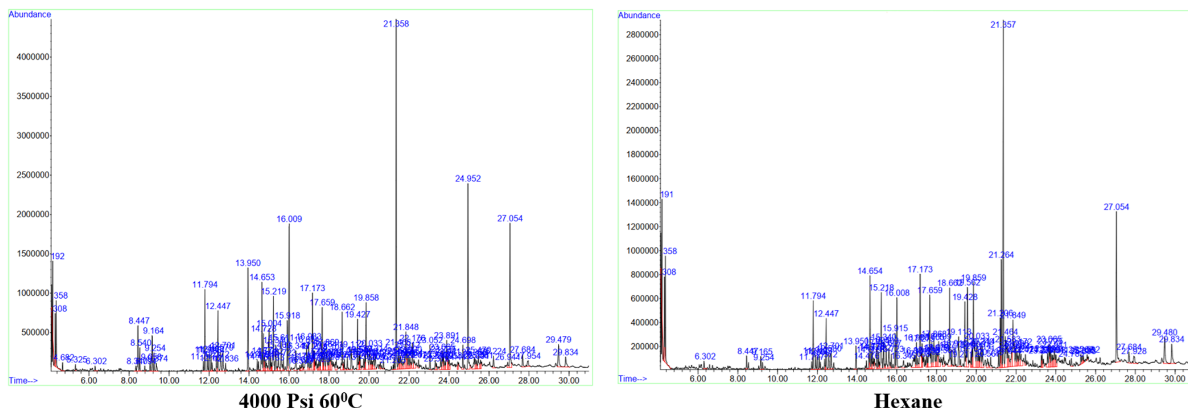
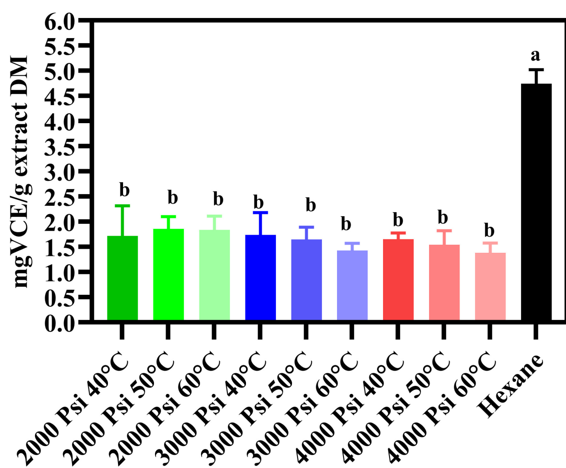


Figure 3. The Chromatograms of GCMS Analysis of Hexane Extract and SCO_2 Extracts with Different Pressures (2000, 3000, and 4000 Psi) and Temperatures (40, 50, and 60°C)



Distinct letters signify notable differences among the extraction treatments at $p < 0.05$.

Figure 4. The Antioxidant Activity of Hexane Extract and SCO_2 Extracts with Different Pressures (2000, 3000, and 4000 Psi) and Temperatures (40, 50, and 60°C), Expressed as mgVCE/g Extract DM

tion (Dall'Acqua et al., 2024). This result might be affected by the broader polarity of hexane used in this study (Yeddes et al., 2012), which yielded more polyphenols and flavonoid concentrations.

3.2 GCMS Analysis

The top 10 predicted compounds are shown in Table 1, and the chromatograms of the extracts are displayed in Figure 3. The highest predicted compound in all the extracts was phytol. Caryophyllene oxide and squalene were observed to have the second and third-highest percentages among most SCO_2 treatments, respectively. However, from the fourth to the ten highest compounds, there were differences among the groups. This result aligns with other studies (Capuzzo et al., 2013; Marques et al., 2022), showing that the terpenoid group yields the highest SPL when extracted with a nonpolar solvent such as hexane solvent. Terpenoid is the primary metabolite compound found in plants, contributing approximately 60% (around 80000 compounds) of metabolite in natural plants (Jiang et al., 2016). Phytol is a diterpene alcohol, which is the essential pigment for photosynthesis (Wang et al., 2010). Caryophyllene oxide is a bicyclic sesquiterpene derived from caryophyllene (Rosado et al., 2022). The third highest content was squalene, a group of unsaturated hydrocarbons with at least one double bond between carbon atoms (Tomovic et al., 2022). Thus, terpenoid offers various health benefits such as antioxidant, antibacterial, and anti-diabetic (Masyita et al., 2022).

This study of SCO_2 extracts resulted in a higher phytol, caryophyllene oxide, and squalene content than hexane extract. A possible explanation is that SCO_2 is more selective in extracting non-polar components due to the CO_2 solubility (Molino et al., 2019). A study found that the phytol compound from horseradish tree (*Moringa oleifera*) leaves was found to be higher in SCO_2 extract than hexane (Silva et al., 2022). Another study using bamboo (*Indocalamus latifolius*) leaves also observed that SCO_2 extraction yielded a higher content of squalene and phytol than conventional extraction using hexane (Chen et al., 2024). Moreover, a study using ten species of Salvia revealed that SCO_2 extraction was superior in yielding squalene and phytol than hexane extraction (Sulniute et al., 2017). In addition, several studies of terpenoid compounds also resulted in a similar finding, showing higher terpenoid compounds in the SCO_2 (Li et al., 2021; Molino et al., 2019; Zizovic et al., 2007). Thus, SCO_2 assists in better extraction of terpenoids compared with hexane, showing that this method could be a preferred method to obtain bioactive compounds.

3.3 Antioxidant Activity

This study applied DPPH as a free stable radical, which could be used to calculate free radical scavenging activity. Inhibition of DPPH at 2500 ppm showed that hexane had the high-

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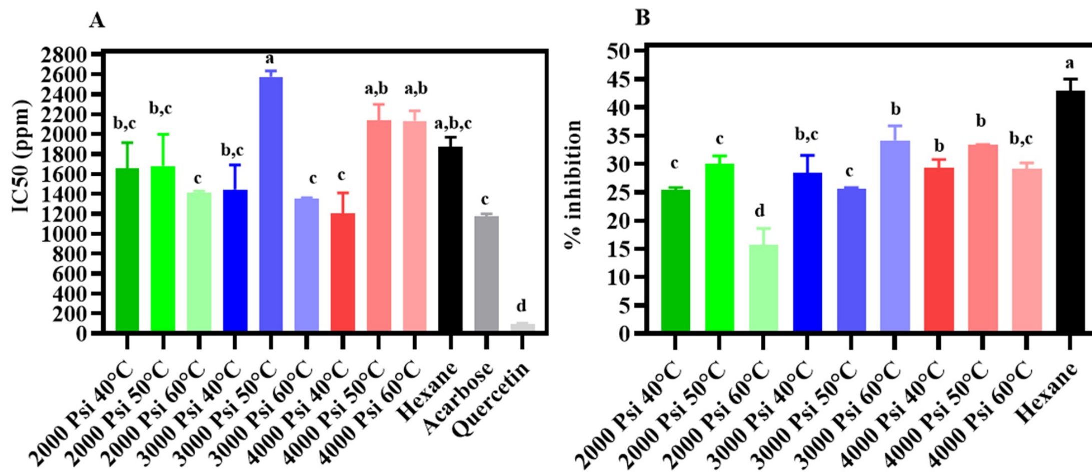

Distinct letters signify notable differences among the extraction treatments at $p < 0.05$.

Figure 5. The Anti-Diabetic Activity of Hexane Extract and SCO_2 Extracts with Different Pressures (2000, 3000, and 4000 Psi) and Temperatures (40, 50, and 60°C), Showing IC_{50} for Alpha-Glucosidase (A) and Percent Inhibition Against DPP4 at 2500 ppm (B)

est activity in scavenging DPPH compared with other groups ($p < 0.05$), resulting in around 60% inhibition. The SCO_2 performed groups were not significantly different ($p < 0.05$) (Figure 4). The total polyphenol and flavonoid concentration might influence the lower antioxidant capacity. A study using different solvents found that the antioxidant activity of SPL was higher in the ethanol 50% and acetone 50% extractions, contributed by high levels of polyphenols and flavonoids (Fu et al., 2016). This study observed higher total phenolic and flavonoid concentrations in hexane extract, which might contribute to its antioxidant activity, almost 3 times higher than all SCO_2 extracts. Phenolics and flavonoids can act as antioxidants because both compounds can donate electrons and neutralize free radicals since they have redox properties (Vladimir-Knezević et al., 2011). This action is contributed by the hydroxyl group (−OH), which inhibits lipid oxidation and stops the development of free radicals (Gulcin, 2020). Moreover, the antioxidant capacity of both compounds is shown beyond in vitro tests. In the biological system, they might affect antioxidant enzyme expression, resulting in increasing the body's defense mechanism against oxidative damage (Kumar et al., 2020).

In addition to phenolics and flavonoids, several terpenoids were found in SCO_2 extracts, known as antioxidant agents, such as phytol, caryophyllene, and squalene. Phytol is a diterpenoid alcohol with an antioxidant that can scavenge free radicals and inhibit oxidative stress (Mohammed et al., 2020). This potency is attributed to its stability in donating electrons to free radicals due to the presence of an allylic alcohol group in its structure (Araujo et al., 2019) that enhances its activity towards free radicals. which might help to prevent cellular damage associated with various diseases, such as cancer, and neurodegenerative disorders (Mohammed et al., 2020). In addition, studies also highlighted that phytol could upregulate the expression of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (Swamy et al., 2017), which also play a crucial role in cellular defense against oxidative stress. Another non-polar compound found is caryophyllene oxide, a bicyclic sesquiterpene. It has been reported that it can scavenge free radicals and reduce oxidative stress in various biological systems (Dowek et al., 2020; Jorge et al., 2017). Studies showed it can activate the nrf2 pathway, a gene encoding antioxidant enzymes, which enhances the cellular capacity of antioxidants and protects against oxidative stress (Adetayo et al., 2023; Dowek et al., 2020). Squalene, the third highest non-polar compound, possesses potent free radical scavenging (Sadiq et al., 2020). Its antioxidant ability is caused by multiple double bonds in its structure, allowing it to donate electrons to free radicals that can neutralize them and prevent cellular damage (Zhang et al., 2017). In biological systems, squalene is primarily attributed to its ability to penetrate the cell membranes, which might protect against lipid peroxidation and maintain the integrity of the membrane from oxidative stress (Xu et al., 2022). In addition, three terpenoids also have a role as anti-inflammatory agents (Jobaer et al., 2023; Mezouara et al., 2024). Therefore, all these compounds can contribute to diabetes management by exerting antioxidant and anti-inflammatory effects.

3.4 Anti-Diabetic Activity

The World Health Organization suggests using oral hypoglycemia drugs to maintain the blood glucose of diabetes mellitus patients (Kalra et al., 2020), including inhibitors of enzymes such as alpha-glucosidase and DPP4. In this study, the extracts of SPL were identified to act as an anti-diabetic agent by inhibiting alpha-glucosidase and DPP4. Alpha-glucosidase is an enzyme that catalyzes the maltose into glucose and galactose (Chiba, 1997). Quercetin, as a standard, exhibited the lowest activity. In addition, anti-glucosidase activity results also

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showed that 4000 Psi 400°C along with 3000 Psi 600°C, 3000 Psi 400°C and 600°C, 2000 Psi 600°C, hexane extraction, and acarbose, exhibited the lowest IC_{50} compared to other groups ($p < 0.05$) (Figure 5A). In contrast, 3000 Psi 500°C was observed as the highest IC_{50} value among the extraction groups ($p < 0.05$) (Figure 5A). Inhibiting this enzyme would be beneficial for lowering blood glucose levels due to delayed carbohydrate hydrolysis and metabolite production during the fermentation of colonic starch (Suzuki et al., 2009).

This study showed that the SCO_2 had an IC_{50} for anti-alpha glucosidase ranging from 1200 to 2600 ppm, which can be attributed to polyphenol and flavonoid values. In hexane extract, higher polyphenols and flavonoid concentration might have an influence compared with other SCO_2 extracts (Corkovic et al., 2022; Sohretoglu and Sari, 2020), which had lower polyphenol and flavonoid concentrations. A study of the polar component of SPL showed that chlorogenic acid and its isomers were responsible for the inhibition of alpha-glucosidase (Nguyen et al., 2021; Suárez et al., 2020). These compounds inhibit the active site of the alpha-glucosidase enzyme at amino acids of aspartate, glutamate, histidine, tyrosine, and serine (Limanto et al., 2019). In addition to both compounds, the activity of non-polar compounds such as phytol, caryophyllene oxide, and squalene has been highlighted to inhibit alpha-glucosidase. In the methanolic extract of Psidium guajava leaves, alpha-glucosidase inhibition was found to be attributed to the presence of phytol, caryophyllene, and squalene (Oo and R, 2019). An in-silico study revealed that phytol and caryophyllene oxide had docking values of -6.8 and -4.55 kcal/mol against alpha-glucosidase (Mahnashi et al., 2022; Nokhala et al., 2020). An in vitro study against alpha-glucosidase of green seaweed Halimeda tuna demonstrated a high inhibition value, with an IC_{50} around 0.05 mg/mL, likely attributed to the phytol content in the extract (Gazali et al., 2023). Moreover, squalene also acts as an anti-alpha glucosidase, with a docking score of -9.1 kcal/mol (Ko et al., 2002). In addition, in vitro analysis showed that the IC_{50} of squalene to inhibit alpha-glucosidase was around 1000 ppm (Morocho et al., 2018).

In addition to its anti-glucosidase activity, DPP4 was evaluated since DPP4 plays a role in lowering blood glucose. DPP4 is a type II transmembrane protein usually found in several organs' endothelium, which can cleave peptides from proteins at the N-terminal position, such as proline or alanine (Barnett, 2006). Due to this action, incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) could be hydrolyzed by DPP4, resulting in insulin secretion and glucagon suppression (Thornberry and Gallwitz, 2009), which helps maintain blood glucose levels. This study revealed that sitagliptin, as mentioned by the manufacturer, exhibited 50% inhibition of DPP4. In addition, this study showed that hexane was the inhibitor with the highest potency to inhibit DPP4. Next, several groups were put together as the second activity: 4000 Psi at 40°C, 50°C, 60°C, 3000 Psi at 40°C, 60°C, and 2000 Psi at 50°C. The lowest activity was observed at 2000 Psi at 60°C (Figure 5B).

Hexane extract contains higher polyphenol, a polar compound, than all SCO_2 extracts. Studies highlight that the majority of polar compounds in SPL are the derivatives of caffeoylquinic acids (CQA), known as DPP4 inhibitors (Osuntokun et al., 2020). However, limited studies have focused on studying the nonpolar extracts of SPL to inhibit DPP4. The nonpolar compounds such as phytol, caryophyllene oxide, and squalene found in both hexane and SCO_2 extracts are potential contributors to inhibit DPP4. In silico studies have shown that phytol binds with DPP4 with docking scores of -5.6 kcal/-mol, suggesting that it interacts with the critical residues of DPP4 (Bouchentouf and Talebi, 2018; Islam et al., 2018). To support the in silico finding, an in vitro study also found that phytol was demonstrated to inhibit DPP4 in a concentration-dependent activity (Islam et al., 2018). Caryophyllene oxide has also been investigated for anti-DPP4 activity, showing an inhibition value of -4.55 kcal/mol in a molecular docking study (Iheagwam et al., 2019). Squalene was also known as an anti-DPP4 agent. A study performed in silico on squalene found that the compound also interacted with the active site of DPP4 (Widyawati et al., 2023). The in vitro finding also found a similar result, showing that squalene had a moderate inhibitory effect on DPP4 (Widyawati et al., 2023). Moreover, an in vivo study also found that the DPP4 level in the diabetic control group was higher than those in the squalene-treated group, with levels of 61.26 ± 15.06 vs. 44.09 ± 5.29 ng/mL ng/mL, respectively (Widyawati et al., 2023).

4. CONCLUSIONS

SCO_2 extraction, an environmentally friendly method, effectively extracts SPL's non-polar content, along with some polyphenol and flavonoid concentrations. This method offers a high yield of non-polar bioactive compounds such as terpenoids. SCO_2 extraction also holds promise as a viable technique for obtaining anti-diabetic agents. However, the limitations of low yield, total polyphenol, and flavonoid concentrations hinder the overall potency of SCO_2 extracts. Future research should explore the broader health potencies of SPL's non-polar bioactive compounds. Additionally, optimizing the SCO_2 extraction process by incorporating co-solvents could increase the yield of polyphenol and flavonoid concentration, improving the overall efficacy of the extracts.

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