SYNTHESIS OF CHOLINE CHLORIDE-ACETIC ACID BASED DEEP EUTECTIC SOLVENT (DES) FOR THE EXTRACTION OF BIOACTIVE POLYSACCHARIDE FROM *GARCINIA ATROVIRIDIS* FRUIT

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by

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LIST OF SYMBOLS

α	Alpha
%	Percentage
°C	Degree Celsius
&	And
~	Approximately
<	Less than
>	More than
+	Plus
X	Times
=	Equal
±	Plus Minus

LIST OF ABBREVIATIONS

μg	Microgram
μL	Microlitre
μm	Micrometer
AA	Acetic acid
ABrC	Analytical Biochemistry Research Centre
ACE	Angiotensin converting enzyme
ACN	Acetonitrile
ANOVA	Analysis of variance
ARA	Arabinose
ACL	ATP-citrate lyase
ATR	Attenuated total reflectance
BBD	Box-behnken design
BSA	Bovine serum albumin
C=O	Carbon-oxygen double bond
CC	Choline chloride
C-H	Carbon-hydrogen single bond
CH_2	Methylene
CH_3	Methyl
cm	Centimetre
C-O	Carbon-oxygen single bond
COO^-	Carboxylate ion

CV	Coefficient of variance
DE	Degree of esterification
DES	Deep eutectic solvent
DSC	Differential scanning calorimetry
DPPH	2,2-diphenyl-1-picrylhydrazyl
e.g.	Exempli gratia (for example)
FC	Foaming capacity
FDA	Food and Drug Administration
FS	Foaming stability
FTIR	Fourier transform infrared
FUC	Fucose
g	Gram
GA	Gallic acid
GAP	*Garcinia atroviridis* polysaccharide
GAP-L	*Garcinia atroviridis* polysaccharide (with pancreatic lipase inhibition)
GAP-A	*Garcinia atroviridis* polysaccharide (with ACE-enzyme inhibition)
GAE	Gallic acid equivalent
GAL	Galactose
GALA	Galacturonic acid
GLU	Glucose
GLUC	Glucuronic acid
h	Hour

HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
i.e.	*Id est* (that is)
IC_{50}	Median inhibition concentration
kDa	Kilodalton
kg	Kilogram
m	Meter
M	Miscible
MAE	Microwave-assisted extraction
MAN	Mannose
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MW	Molecular weight
n	Number of sample
NaOH	Sodium hydroxide
NCD	Non-communicable disease
ND	Not detected
NHMS	National Health and Morbidity Survey
nm	Nanometre
NMR	Nuclear magnetic resonance
O-H	Hydroxyl

OHC	Oil holding capacity
Pa	Pascal
pH	Potential of hydrogen
PL	Pancreatic lipase
PMP	1-phenyl-3-methyl-5-pyrazolone
RG	Rhamnogalacturonan
RG-I	Rhamnogalacturonan-I
RHAM	Rhamnose
RIB	Ribose
Rpm	Revolutions per minute
RSM	Response surface methodology
s	Second
TPC	Total phenolic content
UAE	Ultrasonic-assisted extraction
USA	United States of America
UV	Ultraviolet
UV-Vis	Ultraviolet-vis
v/v	Volume over volume
W	Watt
w/v	Weight over volume
w/w	Weight over weight
WHO	World Health Organization
wt	Weight
X	Actual independent variable

x	Coded variable
XYL	Xylose
y	response

SINTESIS PELARUT EUTEKTIK MENDALAM (DES) YANG BERASASKAN KOLIN KLORIDA-ASID ASETIK UNTUK PENGEKSTRAKAN POLISAKARIDA BIOAKTIF DARIPADA BUAH *GARCINIA ATROVIRIDIS*

ABSTRAK

Buah *Garcinia atroviridis* dikenal pasti sebagai sumber potensi polisakarida bioaktif. Kajian ini meneroka keupayaan polisakarida ini (dikenali sebagai GAP) untuk menghalang lipase pankreas dan enzim penukar angiotensin (ACE), kedua-duanya penting dalam menguruskan obesiti dan hipertensi. Ekstraksi GAP dioptimumkan menggunakan proses berasaskan gelombang mikro yang melibatkan pelarut eutektik mendalam (DES). Kajian ini dijalankan untuk menyediakan pelarut eutektik mendalam (DES) daripada klorida kolin dan asid asetik dalam nisbah 1:2, 1:3, 1:4. Sifat fizikokimia DES yang dihasilkan telah dicirikan sepenuhnya. Interaksi komponen DES melalui ikatan hidrogen disahkan menggunakan spektroskopi inframerah-transformasi Fourier (FTIR) dan perubahan dalam nombor gelombang kumpulan fungsinya. Selain itu, DES ini didapati bersifat hidrofilik dan boleh larut dalam air dengan kepolaran tinggi serta serasi dengan banyak pelarut separa-polar. DES menunjukkan potensi yang sangat baik untuk aplikasi ekstraksi kerana pH rendah dan sifat polaranya. Sifat-sifat ini membolehkan pelarut berinteraksi secara efektif dan melarutkan polisakarida dengan menghidrolisiskannya daripada dinding sel. Oleh itu, DES adalah medium yang menjanjikan untuk mengekstrak GAP. GAP kemudian diekstrak dan keputusan mendapati bahawa hasil eksperimen GAP (24.33%) hampir sepadan dengan hasil yang diramalkan (24.88%), mengesahkan ketepatan model

ekstraksi tersebut. GAP didapati mempunyai berat molekul tinggi (28.15 ± 3.17 kDa) dan kelikatan (3.171 ± 1.23 Pa·s), bersama-sama dengan saiz bercabang yang signifikan (6.39 ± 0.030). Ciri-ciri ini mencadangkan bahawa GAP dapat membentuk struktur kompleks yang dapat menghalang aktiviti enzim dengan berkesan dengan menyekat enzim secara fizikal daripada mencapai substrat mereka, dengan itu memperlahankan fungsi mereka. Dalam ujian makmal, GAP menunjukkan kesan penghalang yang signifikan, mengurangkan aktiviti lipase pankreas sebanyak 79.41% dan aktiviti ACE sebanyak 81.23%. Keputusan ini menunjukkan bahawa GAP mempunyai potensi kuat untuk digunakan dalam membangunkan rawatan anti-obesiti dan anti-hipertensi. Pengoptimuman selanjutnya terhadap bioaktiviti GAP menghasilkan keputusan yang lebih baik, dengan GAP yang dioptimumkan menghalang lipase pankreas sebanyak 87.93% dan ACE sebanyak 95.91%. Nilai eksperimen sepadan dengan nilai yang diramalkan (86.57% untuk penghalang lipase pankreas dan 95.64% untuk penghalang ACE), mengesahkan lagi model tersebut. Oleh itu, GAP menunjukkan potensi yang besar sebagai sumber untuk membangunkan agen efektif dalam menguruskan obesiti dan hipertensi. DES yang dihasilkan juga menunjukkan sifat polar, yang membolehkannya berinteraksi dengan dan melarutkan pelbagai sebatian polar, termasuk polisakarida dan molekul bioaktif lain.

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ABSTRACT

The fruit of *Garcinia atroviridis* was identified as a potential source of bioactive polysaccharides. This study explored the ability of these polysaccharides (referred to as GAP) to inhibit pancreatic lipase and angiotensin-converting enzyme (ACE), both of which are important in managing obesity and hypertension. The extraction of GAP was optimized using a microwave-assisted process involving deep eutectic solvents (DES). The study was promoted to prepare deep eutectic solvents (DES) from choline chloride and acetic acid in 1:2, 1:3, 1:4. The physicochemical properties of the resulting DES were fully characterized. The interaction of the DES components by hydrogen bonds was confirmed using Fourier-transform infrared spectroscopy (FTIR) and changes in wavenumbers of their functional groups. Moreover, these DES were shown to be hydrophilic and could dissolve in water with high polarity while having compatibility with many of semi-polar solvents. the DES demonstrates excellent potential for extraction applications due to its low pH and polar nature. Therefore, DES is a promising medium for extracting GAP. The yield of GAP was then extracted and the result concluded that the experimental yield of GAP (24.33%) closely matched the predicted yield (24.88%), confirming the accuracy of the extraction model. GAP was found to have a high molecular weight (28.15 ± 3.17 kDa) and viscosity (3.171 ± 1.23 Pa·s), along with a significant branching size (6.39

± 0.030). These characteristics suggest that GAP can form complex structures that effectively inhibit enzyme activity by physically blocking the enzymes from accessing their substrates, thereby slowing down their function. In laboratory tests, GAP demonstrated significant inhibitory effects, reducing pancreatic lipase activity by 79.41% and ACE activity by 81.23%. These results indicate that GAP has strong potential for use in developing anti-obesity and antihypertensive treatments. Further optimization of GAP's bioactivity led to even better results, with optimized GAP inhibiting pancreatic lipase (GAP-L) by 87.93% and GAP that inhibits ACE (GAP-A) by 95.91%. The experimental values closely aligned with the predicted values (86.57% for pancreatic lipase inhibition and 95.64% for ACE inhibition), further validating the model. Therefore, GAP shows great promise as a potential source for developing effective agents to manage obesity and hypertension. The suitability of the DES produced as well shown to have a polar nature, making it capable of interacting with and dissolving a wide range of polar compounds, including polysaccharides and other bioactive molecules.

CHAPTER 1

INTRODUCTION

This chapter delves into recent research and categorizing it into four distinct sections. Section 1.1 provides an overview of the current research investigation. Section 1.2 delineates the problem statement that necessitates attention in this study, along with its importance. Section 1.3 underscores the significance of this study in light of the limitations inherent in existing research. Finally, Section 1.4 details the study's objectives individually, aiming to address the identified problem statement.

1.1 Background of the research

Garcinia atroviridis, commonly known as "asam gelugor" in Malaysia, is a native plant and widely distributed in Thailand, Myanmar, Peninsular Malaysia, and India. The fruits of asam gelugor are sliced into thin pieces and sun-dried are called "asam keping" and commercially available in the region. These slices are popularly used as a spice in curries, sour relishes, and fish dressings, imparting acidity to cooked dishes. In Southeast Asian traditional practices, *G. atroviridis* is utilized for postpartum medication, as well as for treating cough, throat irritation, earache, dandruff, and pregnancy-related stomach discomfort. Presently, extracts and bioactive compounds derived from *G. atroviridis* have shown a wide range of biological activities, including antioxidant, antimicrobial, anticancer, anti-inflammatory, antihyperlipidemic, and anti-diabetic effects (Shahid et al., 2022a). These reported therapeutic properties have attracted the attention of numerous research groups over the past decade. The presence of xanthone compounds further contributes to the therapeutic effects of this plant extract. Xanthones are three-membered ring compounds with diverse biological profiles, exhibiting activities such as antihypertensive, antioxidative, antithrombotic, and anticancer properties (Shahid et al., 2022b). It has been revealed that *G. atroviridis* contains (−)-hydroxycitric acid (HCA) and flavonoids, both of which exhibit notable hypolipidemic effects. These compounds have shown the ability to reduce lipogenesis and enhance glycogen formation, thus contributing to weight reduction. In mammals, HCA acts as a metabolic regulator for obesity and lipid disorders (Chuah et al., 2013a). Notably, HCA, the primary acid present in *G. atroviridis* fruits, has been identified as a competitive inhibitor of ATP citrate lyase (ACL) (Chuah et al., 2013a). Hence, this research holds significant importance in exploring the therapeutic potential of bioactive compounds present in *G. atroviridis* as potential treatments for metabolic syndromes such as obesity and hypertension, especially polysaccharides. It should be noted that *G. atroviridis* contains polysaccharide and yet, the extraction of polysaccharide and its characteristics have not been explored.

Polysaccharides are ubiquitous in nature, being present in various living organisms such as seeds, stems, and leaves of plants, as well as in animal body fluids, bacterial cell walls, and fungal extracellular fluids (Khan et al., 2022). They exhibit diverse structures, ranging from linear to extensively branched configurations. Examples include storage polysaccharides like starch and glycogen, alongside structural polysaccharides such as cellulose and chitin (Benalaya et al., 2024). Polysaccharides can be categorized into two main types: hetero-polysaccharides and homo-polysaccharides. Homo-polysaccharides organize monosaccharides in chains of a single type, while hetero-polysaccharides are composed of a range of two or more types of monosaccharides. Homo-polymers of polysaccharides typically have a branched or non-branched structure, as they are made up of the same monosaccharide units. (Qiu et al., 2022a). In both types of polysaccharides, monosaccharides can form linear chains or complex branched structures. It's noteworthy that for a polysaccharide to be considered acidic, it must contain carboxyl, sulfuric or phosphate groups (Ren et al., 2019). Furthermore, in traditional medicine, plants have long been utilized in treating a wide array of diseases and ailments. Modern pharmacological research has revealed that plant-based remedies includes primary constituent that typically consists of alkaloids, terpenoids, flavonoids, saponins and high-molecular weight tannins, proteins and most notably, polysaccharides (Boy et al., 2018). Many of these constituents, polysaccharides have emerged as the significant active ingredients that is responsible in various pharmacological effects that includes, anti-oxidant, anti-hyperlipidemic, anti-diabetic, antiviral, immunostimulatory and anti-hypertensive activities (Ge et al., 2022; Ganesan & Xu, 2019; Xie et al., 2021). Polysaccharides that are found in plant sources are believed to exert effects in their natural state by stimulating the human immune system, scavenging free radicals, inhibits viral replications and prevents oxidation of lipid.

In order to extract bioactive components, a high efficiency solvent is required. Deep eutectic solvents (DESs) represent a novel category of solvents formed by combining two or more compounds, including hydrogen bond donors such as carboxylic acids, alcohols, and amines, with quaternary ammonium salts. DESs exhibit a remarkable reduction in melting point compared to their individual components. Conventional solvents and its derivatives, which are volatile organic compounds, are extensively employed as solvents, but they pose significant risks to the environment and public health (Svigelj et al., 2021).Thus, the use of traditional organic solvents is associated with considerable hazards, prompting the development of environmentally friendly alternatives (Ullah et al., 2023). Green solvents, characterized by their rapid biodegradability, recyclability, low volatility, and reduced flammability, are gaining popularity. They offer a wide range of applications and are considered cost-effective, safe, and user-friendly. Moreover, DESs are highly recommended for use due to their favorable economic and environmental characteristics. They are cost-effective, biodegradable, and employ non -toxic quaternary ammonium salts (Chemat et al., 2019). Additionally, DESs provide a wide range of solubility that can be utilized to generate diverse solvents, depending on their intended applications (Silva et al., 2019). The fact that DES constituents interact via intermolecular forces, rather than covalent or ionic interactions, makes them appealing substitutes for ionic liquids or conventional solvents (Bashir et al., 2023). Solvents play a crucial role in many chemical processes by extracting, separating, and purifying chemicals from natural sources or their mixtures. They are also instrumental in dissolving reagents and maintaining essential molecular connections (Zhang et al., 2018). Moreover, the combination of DESs with innovative extraction technologies like microwave-assisted extraction (MAE) has led to enhanced production of bioactive components from plant sources. The synergy between MAE and DESs enables the effective disruption of cell wall structures, facilitating the release of bioactive substances from within the cells (Bian et al., 2022). This advanced approach offers several advantages over conventional extraction methods utilizing organic solvents, including higher extraction efficiency, reduced processing time, and lower solvent consumption. Hence, the primary objective of this study is to develop a sustainable and effective approach for extracting polysaccharides from plants, aiming to establish an environmentally friendly extraction method. This endeavor will involve optimizing the extraction process while concurrently investigating the chemical properties and bioactivities of the extracted polysaccharides.

Projections indicate a concerning surge in obesity rates within Malaysia over the upcoming years. According to a recent report by the World Obesity Federation (WOF), it is anticipated that by 2035, approximately 41 percent of the country's adults will be classified as obese (Chong et al., 2023). This trajectory raises significant concerns about the impact on Malaysia's healthcare system, economy, and the overall well-being of the population. Currently, according to the National Health and Morbidity Survey (NHMS, 2019), the prevalence of adult obesity stands at 19.7 percent. Consequently, it is concerning to observe that this percentage is forecasted to more than double over approximately 12 years. Obesity, is characterized by a body mass index (BMI) of 30 or higher, while a BMI of 25 or higher is classified as overweight, as defined by the World Health Organization (WHO). Recent data from the World Obesity Atlas (WOA, 2023) suggests that Malaysia is expected to experience a significant annual increase in both adult and child obesity rates in the forthcoming years. The report predicts an annual rise of 4.7 percent in adult obesity rates from 2020 to 2035, with child obesity rates anticipated to increase by 5.3 percent per year during the same period.

Furthermore, according to the most recent Malaysian NHMS, it was revealed that 30 percent of Malaysians aged 18 and above are affected by hypertension (Ismail et al., 2023). Multiple studies conducted across different population groups have consistently shown that optimal blood pressure control is achieved in only around onethird of individuals with hypertension (Chia et al., 2022a). Several factors contribute to this situation, including under-diagnosis, clinician inaction, and the progressive nature of the disease. However, the most significant factor is likely non-adherence by patients, as hypertension is typically asymptomatic in its early stages and requires lifelong treatment. The prevalence of resistant hypertension, a condition in which blood pressure remains uncontrolled despite treatment, has been consistently reported to be between 10 and 20 percent among hypertensive individuals (Chia et al., 2022b). In Malaysia, a cross-sectional survey conducted in a single outpatient department found that 8.8 percent of 1,217 patients had resistant hypertension. The escalating prevalence of Non-Communicable Diseases (NCDs) can be attributed to a range of factors such as urbanization, an aging population, sedentary lifestyles, and increasing obesity rates (Bista et al., 2021). The concerning impact of NCDs on public health necessitates the exploration of urgent alternative treatment options, with traditional medicines often derived from common food sources such as spices, herbs, fruits, and vegetables. Hence, the novelty of this research lies in investigating the potential applications of microwave - DES assisted extraction for extracting bioactive polysaccharides from *G. atroviridis* and examining their potential bioactivities, including anti-obesity, and anti-hypertensive properties. These findings hold promise for potential pharmacological applications.

1.2 Problem statement

Studies have uncovered that *G. atroviridis* harbors (−)-hydroxycitric acid (HCA) and flavonoids, which demonstrate significant hypolipidemic effects. Additionally, the presence of xanthone compounds in *G. atroviridis* enhances the therapeutic properties of this plant extract, showcasing activities such as antihypertensive effects (Lim et al., 2020). However, the study of *G. atroviridis* polysaccharides, which may play a crucial role in these activities, has not been extensively documented. Polysaccharides are often tightly bound within the plant matrix, making their extraction challenging. Nevertheless, the extraction of these compounds from such sources often yields them in low concentrations, necessitating significant advancements in extraction methods and mediums to augment efficiency and maximize the yield of bioactive compounds. The use of conventional solvents in the extraction of polysaccharides can pose several hazards. Many conventional solvents used in extraction processes, such as formic acid, hydrochloric acid and sulphuric acid, that can contribute to environmental pollution. Some conventional solvents, such as acetone, and ether, are highly flammable and can present fire hazards if not handled properly, it is also often toxic and can pose health risks to those involved in the extraction process. Furthermore, the extraction method and parameters, such as duration and temperature, were found to significantly impact the yield of bioactive compounds, as demonstrated by Quitério et al. (2022). Acidic solvents have been recognized as optimal extraction agents for polysaccharides, owing to their ability to dissolve insoluble polysaccharides tightly bound within the plant matrix, consequently resulting in higher yields of bioactive compounds. However, the utilization of a potent acid as an extraction medium also presents a notable drawback, as it tends to yield low quantities of polysaccharides due to the susceptibility of polysaccharide structures to hydrolysis (Bitwell et al., 2023). Other than that, different extraction techniques can influence the structure of polysaccharides in various ways. Conventional extraction techniques suffer from several drawbacks. Firstly, they often require lengthy extraction times, contributing to increased labor and energy costs. Secondly, these methods lack selectivity, leading to the extraction of a broad spectrum of compounds and impurities (Tzanova et al., 2020). Thirdly, they necessitate large volumes of solvents, leading to significant waste generation and environmental impact. Additionally, some techniques involve high energy consumption due to heating or mechanical agitation (Cravotto et al., 2023). Lastly, heat-sensitive compounds may degrade during prolonged extraction, compromising the bioactivity and chemical integrity of the final extract (Usman et al., 2023). Hence, these limitations highlight the need for alternative extraction methods that offer improved efficiency and sustainability.

Obesity and hypertension represent significant public health challenges worldwide, with alarming statistics highlighting their pervasive prevalence and detrimental impact on global health outcomes. According to recent epidemiological data, obesity rates have surged dramatically over the past few decades, with a substantial portion of the global population now classified as overweight or obese. Concurrently, hypertension, characterized by persistently elevated blood pressure levels, has emerged as a leading risk factor for cardiovascular diseases, stroke, and other adverse health outcomes, affecting millions of individuals across diverse demographics and geographical regions (Shariq & McKenzie, 2020). Moreover, these conditions often coexist within affected individuals, exacerbating the risk of cardiovascular complications and mortality. This worrisome trend raises significant apprehensions about its repercussions on Malaysia's healthcare system, economy, and the overall well-being of its population. Typically, synthetic medications like orlistat and angiotensin II receptor blockers are employed for managing obesity and hypertension, respectively. However, the utilization of these pharmaceutical drugs often leads to adverse effects such as kidney dysfunction, heart complications, and gastrointestinal issues (Firus Khan et al., 2022). Furthermore, access to synthetic anti-obesity and anti-hypertensive medications are both costly and limited in certain developing nations. The adverse effects of synthetic drugs on public health underscore the need to urgently explore alternative treatment options. Traditional medicines, often derived from commonly available food sources such as spices, herbs, fruits, and vegetables, offer a promising avenue for investigation. Utilizing active constituents from plants as a potential treatment can hold great promise for addressing various diseases. Importantly, these bioactive polysaccharides derived from plants are biologically safe in comparison to the current synthetic drugs. Hence, the insufficient investigation of the bioactive properties of *G. atroviridis* polysaccharides, in particular their impact on obesity and hypertension, represents a substantial research gap in the field. Although certain parts of *G. atroviridis* have been investigated, nothing is known about the particular polysaccharides and how they work to treat certain medical conditions. By examining the potential of *G. atroviridis* polysaccharides to act as natural inhibitors of important enzymes linked to obesity and hypertension, this study seeks to close that gap and provide a novel approach for public health interventions.

1.3 Significance of study

Choline chloride is approved by the U.S. Food and Drug Administration (FDA) as a food additive. It is commonly used in various food and dietary supplements. Choline is an essential nutrient that plays a crucial role in various physiological functions, including brain health, liver function, and lipid metabolism. Choline chloride is generally recognized as safe (GRAS) when used in accordance with good manufacturing practices and in amounts not exceeding the established limits

(Kansakar et al., 2023). Furthermore, acetic acid is a key component in the production of vinegar, which is used as a flavoring agent, preservative, and condiment in food preparation. It is also used in the production of various food additives and flavor enhancers. Acetic acid is widely used as a chemical reagent in the synthesis of various organic compounds, including acetate esters, acetic anhydride, and vinyl acetate, which are used in the production of plastics, solvents, pharmaceuticals, and dyes (Ho et al., 2017). The production of DES using choline chloride and acetic acid could be use as extraction medium for extraction of polysaccharides. These DES exhibit remarkable properties that make them highly suitable for polysaccharide extraction processes. Firstly, their ability to form hydrogen bonds facilitates the dissolution of polysaccharides, allowing for efficient extraction of these complex molecules from biomass sources. Moreover, DES are typically composed of inexpensive and readily available components, making them cost-effective alternatives to traditional solvents (Sun et al., 2022). The cost effectiveness is primarily due to the availability of their components, such as choline chloride, urea, or glycerol. These materials are cheaper than those used in traditional organic solvents and ionic liquids. DESs are also easy to prepare, requiring only simple mixing and heating, which reduces production costs compared to the more complex synthesis and purification processes needed for ionic liquids. Additionally, DES are considered to be environmentally friendly, as they are derived from renewable resources and are biodegradable, minimizing environmental impact compared to conventional organic solvents. Furthermore, DES can be tailored by adjusting the molar ratio of their constituents, allowing for versatility and customization to optimize extraction efficiency for specific polysaccharide types or sources. Additionally, DES have been found to exhibit low toxicity and pose minimal health risks to operators, ensuring workplace safety during extraction procedures

(Contreras-Gámez et al., 2023). Overall, the use of DES composed of choline chloride and acetic acid as extraction media for polysaccharides presents a promising avenue for sustainable, efficient, and environmentally benign polysaccharide extraction processes with wide-ranging applications across various industries.

Microwave-assisted extraction (MAE) of polysaccharides offers a plethora of advantages over conventional extraction methods, making it an increasingly popular and preferred approach in polysaccharide extraction processes. Firstly, MAE significantly reduces extraction time compared to traditional methods, often achieving higher yields of polysaccharides in a fraction of the time. This accelerated extraction process is attributed to the rapid and uniform heating of the solvent and sample matrix induced by microwaves, which enhances the diffusion of solvent into the biomass and facilitates the release of polysaccharides. Moreover, MAE typically requires lower solvent volumes, resulting in reduced solvent consumption and waste generation, thus contributing to environmental sustainability and cost-effectiveness (Le et al., 2019). Additionally, MAE offers improved extraction efficiency and selectivity, enabling the extraction of a wider range of polysaccharides with varying molecular weights and structures. The controlled heating provided by microwaves also helps to preserve the integrity and bioactivity of extracted polysaccharides, minimizing degradation and ensuring the retention of their functional properties (Yue et al., 2022). Importantly, MAE can be easily optimized by adjusting parameters such as microwave power, extraction time, and solvent composition to tailor extraction conditions to specific polysaccharide sources and target compounds (Hamid Nour et al., 2021). Overall, the advantages of MAE, including rapid extraction kinetics, improved efficiency, reduced solvent consumption, enhanced selectivity, and preservation of polysaccharide quality, underscore its potential as a versatile and efficient technique for polysaccharide extraction across various applications in food, pharmaceutical, and nutraceutical industries, as well as in research and development endeavors.

Renowned for its culinary and medicinal uses, *G. atroviridis* offers a plethora of advantages derived from its rich composition of bioactive compounds. One notable benefit is its potential as a weight management aid, attributed to the presence of hydroxycitric acid (HCA) in its fruit rinds. HCA has been shown to inhibit citrate lyase, an enzyme involved in the synthesis of fatty acids, thereby potentially reducing fat accumulation in the body (Lim et al., 2020). Moreover, *G. atroviridis* exhibits significant antioxidant properties due to its high content of polyphenolic compounds, such as flavonoids and tannins, which help combat oxidative stress and inflammation, consequently promoting overall health and well-being. Additionally, research suggests that *G. atroviridis* possesses antimicrobial activity against various pathogens, including bacteria and fungi, owing to its secondary metabolites like xanthones and benzophenones (Rudrapal et al., 2022). Furthermore, this versatile plant has been explored for its potential anticancer properties, with studies indicating its ability to inhibit cancer cell proliferation and induce apoptosis (Nik Mohamed Kamal et al., 2021a). Moreover, *G. atroviridis* is rich in vitamins, minerals, and dietary fibers, making it beneficial for digestive health and immune function. Nonetheless, natural polysaccharide has its benefits such as low in toxicity, good stability biodegradable as well as hydrophilic (Tudu and Samantha., 2023). However, research concerning the anti-obesity and anti-hypertensive properties of *G. atroviridis* polysaccharide remains scarce. Therefore, further investigation into the efficacy of *G. atroviridis* polysaccharide as a natural anti-obesity and anti-hypertensive agent is warranted to enhance our understanding of its biological and physicochemical characteristics.

1.4 Objective

The main aims of this research were twofold: to develop a novel DES and explore its potential utility as an extraction medium for polysaccharides derived from *G. atroviridis*, a plant known for its anti-obesity and anti-hypertensive properties. The entire research endeavor was structured around three specific objectives, delineated as follows:

i. To synthesize and characterize the DES that consists of the mixture of choline chloride (CC) and acetic acid (AA) at different molar ratios followed by analyzing their physical and chemical properties (i.e., functional group, density, pH and miscibility) as a potential extraction media for the extraction of polysaccharide.

ii. To explore the use of DES in extracting bioactive polysaccharides from G. atroviridis with microwave assistance, optimizing key parameters like microwave power, extraction time, and DES concentration. The study also analyzed the extracted polysaccharide's properties (pH, carbohydrate and protein content, structure) and evaluated its potential as an anti-obesity and anti-hypertension agent by testing its inhibition of pancreatic lipase and ACE.

iii. To optimize the anti-obesity (pancreatic lipase inhibition) and anti-hypertension (ACE inhibition) activities of *G. atroviridis* polysaccharides. The study also examined the physicochemical (pH, carbohydrate, and protein content) and structural (FTIR and sugar analysis) properties to understand their role in the observed bioactivities related to anti-obesity and anti-hypertensive effects.

CHAPTER 2

LITERATURE REVIEW

This chapter presents an extensive literature review pertaining to the subject matter of this study, delineated into several sections. Section 2.1 explores the formation and diverse applications of deep eutectic solvents (DES). Section 2.2 delves into the physicochemical characteristics of DES, encompassing aspects such as pH, viscosity, density, and solubility. In Section 2.3 provides an overview of *G. atroviridis*, a plant with traditional medicinal uses, and recent endeavors in extracting bioactive compounds from it. Moving forward, Section 2.4 scrutinizes the extraction of polysaccharides, shedding light on various parameters that influence extraction efficiency. Lastly, Section 2.5 expounds upon the properties of polysaccharides, elucidating their biological activities of polysaccharides sourced from plants.

2.1 Deep eutectic solvent (DES)

The scientific fascination with DESs is expanding rapidly, driven by their proven efficacy in various applications such as solvents, self-assembly media, cryopreservation, lubrication, catalysis, and more. Despite the considerable interest in DESs, there is still a notable lack of a fundamental understanding, even for the most commonly used DESs, and a limited number of systematic characterization studies have been conducted. At its most fundamental level, DESs are characterized as a combination of a hydrogen bond acceptor and hydrogen bond donor, exhibiting a melting point significantly lower than that of their individual components at a specific ratio (Figure 2.1). In the past, DESs have been mistakenly classified as a subset of ionic liquids. However, this is inaccurate, and instead, DESs should be regarded as a distinct category of neoteric solvents that share certain similarities with ionic liquids. The unique feature of DESs lies in their high tunability, allowing for the mix-and-match of various hydrogen bond donors and acceptors. Notably, DESs can be crafted from food-grade ingredients, rendering them inherently environmentally friendly, non-toxic, and cost-effective (Zhao et al., 2021). It's crucial to acknowledge that, akin to ionic liquids, the diversity within the realm of DESs prevents sweeping generalizations about properties such as volatility; rather, these must be empirically determined for each specific subset of DESs. The synthesis of DESs is considerably simpler compared to that of ionic liquids, involving straightforward mixing with some heating, typically not exceeding 80 °C, and achieving 100% atom efficiency.

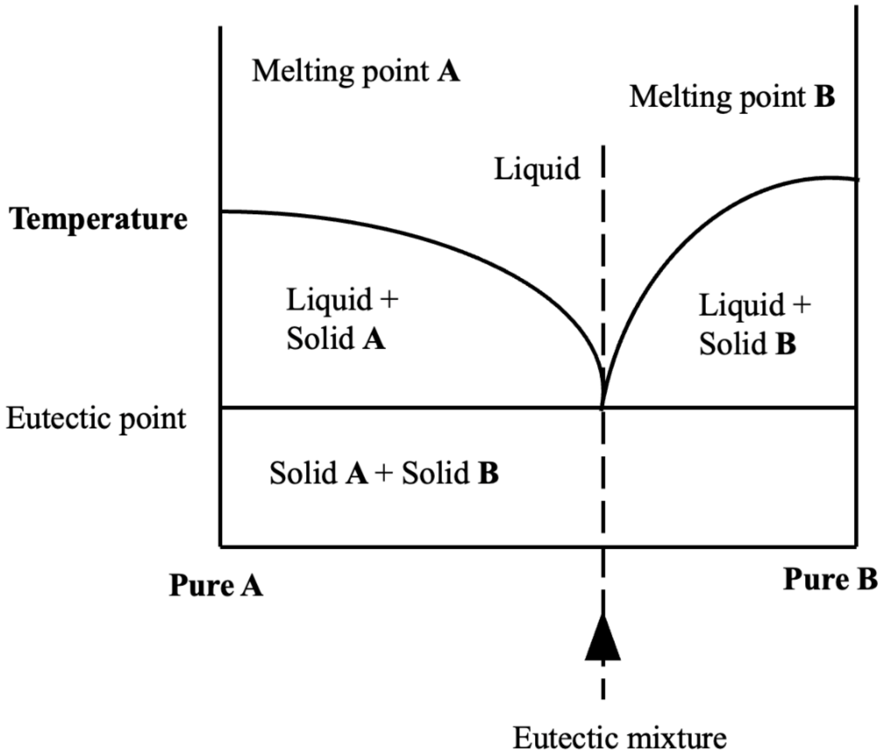

Figure 2.1 Phase diagram indicate the formation of eutectic mixture

Preparing DESs is typically a straightforward process, often guided by personal preferences, available equipment, and the aim to minimize water content. The most common method involves heating and stirring the DES constituents together under an inert atmosphere until they form a homogeneous liquid. This approach eliminates the need for additional solvents or traditional reaction steps, thus bypassing the necessity for purification procedures. Such simplicity enhances their potential as economically feasible alternatives to conventional organic solvents and ionic liquids. Alternative methods for DES preparation include vacuum evaporation, grinding, and freeze-drying. In the evaporative method, the components are dissolved in water, and the majority of water is subsequently evaporated under vacuum (Hammond & Mudring, 2022). Subsequently, the final mixture is stored in a desiccator with silica gel until it reaches a constant weight. In the grinding method, the two solid components are added to a mortar and ground until a clear, homogeneous liquid forms, typically under a nitrogen atmosphere and/or within a glovebox. In the freeze-drying method, both the hydrogen bond donor and hydrogen bond acceptor are dissolved in approximately 5 wt% of water. These solutions are mixed, frozen, and then freeze-dried to produce a clear, homogeneous liquid. Given the well-recognized advantages of DESs, they find applications across various fields, including synthesis of nano-particles, synthesis of nanosheets, design of cryoprotective agents, enzyme catalyzed hydrolysis, food analysis and extraction (Balaji & Ilangeswaran, 2022; Krishnan & Chipatecua Godoy, 2020; Zhang et al., 2020; Arnodo et al., 2023, Kostag et al., 2018; Kaumbekova & Shah, 2023). Numerous examples of DES and their applications are outlined in Table 2.1, as discovered by other researchers. However, despite the extensive use of DESs, the synthesis of DES from choline chloride as a hydrogen bond acceptor and acetic acid as a hydrogen bond donor remains largely unexplored.

Therefore, it is proposed that further investigation into the synthesis of DES from these combinations, along with their potential applications, is warranted.

Table 2.1 Example of combination between HBA and HBD in DES and their applications

Hydrogen bond acceptor	Hydrogen bond donor	Molar ratio	Applications	References
Choline bicarbonate	Geranic acid	1:2	Antimicrobial activity of DES	Ko et al. (2021)
Choline chloride	Urea	1:2	Synthesis of nano-particles	Balaji & Ilangeswaran (2022)
Choline chloride	Urea	1:2	Synthesis of nano-sheets	Krishnan & Chipatecua Godoy (2020)
Choline chloride	Urea	1:2	Controlled DNA folding	Sanchez-Fernandez et al. (2022)
Trehalose	Glycerol	1:1	Design of cryoprotective agents	Zhang et al. (2020)
Choline chloride	Formic acid	1:2	Biomass pretreatment and hydrolysis	Ling et al. (2021)
Choline chloride	Imidazole	Various	Solubility of cellulose	Kostag et al. (2018)
Choline chloride	Urea	1:2	Egg white lysozyme stability	Kaumbekova & Shah (2023)
Choline chloride	Ethane-diol	1:2	Enzyme-catalyzed hydrolysis	Arnodo et al. (2023)
Silver triflate	Acetamide	1:4	Synthesis of nano-crystals	Adhikari et al. (2019)

Choline chloride	Proline	1:2	Biomass pretreatment and hydrolysis	Zhang, et al. (2022)
Choline chloride	Boric acid	5:2	Biomass pretreatment and hydrolysis	Kalhor & Ghandi (2019)
Choline chloride	Glycerol	1:3	Solar cell testing	Boldrini et al. (2022)

Due to its numerous advantages, DESs have found extensive applications in separation, absorption, purification, and extraction processes. The non-toxic, biodegradable nature, coupled with its ease of production, has led to growing interest in utilizing DES as an extraction medium. Recent research indicates that DES has been increasingly employed in extracting bioactive constituents, yielding higher extraction yields compared to conventional solvents. This can be explained from a study by Coppola et al., (2021) conducted an extensive study employing DES for extracting proteins from waste streams generated during sardine processing. The UV-visible spectroscopy method was used to determine the total protein content, by measuring absorbance at 750 nm parameter, and the concentration of protein was assessed against the Bovine serum albumin (BSA) as standard. DES resulted in a slightly higher extraction yield than the water extraction (145.7 mg/g) the water, propylene glycol and betaine extraction (162.2 mg/g). Another observation is the extracted proteins displayed strong antioxidant and antibacterial properties, over 250-fold compared with water extraction. The high antioxidant activity was probably due to the profusion of hydrophobic amino acids in the extracts, and this low polarity DES was responsible for this (Coppola et al., 2021). Additionally, the antimicrobial properties of the DES were found to enhance the bioactivities of the extracted proteins.

Other than that, DESs were also investigated as potential extraction solvents for polysaccharides derived from *Poria cocos* (PCPs) by Zhang et al. (2020). Six types of DESs were synthesized, with the choline chloride and oxalic acid composition proving to be the most suitable. Under optimized extraction conditions, the yield reached 46.24% ± 0.13%, representing an 8.6-fold increase compared to hot water extraction. Reusability testing demonstrated the DES's effectiveness over six consecutive runs, with the PCP extraction yield remaining at 38.40% ± 0.23% without the need for additional chemicals. Furthermore, analysis of the resulting PCP revealed molecular weight distributions predominantly ranging from 753 to 3578 g/mol (Zhang et al., 2020). In another study by (Fatahi & Tabaraki, 2023) Evaluation took place for the extraction of polysaccharides and antioxidants from *Persian manna* (Taranjabin) using 16 deep eutectic solvents and four conventional solvents. One of them is choline chloride-xylitol, which was found to be the most efficient solvent. The extraction yield and carbohydrate were calculated to be 55.5% and 2.5 mg/gddm, respectively. Analysis of monosaccharide composition revealed the presence of rhamnose, arabinose, glucose, and galactose in the extracted polysaccharides, with a molar ratio of 1.68:0.69:1.00:2.16. There were many antioxidant assays carried out. These included total flavonoids and phenolic content, , trolox equivalent antioxidant capacity reducing power assay ferric-reducing antioxidant power, and DPPH assay. The total phenolic content (TPC) was analyzed using the experimental method at a value of 2.15 mg GAE/gddm. The extraction ability of DES-based ultrasonic method was found to be 2.50 mg Glu/gddm and 2.15 mg GAE/gddm, respectively, for total carbohydrates and phenolic content. The DES (choline chloride-xylitol) based extraction purity is approximately 26% higher in the total carbohydrate content (1.057) It is 167% higher in antioxidant capacity (3.94 vs. 1.47 mg GE/gddm) and 56% more in total phenolic content (0.53 vs. 0.34 mg GAE/gddm) compared to water. These findings highlight DESs as a promising alternative solvent for efficient extraction of polysaccharide, offering enhanced yields and demonstrating recyclability, thus presenting a viable and sustainable extraction method.

In order to extract the polysaccharide, the latest technology which uses deep eutectic solvents (DES) as extraction buffer with assistant of the microwave to enhance the extraction yield was proposed. The DES was formed using choline chloride with acetic acid, which has never been reported elsewhere. During the preliminary work, the DES was successfully characterized and due to its high acidity and solubility characteristics (communicating work), it was therefore proposed to be used as the extraction buffer and optimization of the extraction condition was required. Similar works have been reported. For example, a study by Tien et al. (2022) obtained polysaccharide from dragon fruit (Hylocereuspolyrhizus) peels via microwave-ultrasound-assisted extraction (MUA) and DESs. The study used a solvent mixture of choline chloride:glucose:water in a 5:2:5 ratio. Under ideal conditions, which involved a microwave irradiation for 14.26 minutes at 240 W, subsequent ultrasonication for 46.07 minutes, a water/DES ratio of 3.37 mL/mL and liquid/solid ratio of 35.25 mL/g, the polysaccharide yield reached up to 19.39%. Another research focused on extracting polysaccharide from *Dioscorea opposite* Thunb using DES consisted of 1,4-butanediol and choline chloride with the optimal extraction conditions of 94°C extraction temperature, and 44.74 minutes of extraction time. These optimized conditions resulted in higher extraction yield (15.98% ± 0.15) compared to conventional hot water extraction and water-based ultrasound-assisted extraction methods (10.51% and 12.19%, respectively). It was strongly believed that DES is an outstanding alternative solvent for extracting polysaccharides (Suthar et al., 2023). Moreover, microwave-assisted extraction (MAE) is also an innovative and practical method that has emerged as a highly efficient technique for obtaining target bioactive compounds. This method offers several advantages, including reduced solvent and energy consumption, enhanced functionalities of food products, shortened extraction time, and accelerated processes (Mirzadeh et al., 2021). By combining both DES and microwave would therefore improve the extraction yield.

2.2 Physiochemical properties of DES

The pH, density and miscibility of DES are crucial for application as an extraction media. Thus, the study on its physiochemical properties is significant prior to utilizing in any applications

2.2.1 pH of DES

The pH, which measures the acidity of a solution, stands out as a crucial property in the development of DESs. As previously discussed, DESs are typically defined as systems formed from a combination of Lewis or Brønsted acids and bases, highlighting the significance of pH in DES formulation (Álvarez et al., 2023). The pH level varies depending on the relative acidity of the mixed anionic and cationic species. Not only does pH define various characteristics of a system, but it also holds paramount importance in various applications The acidity or alkalinity of a solvent, including DESs, can be impacted by the variety of hydrogen bond donors and hydrogen bond acceptors present in the solvent system. The pH of a solution, denoting its acidic or basic nature, is determined by the concentration of hydrogen ions (H^+)

within the solvent (Jančíková et al., 2022). The presence of hydrogen bond donors and hydrogen bond acceptors in DESs influences the dissociation of water molecules and the equilibrium of proton transfer reactions, thereby affecting the pH of the solvent. In DESs, hydrogen bond donors and hydrogen bond acceptors typically comprise organic compounds like quaternary ammonium salts, organic acids, and hydrogen bond acceptors such as urea, choline chloride, or carboxylic acids. These constituents engage in hydrogen bonding interactions with water molecules, leading to alterations in the concentration of H^+ ions and consequent changes in the pH of the DES. For instance, in DESs containing strong acids as hydrogen bond donors, like hydrochloric acid, proton donation to water molecules increases the concentration of H^+ ions, resulting in a lower pH and heightened acidity (Kivelä et al., 2022a). Conversely, DESs with weak acids as hydrogen bond donors, such as acetic acid, experience an equilibrium between the acid and its conjugate base, influencing the solution's pH. Similarly, the presence of hydrogen bond acceptors like urea or choline chloride can impact pH by affecting water molecule dissociation and subsequent H^+ ion formation. Moreover, the pH of DESs is influenced by the stoichiometry and composition of the solvent. Different combinations of hydrogen bond donors and hydrogen bond acceptors can yield varying degrees of acidity or alkalinity. Additionally, the water concentration in DESs can influence pH, as water acts as both an hydrogen bond donor and hydrogen bond acceptor in hydrogen bonding interactions (Swebocki et al., 2023). In summary, the pH of a deep eutectic solvent is shaped by the nature and potency of its hydrogen bond donors and acceptors, alongside their interactions with water molecules and the resultant equilibrium of proton transfer reactions. Comprehensive understanding of these factors is imperative for pH control in DESs across various applications, encompassing catalysis, extraction, and synthesis. The pH of DES can as