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**EXTRACTION AND BIOACTIVITY EVALUATION OF ANTIOXIDANT
COMPONENTS FROM TEUCRIUM STOCKSIANUM BIOS
COLLECTED FROM UAE**

Ahmad Ayman Al Salloum

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EXTRACTION AND BIOACTIVITY EVALUATION OF
ANTIOXIDANT COMPONENTS FROM *TEUCRIUM*
STOCKSIANUM BIOS COLLECTED FROM UAE

Ahmad Ayman Al Salloum

This thesis is submitted in partial fulfilment of the requirements for the degree of
Master of Science in Chemical Engineering

Under the Supervision of Dr. Naeema Al Darmaki

June 2021

Declaration of Original Work

I, Ahmad Ayman Al Salloum, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “*Extraction and Bioactivity Evaluation of Antioxidant Components from Teucrium Stocksianum Bioss Collected from UAE*”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Naeema Ibrahim Al Darmaki, in the College of Engineering at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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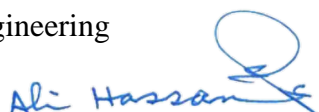
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Abstract

Recently, the industry of Natural Bioactive Compounds (NBAC) is expanding dramatically due to its high medical potentials. High bioactivity of natural extracts from a wide range of plants is evident from the reported literature. For instance, NBACs show antibacterial, antifungal, antimicrobial, anti-inflammatory, anticancer and antioxidant activities. *Teucrium stocksianum* Boiss. from the *Lamiaceae* family is a known herb in the traditional medicine in United Arab Emirates. Multiple bioactivities of *T. stocksianum* were recorded such as anti-inflammation, analgesia, antioxidation, anticancer, antinociception and antimicrobial bioactivity. In this thesis, the extraction of NBACs from *T. stocksianum* was studied with Microwave-assisted extraction using Deep eutectic solvent (MAE-DES). The resultant yield of extraction and antioxidant capacity were compared with the results of Ultrasound-assisted extraction with water, Hydrodistillation and Soxhlet with four different solvents (methanol, ethanol, Diethyl ether and n-hexane). MAE-DES showed higher results in term of yield (54.18%) and antioxidant bioactivity (21.67 mg Trolox equivalent/g dry weight). The effects of operating parameters for MAE-DES method on the total antioxidant capacity of extracts and the yield of extraction were investigated. Four independent variables, solid–liquid ratio (1:10, 1:30, and 1:50 ratio of raw material to DES, w/v), microwave power (220, 400, and 800 W), microwave time (60, 120, and 180 s), and DES concentration (30%, 50%, and 70%)—were used, and the optimized conditions were found to be 1:50, 400 W, 180 s and 30%.

Keywords: Herbal extraction, pharmaceuticals, essential oil, extraction solvents, extraction methods, bioactivity.

Title and Abstract (in Arabic)

الاستخلاص و التقييم لفعالية مضادات الأكسدة لمكونات نبتة الجعدة في دولة الإمارات العربية المتحدة

الملخص

في الآونة الأخيرة، أصبحت الصناعات القائمة على إستخلاص المواد الفعالة الطبيعية في حالة تضخم كبير بسبب أهمية هذه المواد في المجالات الطبية. حيث رصدت الدراسات هذه الفعالية العالية لدى المستخلصات من أنواع كثيرة من النباتات. فعلى سبيل المثال، المستخلصات الطبية النباتية أظهرت فعالية مضادة للبكتيريا و الفطريات و الميكروبات و فعالية مضادة للإلتهابات و السرطانات و الكثير غيرها. و في دولة الإمارات العربية المتحدة، تتواجد أحد النباتات الطبية التي تم إستعمالها في الطب الشعبي كمضاد للإلتهابات و كمضاد للفطريات ألا و هي نبتة الجعدة (*Teucrium stocksianum* Bioss.). الهدف من هذه الأطروحة هو الدمج بين أسلوب إستخلاص المواد الفعالة من النبات باستخدام الأشعة الميكروية (Microwave) مع استعمال سائل خليط منخفض الانصهار (Deep eutectic solvent) و تجربة نبات الجعدة مع هذه الدمج المقترح. تمت مقارنة معدل الإستخلاص الناتج عن هذه الطريقة مع أساليب إستخلاص أخرى كإستعمال الموجات فوق-صوتية مع الماء كسائل إستخلاص، أيضا تمت المقارنة مع عملية التقطير و عملية (Soxhlet) للإستخلاص باستعمال أربعة سوائل مختلفة. طريقة الأشعة الميكروية مع إستعمال سائل خليط منخفض الانصهار أظهرت نتائج مرتفعة من ناحية معدل الإستخلاص و الفعالية المضادة للأكسدة. لقد تم قياس الفعالية المضادة للأكسدة باستعمال التحليل الضوئي (Spectrophotometry). و من ثم، تم تحديد أربعة عوامل في أسلوب الإستخلاص المدمج تؤثر على معدل الاستخلاص و الفعالية المضادة للأكسدة. هذه العوامل هي نسبة سائل الاستخلاص إلى كمية النبات المستعملة وقوة الأشعة الميكروية ومدة التعرض للأشعة و تركيز السائل المنخفض الانصهار.

مفاهيم البحث الرئيسية: الاستخلاص العشبي، المستحضرات الصيدلانية، الزيوت العطرية، مذيبات الاستخلاص، طرق الاستخلاص، النشاط الحيوي.

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Dedication

To my beloved parents and family

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List of Abbreviations

BAC	Bioactive Compound
DES	Deep Eutectic Solvent
EO	Essential Oil
GC-MS	Gas Chromatography- Mass Spectroscopy
HAE	Heat Assisted Extraction
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HD	Hydrodistillation
HS-SPME	Headspace Solid Phase Microextraction
IL	Ionic Liquids
MAE	Microwave-Assisted Extraction
MA-HD	Microwave-Assisted Hydrodistillation
MA-SE	Microwave-Assisted Soxhlet Extraction
MW	Microwaves
NBACs	Natural Bioactive Compounds
OAHD	Ohmic-Assisted Hydrodistillation
SCE	Supercritical Extraction
SEM	Scanning Electron Microscope
SFME	Solvent-Free Microwave-Assisted Extraction
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
UAE	Ultrasound-Assisted Extraction
UA-HD	Ultrasound-Assisted Hydrodistillation

UA-SCE Ultrasound-Assisted Supercritical Extraction

UA-SE Ultrasound Assisted Soxhlet Extraction

Chapter 1: Introduction

1.1 Overview

Natural Bioactive Compounds (NBACs) extracted from medicinal herbs showed numerous favorable biological activities such as antibacterial [1]–[3], antifungal [1], [2], [4], antimicrobial [1], [3], [5], anti-inflammatory [6]–[8], anticancer [9]–[11] and antioxidant activities [5], [12], [13]. Bioactivities are caused by the chemical constituents present in the herb. Such chemicals are of great significance in pharmaceutical, food preservation, cosmetic and fragrance industries [14]. For example, the Essential Oil (EO) of *Digitalis lanata* is used to produce *Digoxin*, one of the most commonly used medicines for heart diseases [15]. Studies show that the global market of NBACs is expected to reach around 189.93 AED billion by 2024 [16].

With the increasing demand for natural bioactive materials, the demand for better extraction methods increased which resulted in designing various new extraction methods [17]. Some of the most common conventional methods used in recovering bioactive materials include hydrodistillation [14], [18]–[21], maceration [4], [22], [23] and Soxhlet extraction [14], [24]–[26]. Other newer but broadly used methods are supercritical fluid extraction [14], [24], [25], [27], ultrasound-assisted extraction [13], [20], [28], [29] and microwave-assisted extraction [18], [25], [28], [30]. To achieve maximum yield out of the extraction processes, a deep understanding of three main cornerstones is required. First: the nature of the bioactive compound. Second: the extraction method to be employed. Third: the solvent to be used. Therefore, the

methodology adopted in this thesis is based on combining two innovative aspects of extraction which are microwave assisted extraction and Deep eutectic solvents.

1.2 Statement of the Problem

Medicinal herbs are used for the treatment of diverse diseases in traditional medicine. The NBACs found in the extracts of these herbs have been found to have applications in pharmaceutical industry and food industry. In United Arab Emirates, the herb *Teucrium stocksianum*, known locally as Alja'ada, has been used traditionally for several generations to cure flue, renal, diarrhea, cough, jaundice, and abdominal pain. Also, it was a good disinfectant for wounds and topical inflammation. Moreover, the benefit of this rich plant is proven, and studies show its medicinal effects on diabetes and many other illnesses. The aim of the research carried out in this thesis is to prepare extracts of *Teucrium stocksianum* using different extraction methods. The main extraction method applied and studied is Microwave-assisted extraction using Deep eutectic solvent (MAE-DES). Soxhlet extraction, hydrodistillation, and ultrasound assisted extraction were carried out for comparison purposes in order to validate the efficiency of the new suggested method and initial optimization of the four different parameters that affects the extraction technique was done.

All the previously used extraction methods with *T. stocksianum* are conventional and none of the modern, green extraction techniques are used. Furthermore, it was noticed that optimization of extraction method was always ignored, and research were more focused on the bioactivity of the herb's extracts which limits the possibility of scaling up the extraction process to the industrial field. In this study, microwaves-assisted extraction using deep eutectic solvents, a green innovative

extraction technique, is carried out with initial optimization of the extraction parameters.

1.3 Relevant Literature

Understanding the type and the constituents in plant extracts is of crucial significance when extraction is to be carried out. They are, from an industrial perspective, the separated products from various plants, and they usually have distinguished flavor, aroma and bioactive effect [31]. Methods of plant extraction are numerous. Each method has its own advantages and disadvantages which require extensive studies in order to select the best method for the herb in hand. The goal, ultimately, is to design an optimum method for industrial application. To do so, the nature of plant extracts and extraction methods are reviewed below.

1.3.1 Natural Bioactive Compounds (NBACs)

To classify NBAC, the chemical compounds involved in metabolism, which are called metabolites, should be identified. Metabolites, from a biological perspective, are divided into two categories, primary metabolites and secondary metabolites. Primary metabolites are directly related to growth, cell division, storage, respiration, development and reproduction of the organism, thus they are more or less similar in all living cells [15]. Some of the primary metabolites are sugars, amino acids or proteins. On the other hand, secondary metabolites are not involved clearly in primary functions, rather in ecological functions, immune system of plants and other desired bioactivities in the organism [32], [33]. Importantly, the secondary metabolites present in medical plants are the desired product of the extraction processes. They can be classified, based on their biosynthetic origins, into five main groups:

Phenylpropanoids, Alkaloids, Terpenoids, Quinones and Steroids [33]. While extraction processes and relevant solvents are the main scope of this thesis, the biosynthetic origin of secondary metabolites may not be of much importance. Rather, a classification of the secondary metabolites based on the structure of their chemical compounds is more beneficial because the chemical structures directly indicate the solubility of the compound in various solvents. According to the chemical structure and functional groups, secondary metabolites could be divided into four main families, phenolic compounds, alkaloids, saponin and terpenes [15]. Phenolic compounds share the presence of one or more phenol groups as a common characteristic ranging from simple structures with one aromatic ring to highly complex polymeric substances. Alkaloid compounds contain at least one nitrogen atom in a heterocyclic ring. Their definition is problematic, as they do not represent a homogeneous group of compounds from chemical, biochemical, or physiological standpoints. Saponin are compounds that possess a polycyclic aglycone moiety with either a steroid (steroidal saponins) or triterpenoid (triterpenoidal saponins) attached to a carbohydrate unit (a monosaccharide or oligosaccharide chain) [15]. Terpenes are chemically derived from 5-carbon isoprene units assembled in different ways. They are divided into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes and triterpenes [15]. Terpenes can react with oxygen to form terpenoids. Terpenes, terpenoids and some of their derivatives are the main constituents of what is called “Essential Oils (EO)” [34].

Understanding the chemical structures of the various families of bioactive materials helps in linking the family of plants/herbs to the exact bioactive compounds. It is reported that bioactive compounds could be found in various genera of plants distributed over sixty families [31]. The major plant families known for their ability to

produce compounds of medicinal and industrial values include *Alliaceae*, *Apiaceae*, *Asteraceae*, *Lamiaceae*, *Myrtaceae*, *Poaceae* and *Rutaceae*. If a family of plants is known to be associated with a certain bioactive material, an optimized extraction method used to extract that material could be generalized all over its plant family. Table 1 classifies the secondary metabolites and the possible plant families that produce it. For example, saponins could be found in sufficient amounts in *Alliaceae* and *Lamiaceae* [35]. However, the concentration of the bioactive compounds depends on which part of herb is used for extraction; Pandey et al. [4] used different parts (i.e., leaves, stem, bark, etc.) of the herb as a feedstock for NBACs extraction and reported significant differences in contents extracted from each feedstock.

Table 1: Classification of secondary metabolites based on their molecular structure

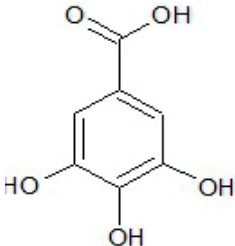
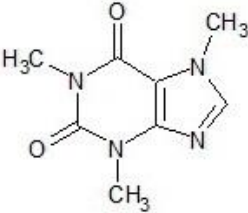
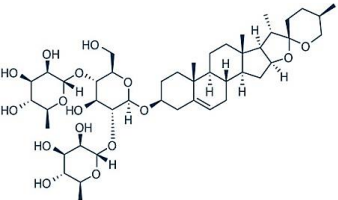
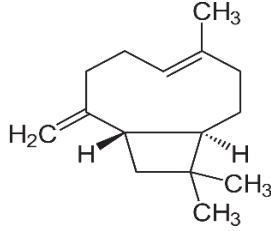
Family	Origins [15]	Examples [15]	Subdivisions [15]	Suggested Solvents
Phenolics	Compounds with a phenol ring or more. Plant families: Lamiaceae [36], Brassicaceae [37], Rutaceae [38], Caryophyllaceae [39], Araliaceae [40]	Gallic acid 	Simple phenolics	Acetone Methanol
			Tannins	Methanol Ethanol Water
			Coumarins	Ether
			Flavonoids	Acetone Ethanol Chloroform
			Xanthones	Methanol
Alkaloids	Nitrogen-Containing compounds Plant families: Lamiaceae [36] Rutaceae [38], Ranunculaceae [41], Araliaceae [40]	Caffeine 	Acridones	Ether
			Indoles	Ethanol
			Ergots	
			Imidazoles	
			Oxindoles	
Saponins	Compounds with a polycyclic aglycone moiety with steroid or triterpenoid Plant families: Alliaceae [42], Lamiaceae [36], Caryophyllaceae [39], Araliaceae [40]	Dioscin 	Steroidal Saponins	Methanol
			Triterpenoidal saponins	Ethanol Ether Water Chloroform

Table 1: Classification of secondary metabolites based on their molecular structure (continued)

Family	Origins [15]	Examples [15]	Subdivisions [15]	Suggested Solvents
Terpenes	Compounds derived chemically from 5-carbon isoprene units assembled in different ways. Plant families: Lamiaceae [36] Vitaceae [43], Ranunculaceae [41], Caryophyllaceae [39]	Caryophyllene 	Hemiterpenes	Methanol Ethanol Ether
			Monoterpenes	
			Sesquiterpenes	
			Diterpenes	
			Triterpenes	

As evident from Table 1, when the bioactive material is identified to be of a specific compound family, the listed solvent(s) could be used, which are suggested based on the molecular structure. However, possible modification and alteration of the compound(s) also should be accounted for. For instance, some compounds may not have good thermal stability; hence if a bioactive material is thermally unstable, it needs to be extracted under minimum heat exposure.

NBACs of a plant might face multiple alterations and changes when exposed to light, heat or oxygen [44]. In most cases, reactions that change the chemicals of NBACs are unfavorable but sometimes the product of those reactions may be the sought-after bioactive chemicals. For instance, Bounatirou et al. [45] reported that the essential oil of *Thymus Capitatus*, when stored at 60°C in complete darkness for 37 days showed higher percentages of *p-cymene* while *γ-terpinene* was decreasing. Bounatirou et al. [45] suggested that heating the sample promoted the aromatization of *γ-terpinene* generating *p-cymene*. Another example, terpenes are simple hydrocarbons, while terpenoids are modified class of terpenes with different functional groups and oxidized methyl group moved or removed at various positions [46]. Many terpenoids provide anticancer activities. Hence, it is better if the extraction conditions could enhance the oxidation of terpenes to increase the percentage of terpenoids. However, if there is not enough knowledge about the bioactivity of a herb, a sample BACs needs to be treated without direct exposure to the reaction triggers, as an initial assumption until proved otherwise.

1.3.2 Extraction Methods of NBACs

Extraction is a very common industrial process where the material of interest is separated from a mixture. Liquid-liquid extraction and solid-liquid extraction are

some of the categories of extraction processes. Herbal extraction is an example of solid-liquid extraction and it proceeds through the following steps [23]: (1) The solvent penetrates the solid matrix of the herb. (2) The solvent dissolves some or all the chemical constituents. (3) Diffusion of the solution of dissolved chemical constituents from the solid matrix to the surrounding medium. (4) Collection of the extracted solutes. There are numerous extraction methods used for extracting NBACs, such as, Hydrodistillation, steam distillation, organic solvent extraction, expression, enfleurage, microwave-assisted distillation, microwave hydro diffusion and gravity, high-pressure solvent extraction, supercritical carbon dioxide extraction, ultrasonic extraction, solvent-free microwave extraction, and the phytonic process [31]. The most commonly used extraction methods are discussed below.

1.3.2.1 Soxhlet Extraction

Soxhlet extraction is one of the oldest extraction methods [14]. It is considered as a standard method to compare other methods to [4], [14], [24]–[26]. In theory, Soxhlet extraction keeps the concentration gradient between the extraction solvent and the herb as strong as possible by a siphon mechanism allowing the mass transfer to happen at a faster rate which results in a higher extraction yield. However, the selectivity of Soxhlet extraction is low because it removes most of the chemical constituents with the repeated siphon cycles. It is also evident that yield and selectivity are inversely related.

A scheme of Soxhlet extraction is shown in Figure 1 (A). The solvent evaporates from the distillation flask and condenses above the thimble containing the sample. Mass transfer occurs, and when the level of solvent is high enough; the siphon mechanism is triggered through the return tube taking the solvent back to the

distillation flask which is heated enough to evaporate only the extraction solvent but not the chemical constituents. The main advantages of Soxhlet method is that it gives the highest yield, removing all the content from the herbs [14]. The temperature is kept relatively high in this method, which is favorable in case of thermally stable bioactive components. Another advantage is the low cost of Soxhlet extraction [47]. However, toxic, flammable and relatively expensive organic solvents are usually used in Soxhlet which represents the drawbacks of this method. Also, this method is relatively time consuming [25], cannot provide agitation [47], and the isolation of extract from the extraction solvent could be challenging. Another major disadvantage of Soxhlet method is the total dependence of selectivity on the extraction solvent while the method itself has no effect on the selectivity [47]. Despite having some disadvantages, Soxhlet method is the most commonly used extraction methods to extract EO from herbs [4], [14], [24]–[26].

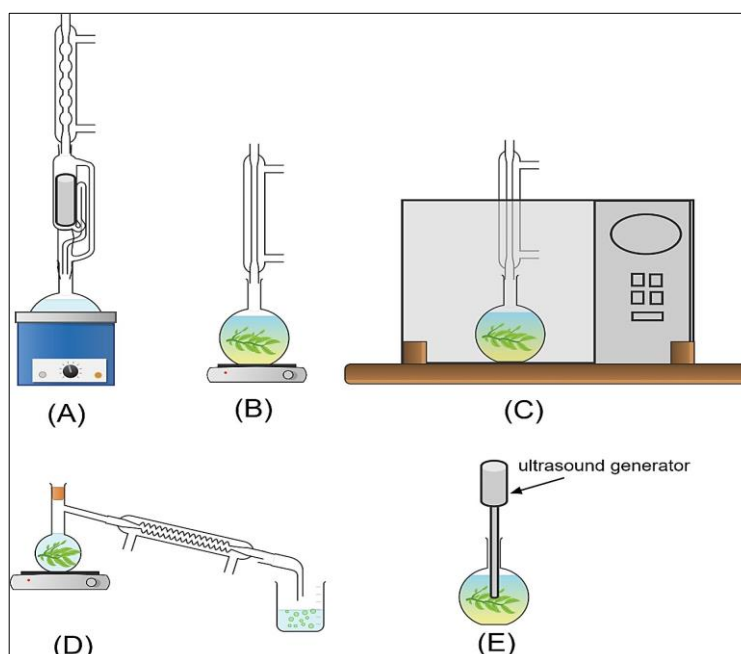


Figure 1: Extraction methods used. (A) Soxhlet Extraction (B) Reflux distillation (C) Microwaves-assisted Extraction (D) Steam Distillation (E) Ultrasound-assisted Extraction

Very recently, Gu et al. [24] employed Soxhlet method to extract *Xanthoceras sorbifolia* using n-hexane, then compared it with supercritical extraction using CO₂ and subcritical n-butane extraction. Soxhlet extraction recorded a yield of 60.43% which was the highest among the other methods. However, the bioactivity of subcritical n-butane extract was found to be higher than that of the Soxhlet extraction, which was attributed to the difference in solvents where butane, according to Gu et al. [24], is better in dissolving Tocopherols.

Pandey et al. [4] reported the extraction of *Taxus wallichiana Zucc* herb using Soxhlet extraction and Maceration with seven different solvents, such as, methanol, ethanol, acetone, chloroform, ethyl acetate, dichloromethane and petroleum ether. They focused on the bioactivity of the extracts instead of the yield. Maceration extracts using ethanol showed the highest antibacterial activity and Soxhlet extracts using ethyl acetate showed the highest antifungal activity [4]. It may be understood that the antibacterial chemicals present in this herb are mostly affected by the heat used in Soxhlet extraction; on the other hand, the chemical compounds responsible for the antifungal activities are thermostable.

Alara et al. [48] used Soxhlet extraction to extract phenolic compounds from *Vernonia cinerea* leaves and tested its antioxidant activity. Optimization of extraction time, feed to solvent ratio and other conditions were included in the study that could be taken as an example to follow in case of using Soxhlet extraction. The highest yield (10.01%) was achieved using an extraction time of 2 h (1 h–4 h), feed to solvent ratio of 1:20 g/mL (1:10-1:25 g/mL) and ethanol concentration of 60% v/v (20-80% v/v).

Mohammadpour et al. [49] used ultrasound-assisted extraction to extract essential oil from *Moringa peregrina* and tested its bioactivity while using Soxhlet extraction as a reference method for comparison. Ultrasound assisted extraction was advised as an extraction method with this herb because it exhibited better results than Soxhlet in terms of yield and quality of the extracted oil. But this indeed indicates the significance of Soxhlet extraction where it is used as the benchmark to measure the success of other methods.

1.3.2.2 Conventional Distillation

The term ‘Conventional distillation’ is usually used to describe Hydrodistillation (HD) and its other improved versions. In HD, the herbs are boiled in water to supply the required energy to penetrate the biomass structure and to dissolve the chemical compounds of interest [14], [18]–[21]. HD has some critical disadvantages, mostly related to the low extraction yield and long extraction time [20]. However, HD is very simple and cheap compared to the other extraction methods [14].

The high temperature needed to boil water could decompose the thermolabile chemicals. This have always been marked as a big disadvantage, but if the desired compounds are thermostable, then decomposing the undesired thermolabile compounds could increase the concentration of the bioactive material in extracts. HD could trigger oxidation reactions and extensive hydrolysis [14].

Reflux distillation and steam distillation are the upgraded versions of HD. These distillation methods follow the same principles of HD, but they differ in mechanism which solves some of the disadvantages of regular HD. In reflux

distillation (known also as cohobation [50]), shown in Figure 1 (B), water is condensed and refeed to the extraction chamber. The result is a higher extraction yield and a concentrated extract with less solvent consumption.

In steam distillation [shown in Figure 1 (D)] [51], many advantages are introduced which include the extraction of water-insoluble components and better thermolabile components extraction. The explanation behind the ability of water to dissolve water-insoluble components in this method lies in the thermodynamics of immiscible mixtures. An immiscible mixture will apply vapor pressure that is equal to the total of both pure components vapor pressures under the condition of continuous stirring and enough agitation [52]. If the mixture is left to settle down and form layers, then the total vapor pressure will be equal to the vapor pressure of the upper layer only. Once the vapor pressure exerted by a mixture is above the atmospheric pressure, it will evaporate. This means that even a component with a high boiling point (such as, essential oils) could boil at a much lower temperature if present in a mixture [52]. Going back to the case of steam distillation, steam passes by the biomass, transfers heat then carries the water-insoluble components, because they are present in a mixture that exerts pressure more than the atmospheric pressure. Ideally, steam should be passed through the biomass but that is not the case in Figure 1 (D). Instead, biomass is mixed with water. To activate the thermodynamics of boiling an immiscible mixture at a lower boiling point, both pure components should be exposed to the atmosphere so they can exert their vapor pressure. This could be done in the case of biomass and water mixture by keeping the level of water low enough that it cannot cover the whole biomass. Even though steam distillation reduces the decomposition of thermolabile chemical constituents, it still applies some modifications to essential oils [31] and suffers from relatively lower extraction yield and long-term consumption [20].

Research used distillation in all of its forms for extraction purposes. Nejia et al. [53] used conventional distillation, mainly five components (α -pinene, myrcene, piperonal, and trans-4 and 5-epoxy-(E)-2-docenal) were extracted from *cupressus sempervirens* [53], while the essential oil was proven to contain other chemicals (mainly: manoyl oxide, trans-totarol, α -acoradiene, and cis- α -santalol) [53]. Those chemicals have low heat tolerance which explains their absence in the conventional distillation extract.

Manouchehri et al. [21] reported the total extraction time of essential oil from damask rose using solvent-free microwave-assisted extraction (SFME) and ohmic-assisted hydrodistillation (OAHD) then compared the results to conventional hydrodistillation. It was found that SFME and OAHD needed less than 3 min and less than 18 min, respectively while more than 40 min were needed to accomplish conventional hydrodistillation extraction. This shows the disadvantage of high consumption of time and energy in hydrodistillation.

Camelo et al. [54] extracted the essential oil of *Plectranthus grandis* by hydrodistillation and headspace solid phase microextraction (HS-SPME). They reported that hydrodistillation extracts showed higher yields than HS-SPME. Moreover, hydrodistillation was better in extracting the higher molar mass compounds while HS-SPME was better in extracting the volatile components. This could be because of the high temperature applied to hydrodistillation process which enhances the extraction but damages the volatile thermolabile components.

Damyeh et al. [55] used Ohmic-assisted Hydrodistillation (OAHD) to extract essential oils from *Prangos ferulacea Lindle* and compared the extracts obtained with HD as a reference method. OAHD required only half the time and half the energy

needed in HD to achieve the same yield. They also reported that OAHD showed better selectivity for *limonene* and *(E)- β -ocimene* but lower bioactivity. However, the HD extracts showed higher antibacterial and antioxidant activity.

Stanojević et al. [50] obtained essential oil from Dill seeds using HD with slight modifications. They modified the HD technique in four different ways to produce extracts from the Dill seeds (i.e., 1. classical Clevenger- type HD, 2. condensate water combined with fresh water, 3. residual water from previous run mixed with new amount of water 4. condensate water mixed with residual water from previous run combined with fresh water). According to the GC-MS results, the extracts showed similar composition but in different amounts. This work suggests that the HD could be affected by those slight changes, and it still could be improved and redesigned.

1.3.2.3 Maceration Extraction

Maceration is the simplest method of extraction where the solid matrix is soaked in an extraction solvent under normal conditions. Although it has low extraction efficiency, and it needs longer extraction time, it is often favored because of its ability to extract thermolabile chemicals without any decomposition [23]. Multiple studies [22], [23] reported that maceration extracts have higher effectiveness in terms of bioactivity. Pandey et al. [4] compared between Soxhlet extraction and maceration to extract bioactive components from *Taxus wallichiana Zucc* herb. Soxhlet extraction gave higher yields, but the macerated extracts showed better antimicrobial activities [4]. Since it is carried out at room temperature, not enough heat is available in the maceration process to provide sufficient energy to dissolve all the chemicals present in the herb, which enables the solvent to dissolve more of the obvious soluble active constituents (i.e., better selectivity). These obvious soluble

active constituents might be decomposed at higher temperatures, causing the reduction of antimicrobial effect in Soxhlet extracts.

Percolation, shown in Figure 2 [56], could be considered as an upgrade of maceration. In percolation, the saturated solvent is replaced by a fresh solvent to elevate the extraction efficiency [23].

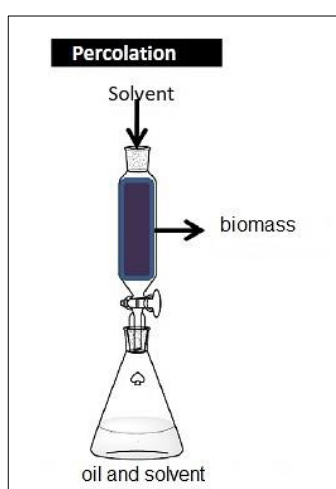


Figure 2: Percolation extraction [56]

Vieitez et al. [57] compared between the bioactivity of macerated and supercritical extracts of six different herbs (*Rosmarinus officinalis*, *Peumus boldus*, *Aloysia citrodora*, *Maytenus ilicifolia*, *Ilex paraguariensis*, *Eugenia uniflora*). Maceration with 75% ethanol achieved higher extraction yields than supercritical for all these herbs but bioactivities of the extracts varied depending on the type of herb and the method of extraction chosen.

Jovanović et al. [58] compared between maceration, heat-assisted extraction and ultrasound-assisted extraction of *Thymus serpyllum L.* and then optimized their conditions. The methods were ranked according to the total polyphenols yield in the order of Ultrasound assisted extraction > HAE > Maceration. HAE attained an

extraction yield in 5 min, which is equal to that attained by maceration in 1 hour.

Chen et al. [59] applied maceration as a pre-extraction step with many Chinese herbal medicines then extracted the herbs using heat-assisted ethanol extraction. The maceration step enhanced the extraction yield for some of the herbs. Chen et al. claims that maceration, in this way, enhanced the extraction by dissolving the unwanted chemical constituents that could trap the sought-after chemicals present in the herb(s). Also, the type of solvent selected for maceration affected the extraction yield depending on the ability of the solvent to remove the unwanted chemical constituents.

1.3.2.4 Supercritical Extraction (SCE)

Supercritical Extraction (SCE) is one of the most widely used green technologies. The operating conditions in this method are maintained above the critical point of the solvent. A solvent in its critical phase (above the critical point) possesses desired physiochemical properties, diffusivity and density that facilitate herbal extractions. The commonly used solvent in SCE method is carbon dioxide. Critical point of carbon dioxide, shown in Figure 3, is at 304.21 K and 7.3915 MPa [60].

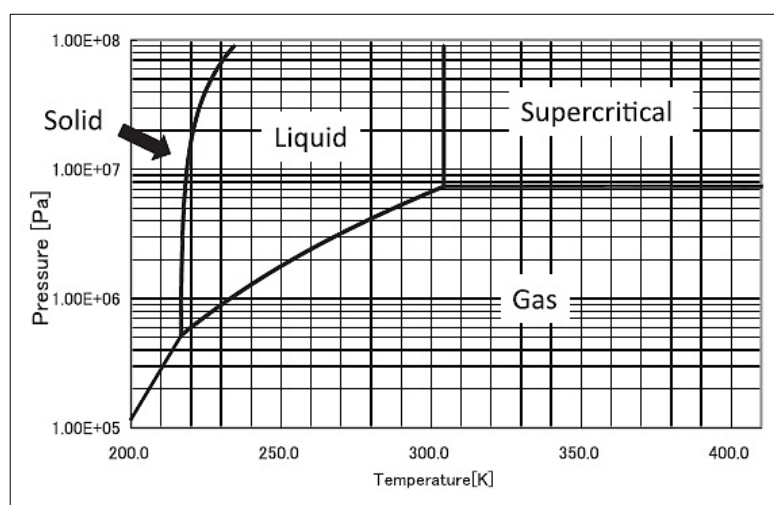


Figure 3: Phase diagram of carbon dioxide [55]

CO₂ is a non-flammable, non-toxic, non-explosive solvent that is available in high purity, and can be removed after the extraction by simply releasing the pressure converting it into gas [27], [31]. Also, most of the polar components of essential oils have limited solubility in carbon dioxide. Due to this, higher selectivity is achieved in SCE method. The main disadvantages in carbon dioxide include the inability to dissolve polar substances [14], high equipment investment and the danger of high pressures [24].

To understand the mechanism of supercritical extraction, supercritical phase must be studied. Pushing a substance over its critical point produces a phase that could be described to be in-between the properties of liquid and gas. Supercritical phase has higher diffusivity than liquids and higher density than gases. High diffusivity is favorable in extraction processes because it ensures that the extraction solvent would reach the bioactive material(s) of interest and extract it. Similarly, higher density is also favorable so that the solvating power of the solvent increases, and the solvent molecules can carry the extract with it. Furthermore, these properties could be easily altered in the case of supercritical phase just by changing the temperature and pressure. However, supercritical extraction is affected by many parameters, ranging from the pressure and temperature to the flow rate of solvent, size of sample and co-solvents [14].

Yousefi et al. [14] explained the effect of temperature, pressure, sample size, flowrate of solvents and modifiers (co-solvents) on the quality of supercritical extracts in details. Table 2 summarizes the effect of these parameters and explains how they can alter the extraction.

Table 2: Parameters affecting SCFE. [14]

Parameters	Effect	Explanation	
Temperature	Complicated effect: increased yield with increased temperature until it reaches a limit at which it reaches a constant extraction yield and starts damaging the thermolabile chemicals.	Increased temperature increases the solubility of EO (positive effect) but decreases density of extraction solvent (negative effect).	
		If the positive effect is more dominant than the negative effect, better extraction.	If the positive effect is equal to the negative effect, decomposition of thermolabile chemicals and waste of energy.
Pressure	Proportional effect: Elevated pressure increases the solvation power. At higher pressure, unwanted materials could be co-extracted. High pressure gives better extraction but lowers the selectivity.	The impact of compression disrupts the biomass and improves mass transfer. When it reaches a higher pressure, selectivity drops, and solvents removes everything (even the undesired materials). Solution: Fractionation system with at least two Separators to isolate EO.	
Particles Size and grinding	Inversely proportional effect: decreased particles size increases the surface area thus increasing the mass transfer. If excessive grinding is carried out, unwanted re-adsorption could happen, and pressure drop might occur.	Mass transfer is affected by the structure of plant matrix, particle size, surface area, shape and porosity. Changing these aspects changes the resistance faced by the solutes.	

Table 2: Parameters affecting SCFE (continued). [14]

Parameters	Effect	Explanation
Flow rate	Complicated effect: increased flow rate increases the extraction yield until the saturation is obtained, any further increment decreases the residence time and deviates the system from equilibrium.	The fluid penetrates the plant material and solvates the needed compounds. Smaller amount of fluids may not be enough to dissolve the compounds, limiting the equilibrium. On the other hand, excessive fluids may bypass the plant matrix leading to poor extraction.
Modifiers (co-solvents)	Proportional effect: increased percentage of modifier usually increases the yield of extraction. On the other hand, the use of modifiers requires the addition of a separation step.	Carbon dioxide lacks the polarity needed for many solutes. Addition of polar co-solvents (i.e., methanol, ethanol, water) enhances the solubility of the method dramatically.

Elgndi et al. [61] extracted the essential oil from four aromatic herbs using supercritical extraction with carbon dioxide. The herbs used in the study were *Satureja montana*, *Coriandrum sativum* and *Ocimum basilicum*. The extraction was carried out at two different pressures (100 bar and 300 bar) while fixing the temperature. The lower pressure extracted the easily accessible components while the higher pressure extracted the total extract. Elgndi et al. [61] noticed huge increment in the yield of extraction with higher pressure as such, the yield increased from 1.5-2% with 100 bars to 2.07- 5.6% with 300 bars.

Xiong et al. [62] optimized the operating conditions of SCE using CO₂ for *Pogostemon cablin*. The effect of temperature, pressure, extraction time and particles size on the extraction yield was studied. The maximum yield obtained was 2.4356% at 47°C, 24.5 MPa and 119 min.

Shahsavarpour et al. [63] used SCE-CO₂ to extract the essential oil from *Mentha spicata L.* and then optimized the extraction conditions after thorough examination (42.6°C, 119.7 bar, 0.169 g/min and 0.248 mm). It was found that the yield was directly proportional with the extraction pressure, but temperature and flow rate showed complex trends in such a way that the yield increased in the beginning and then started decreasing with further increment of the temperature and flow rate of the solvent. This result is compatible with what Table 2 presented.

Morsy [64] compared SCE-CO₂ with HD in extraction of essential oils from *Carum copticum L.* and *thymus vulgaris*. SCE selectively extracted the non-polar compounds in the herb with increasing yields at higher pressures. The yield for *Carum copticum L.* using HD was found to be 2.80% while SCE recorded a yield of 1.38% at 10.4 MPa and 2.78% at 16.7 MPa. For *thymus vulgaris*, HD achieved 1.00% yield

while SCE recorded a yield of 0.32% at 10.4 MPa and 1.28% at 16.7 MPa. Considering the high selectivity of SCE, the recorded yield is high because when selectivity increases, the yield drops down.

The scale-up of SCE process could be carried out using different approaches [65]. The complexity of the scale-up of SCE is relatively higher than other extraction methods where it could be directly related to one of the extraction criteria. Hassim et al. [66] reported that solvent to feed ratio (S/F) was found to be the best scale-up criterion for model verification and economic analysis of SCE process, because it would produce consistent yields at larger scale capacities.

1.3.2.5 Ultrasound-Assisted Extraction

The ultrasound-assisted extraction, shown in Figure 1 (E) is carried out with the help of sound waves that propagate through the solvent. The sound waves shake the structure of the plant cells and increase the movement frequency of solvent molecules [20], speeding it up and increasing the yield [13]. After the ultrasound assisted extraction, the biomass observed under Scanning Electron Microscope (SEM) was found to be crushed into tiny particles, which explains the mechanism of this extraction technique [20]. This method is considered to be as one of the green and recent extraction techniques because it decreases the consumption of solvent and reduces the time required for the extraction [28]. The disadvantage of this method lies in the possibility of building up a cavitation effect [29]. Due to the ultrasonic waves, bubbles are formed. The bubbles collapse after a while releasing huge amounts of energy. The cavitation effect makes temperature control more difficult, and due to the energy release followed by bubble collapse; it could break down some of the larger molecules. Shang et al. [29] reported that, ultrasound waves helped in the

diversification of the molecular weight distribution. Other studies reported that the cavitation effect enhances molecular diffusion where it shakes the system strongly [67]. Moreover, ultrasound waves may sometimes cause the formation of free radicals followed by oxidation, which may be accounted as another disadvantage to consider [67].

Luo et al. [68] optimized the parameters of ultrasound-assisted extraction when applied in isolating polyphenolics compounds from *Sorghum bicolor* L. and tested its bioactivity. The optimized conditions were found to be 21 min of ultrasound time, 53% of ethanol concentration and 52:2 mL/g solvent to solid ratio. These conditions exhibited a yield of 49.743 mg Gallic acid equivalent per gram (GAE/g) [69].

Živković et al. [70] found the optimum operating conditions to isolate polyphenolics compounds from pomegranate peel using ultrasound assisted extraction . The factors affecting the yields were reported to be the extraction time, ethanol concentration, solid to solvent ratio, and extraction temperature. The optimal extraction conditions were found to be 25 min extraction time, 59% ethanol concentration, 1:44 solid to solvent ratio and 80°C.

Mohammadpour et al. [49] used ultrasound-assisted extraction to extract essential oil from *Moringa peregrina* while using Soxhlet extraction as a reference method. The maximum yield (53.101%) was achieved at 26.3 min for extraction time, 17.8 mL/g liquid to solid ratio, 348 W ultrasound power and 30°C.

The scale-up potential of Ultrasound assisted extraction was extensively studied in previous studies [65]. Some pilot-scale setups showed slightly lower yields than the lab scale [71], but the extraction was efficient overall. In other studies, it was

found that the yield exhibited in the laboratory level and the pilot level were very similar [72]. Those variations between different references could be due to the herbal feedstock.

1.3.2.6 Microwave-Assisted Extraction (MAE)

Microwave-assisted extraction (MAE) method, shown in Figure 1 (C), is employed as an alternative to traditional heating extraction methods since it is an environmentally friendly and energy saving process [28]. Microwaves are electromagnetic waves (300 MHz to 300 GHz) that does not cause ionization of the molecules. Those waves increase the temperature in a localized way by vibrating organic molecules [25]. This causes heat transfer from inside the herb to the outside, which is opposite to the regular heating techniques [18]. This phenomenon supports mass transfer in the same direction of the heat transfer [28]. MAE reduces solvent consumption and extraction time while increasing the yield of extraction [30]. It also increases the selectivity of extraction by limiting the presence of undesired chemicals [18]. Another unique advantage of MAE is the ability to carry out the extraction without a solvent [65]. The main reported disadvantage of this method lies in the degradation of desired chemicals under the circumstances of excess microwave irradiation [28], [30]. Moreover, microwave-assisted extraction is very hard to scale up to the industrial level and would require huge investments [35].

The absorbance of microwaves differs from one solvent to another. The dielectric constant and the dielectric loss are used to measure the absorbance which could be translated into, the efficiency of converting the microwave radiation to heat. At 2.45 GHz and 30°C, ethanol showed better absorbance than methanol. This means that the waves are converted to heat in ethanol at a faster rate. Ethanol delivers the heat

to the biomass where it gets absorbed by the essential oil promoting a quicker rupture of the herb materials and increasing the diffusion of desired compounds [28]. This effect includes a new criterion to be considered while selecting the extraction solvent which, in turn, increases the complexity of the method.

Kaderides et al. [28] found that microwave-assisted extraction is more efficient than ultrasound-assisted extraction in isolating phenolics compounds from pomegranate peels. They reported that the yield achieved by MAE in 4 minutes processing time is 1.7 times higher than that by Ultrasound assisted extraction in 10 minutes. The optimal conditions were found to be 50% aqueous ethanol, 60:1 mL/g solvent to solid ratio and 600 W microwaves power.

Lopes et al. [73] used MAE to produce coffee extracts with yields sufficient for commercial applications. The study focused on the effect of extraction time, temperature and solid-to-solvent ratio on coffee extracts. The use of MAE reduced the required processing time to carry out the extraction due to the sudden increase of temperature in the solid residue. Lopes et al. [73] claimed that the mechanism of MAE is best utilized in closed-vessel systems under high pressure allowing the boiling point to rise up.

1.3.2.7 Integration between Extraction Methods

Many studies [21], [74]–[82] showed that better extraction could be accomplished by combining some existing extraction methods together. The combined technique usually shows better results than the individual or single ones. Some methods could be easily integrated to other methods, especially conventional methods with simple mechanisms. For instance, instead of using normal distillation with a

regular heating source, the heat source could be changed to a microwave source which will incur multiple desired benefits to the extraction. Also, the ultrasound wave rods could be used with any simple conventional methods to enhance the effectiveness of the technique(s) in hand. In principle, it could be said that using microwaves is not an extraction method by its own rather a method of applying heat to the system. Thus, it could be, theoretically, integrated with any technique that depends on raising the kinetic energy of the chemical constituents to enhance its mass transfer. The same could be done with ultrasound waves. Ultrasound waves shakes the structure and cracks the cell walls. It increases the mass transfer, not by increasing the energy of the system, but by removing the resistance that sabotages the desorption of the desired compounds. The following methods are the most recently researched combinations used for herbal extractions.

- Ultrasound-assisted Soxhlet Extraction (UA-SE)

Ultrasound waves in a water bath were combined with a Soxhlet apparatus [47], [77] to give the first ultrasound-assisted Soxhlet extraction (UA-SE) in 2004 (shown in Figure 4 (B)). The prototype was later redesigned in 2013 to insert the ultrasound rod directly in the extraction area in a Soxhlet extractor (shown in Figure 4.A). This combination reduces the time of extraction significantly [77] and increases the yield of extraction while applying a decompaction effect to the Soxhlet. The dripping solvent above the herb sample makes it more compressed forming a layer of biomass that could trap some of the solvent, the ultrasound waves break this formation thus granting an easier flow and a better extraction mechanism. Looking at the negative side effects, the ultrasound could affect the EO extracts and promotes oxidation, which may be more dominant in the set up where the generator is immersed in the sample-

extraction chamber [47], shown in Figure 4 (A).

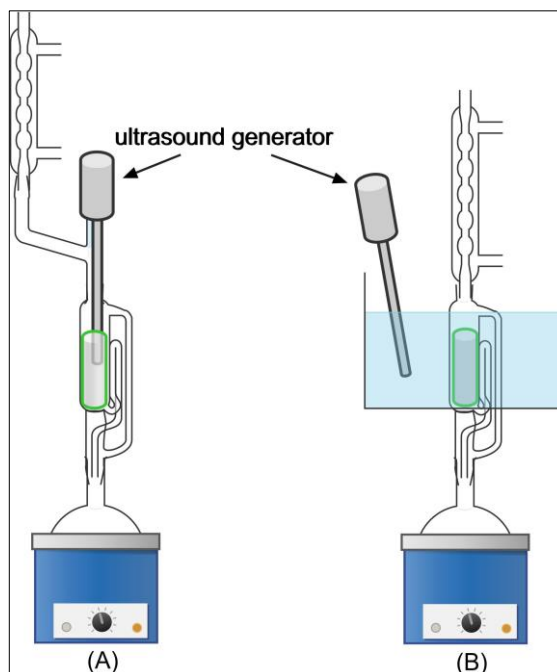


Figure 4: Ultrasound-assisted Soxhlet extraction prototypes [84]

- Microwave-assisted Soxhlet Extraction (MA-SE)

López-Bascón and Luque de Castro [47] stated that the addition of microwaves is the greatest improvement to the Soxhlet process. Multiple prototypes were introduced in the last 20 years [47]. Microwave's irradiation is applied to the thimble-sample area where the chemical constituents are heated up and diffused out of the biomass matrix. The required time for Soxhlet extraction was drastically reduced when microwaves were used and a possibility for automation was added. The design still could suffer from the possibility of thermolabile compounds degradation as the EO-extraction solvent mixture is recycled back to the distillation flask where it would receive heat from an electrical source. López-Bascón and Luque de Castro [47] claimed that the degradation of thermolabile components may be reduced since the extraction time is shortened, but this could be argued because in conventional Soxhlet,

the thimble area is never heated. Therefore, even if the extraction time is reduced, risk of altering the bioactive volatiles may still be significant because they will be subjected to microwaves and then will be heated again in the distillation flask.

- Ultrasound-assisted Hydrodistillation (UA-HD)

The ultrasound waves generator could be brought in contact with herb-water mixture to assemble the ultrasound-assisted hydrodistillation apparatus (Figure 5). The combination of the methods enhances the extraction yield remarkably and decreases the time required for extraction [20]. It can be claimed that the scale up of such a method to the commercialized level could be simpler than other techniques.

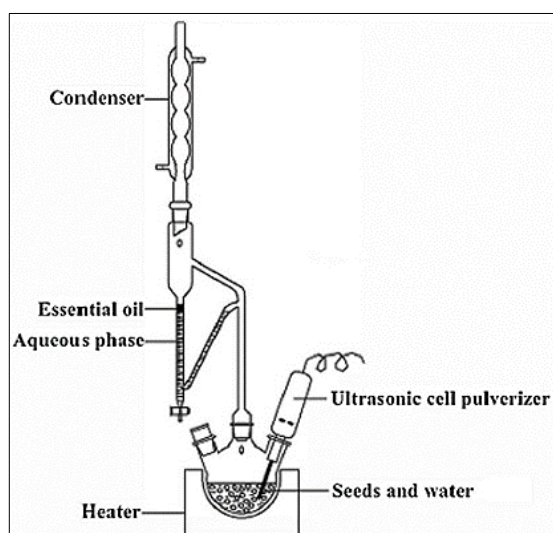


Figure 5: Schematic diagram of apparatus with UA-HD [19]

- Microwave-assisted Hydrodistillation (MA-HD)

Bustamante et al. [80] suggested microwave-assisted hydrodistillation as a better method for extracting essential oil from wet citrus peel waste. The method reduces the required time and energy for extraction while claiming easier scale-up options but does not have significant improvement on extraction yield compared to

HD [80]. This method differs from the conventional hydrodistillation just by the heat source, which definitely changes the mechanism of the extraction; but it does not add any new stimulator that promotes mass transfer. On the other hand, the previously mentioned UA-HD method uses heat and molecular shaking to reduce the mass transfer resistance, which, as a result, enhances desired mass transfer while MA-HD only uses a different source of heating.

- Ultrasound-assisted Supercritical Extraction (UA-SCE)

Dassoff and Li [75] found that the combination of ultrasound with supercritical extraction for herbal applications provides higher yields and reduces extraction time while carrying out the extraction under milder conditions relative to conventional solvent extraction methods. The coupled processes increase the already-high initial capital costs and the complexity of the method. The advantages of this combination are huge if the process is well-understood. However, this could be very challenging as there are many parameters to be set/considered and studied. The Ultrasound waves affects the temperature and makes it hard to control. On the other hand, this combination shortened the extraction time drastically, and reduced the required pressure. Other advantages and disadvantages of the process were also examined by Dassoff and Li [75].

1.3.3 Solvents Used for Extraction Purposes

1.3.3.1 Organic Solvents

Organic solvents have always been used for extraction purposes. Methanol, ethanol, acetone, chloroform, ethyl acetate etc. are typically used in Soxhlet Extraction [4], [24], [83], maceration and percolation extraction [4], [23], [24] and in supercritical

extraction as co-solvents [17], [75], [83]. These solvents have low viscosity which enhances the mass transfer, high volatility which facilitates the separation of NBACs from the extraction solvent, and wide polarity range that increases the solvation power leading to the repeatability of high extraction yields. These advantages made the use of organic solvents in extraction extremely favorable as an initial screening process. On the other hand, most of the organic solvents are hazardous, flammable and toxic. Also, they are considered expensive and difficult to recycle. Organic solvents are environmentally harmful too, if not treated or recycled. They may cause severe damages to the aqua life and the underground water resources.

1.3.3.2 Green Solvents (Ionic Solvents and Deep Eutectic Solvents)

A green solvent is a solvent that impacts the environment mildly or does not affect the environment at all. Researchers, all over the world, are trying to come up with an ideal solvent that achieves both environmental and industrial efficiency. Recently, some ionic liquids and deep eutectic solvents became the most commonly used green solvents for extraction.

Ionic liquids (ILs) are defined as compounds completely made of ions with relatively low melting points [84]. Sometimes it could be defined as liquid salt mixtures [85]. Some ILs are considered as green solvents due to their lower vapor pressure and high thermal stability [86]. But the range of physicochemical properties of ILs range in terms of volatility, flammability and stability [84]. Therefore, some ILs were capable of replacing organic solvents in many applications because of their unique physicochemical characteristics such as high conductivity, wider range of electrochemical properties that covers both hydrophilic and hydrophobic features, biodegradability, recyclability and their improved safety, as ILs have high chemical

and thermal stability [87]. Some ILs are capable of dissolving the cellular structure of plants which is usually made of cellulose, facilitating the release of components from inside the plant [86], [88]. On the other hand, ILs are relatively expensive, toxic and complicate the process more where a second separation step for the extract from the ILs is needed [89].

Deep Eutectic Solvents (DES) are a new type of solvents used for extraction purposes. Unlike ILs, DESs are made of a mixture of a Hydrogen Bond Donor (HBD) and a Hydrogen Bond Acceptor (HBA). However, defining DES based on that solely is not enough because simple eutectic solvents are also made of HBD and HBA [90]. From a thermodynamic point of view, DESs have a eutectic point temperature below that of an ideal liquid mixture [90]. Also, DESs should not be generally labeled as green solvents, where this feature is dependent on the types of HBD and HBA. Despite that, when compared to ionic liquids, most DESs have desirable advantages because of their lower cost and non-toxicity [89]. In addition to that, DES could be easily tuned and prepared. For example, by simply mixing choline chloride (HBA) and urea (HBD) with a ratio of 1:2, a deep eutectic solvent can be prepared. The mechanism of its extraction is similar to ILs; some DESs can dissolve the cellulose in the wall of the plant cells, promoting the mass transfer of the desired components [86]. The mixture has a melting point far less than the original individual components. DES could be classified as ionic and non-ionic. Choline Chloride with urea is considered an ionic DES while glucose with sucrose is classified as non-ionic DES [91]. Huge numbers of DESs were used in NBAC extraction. DESs were prepared by mixing and heating of the HBA and HBD with the needed molar ratio. The combination used in this research is of choline chloride as a HBA and urea HBD.

DES were tested to extract various bioactive components all over the polarity range [91]. They were tested to extract flavonoids, isoflavonoids, phenolic compounds, phenolic acids, terpenoids, anthraquinones, and alkaloids [91]. Variation of extraction efficiency was observed with the change of HBDs [91]. Those variations could be the resultant of the intermolecular electromagnetic differences.

The main disadvantage of using ionic liquids and deep eutectic solvents in plant extraction applications is the need for an additional step to separate the BACs and recover the solvent for recycling, because of the difficulty of total evaporation of such liquids. Many approaches were investigated, such as, using antisolvents, microporous resins [91] and liquid-liquid extraction [92]. When an anti-solvent of a mixture is added to a solution, it reacts or dissolves some of the compounds leaving the desired compounds to precipitate. Therefore, the antisolvent of a mixture does not dissolve the desired material. De Faria et al. [93] used water as an antisolvent to separate *Cynaropicrin* essential oil extracts from the mixture of a deep eutectic solvent (i.e., 6 different DES were tested). Once water is added, *Cynaropicrin* extract precipitate, and the mixture would be centrifuged and filtered. Carneiro et al. [94] studied sixteen different antisolvents and their effect on the extraction; the results showed that the ratio of IL/antisolvent is significant in addition to the antisolvent type. Smink et al. [92] suggested that the use of antisolvent is not very efficient where the amount of water (i.e., the selected antisolvent) were very large, thus liquid-liquid extraction was preferred. Despite the increase of complexity of extraction technique when using ILs or DES because of the extract/solvent separation step, still the advantages are much more, especially the biodegradability and recyclability aspects of these solvents.

1.4 Suggested Extraction Method for *T. stocksianum*

One of the most advanced combinations of herbal extraction is Microwave-assisted extraction using deep eutectic solvent (MAE-DES). The method was carried out by different researchers and proved high efficiency [95]–[98]. This combination provides many favorable aspects. The DES is proven to dissolve the cellulose structure of plants which promotes the diffusion of NBACs. In addition, MW provide internal heating for the DES which solves the problems of heat transfer caused by the high viscosity of DESs [98]. MAE-DES will be used to extract the bioactive compounds from *T. stocksainum* and the optimization of the method will be done. *T. stocksianum* had been extracted by other researchers using conventional methods and different bioactivities of the herb was studied. In this study, *T. stocksianum* is extracted using MAE-DES for the first time, according to the knowledge of author. Also, the optimization of four different parameters of the extraction method was conducted which was not done for any extraction method used with this herb in previous research. The feasibility of using this method with *T. stocksianum* was analyzed. The effects of four different parameters, Solid-liquid ratio, MW power, MW time and DES concentration, were examined.

Chapter 2: Methods

In this study, *T. stocksianum* was extracted using MAE-DES method for the first time according to the knowledge of authors. Other extraction methods were carried out to compare the results of the new selected method. The antioxidant bioactivity was selected as the sought-after biological bioactivity, but the extract could have other bioactivities that could be investigated in future research.

2.1 Plant Materials

Whole plant of *T. stocksianum* was collected from Khor Fakkan in UAE near Alrabi ancient tower in the beginning of January and at the end of February of the year 2020. The plant was authenticated by the herbarium in the United Arab Emirates University and a sample of the plant is deposited there for future references (voucher number: 14671). The collection showed plants with flowering phase. Plants were washed with fresh cool water, dried in shade for two weeks then the leaves and flowers were picked, crushed, and freeze dried using (TELSTAR Technologies model LYOQUEST, 2013, Spain) for accurate measurement of water content. Moisture was found to be 1.445% of herb after shade drying. Plant material was stored in the dark at room temperature for future use.

2.2 NBACs Extraction Methods

Four different extraction methods were carried out to extract the NBACs from *T. stocksianum*, Soxhlet extraction, HD, Ultrasound assisted extraction using water and MAE using one DES (choline chloride:urea (1:2)). The solvents of extraction used

in this research are selected to be of different polarities in order to discover which chemical compounds in the extract are likely to be involved in the bioactivity effect.

2.2.1 Soxhlet Extraction

A sample of *T. stocksianum* leaves (10 g) was weighed into a Soxhlet extractor paper thimble and placed in the extraction apparatus (150 mL). 200 mL of extraction solvent (methanol, ethanol, n-hexane and diethyl ether) was refluxed for 3 hours of extraction time using a heating mantle. After the extraction was finished, the extract solution was cooled down and then filtered twice with a filter paper (Whatman No.1). Rotary evaporation was used to remove extraction solvent completely. The yield of extraction was determined afterward by Equation (I). The experimental procedure was replicated thrice and the mean \pm standard deviation was recorded.

$$\text{Extraction yield(\%)} = \frac{\text{mass of total extract}}{\text{mass of dried herb}} \times 100 \quad \text{Eq. (I)}$$

2.2.2 Hydrodistillation

Dried leaves of *T. Stocksianum* were grounded and submitted to reflux HD. 10 grams of the herb were boiled in 200 mL of distilled water for 3 hours. Then, the sample was filtered twice with Whatman filter paper (No.1) to remove solid particles and the paper was dried and weighted to measure the reduction in herb sample then calculate the yield of extraction using Equation (I).

2.2.3 Ultrasound-Assisted Extraction

Dried leaves of *T. Stocksianum* were grounded and submitted to Ultrasound-assisted extraction using (QSonica model Q55, USA). 10 grams of the herb were soaked in 200 mL of distilled water for 3 hours at the maximum power of device (55

W). Then, the sample was filtered with Whatman filter paper (No.1) to remove solid particles and the paper was dried and weighted to measure the reduction in herb sample then calculate the yield of extraction using Equation (I).

2.2.4 Microwave-Assisted Extraction using Deep Eutectic Solvent (MAE-DES)

DES was used with MAE to extract NBACs from *T. Stocksianum* using commercial Panasonic microwave oven (NNST34H model). 1 g of herb was mixed with different amounts of solvent with different parameters. The effects of operating parameters on the total antioxidant capacity of extracts and the yield of extraction were investigated. Four independent variables, solid–liquid ratio (1:10, 1:30, and 1:50 ratio of herb to solvent, w/v), microwave power (220, 400, and 800 W), microwave time (60, 120, and 180 s), and DES concentration (30%, 50%, and 70%), were used. Herb was soaked in the solvent and immediately put in the oven according to the parameters. The sample was filtered twice using Whatman filter paper. Filtrate was stored in brown bottles at 4°C for future antioxidant analysis. Residue biomass was washed with water to remove the remaining DES because, without the washing step, it was extremely difficult to dry the residue. Difference in weight of the herb before and after the extraction was measured in order to calculate the total yield of extraction.

2.2.5 Supercritical Fluid Extraction Using Carbon Dioxide

It is worth mentioning, that Supercritical fluid extraction using carbon dioxide was carried out in the preliminary stage of this research using a SCF Extractor (Supercritical Fluid Technologies, INC. USA. SFT110 Model) under 100 bars, 40°C and five cycles of 20 min (10 min static and 10 min dynamic with flowrate of 24 mL/min). The extraction yield was relatively low (1.016%) and antioxidant bioactivity

was not observed which is why it was discarded as an extraction method in this thesis. Nonetheless, the SCF could show different desired bioactivities which could be studied in the future.

2.3 Determination of Antioxidant Activity

The extracts of *T. stocksianum* were prepared with different extraction methods and examined for antioxidant bioactivity using Total antioxidant assay kit (Sigma-Aldrich #MAK334). The Antioxidant Assay Kit measures Total Antioxidant Capacity (TAC) in which Cu^{2+} is reduced by antioxidants to Cu^+ . The resulting Cu^+ specifically forms a colored complex with a dye reagent. The color intensity at 570 nm is proportional to TAC in the sample. The kit uses 20 μL of sample and has a linear detection range from 1.5 to 1000 μM Trolox equivalents. A standard curve of Trolox was generated and used in calculating the total antioxidant capacity of the samples in hand by measuring the absorbance at the indicated wavelength using Microplate Reader device (Platos R496), according to Equation (II):

$$TAC(\mu M) = \frac{(A_{570})_{sample} - (A_{570})_{blank}}{\text{Slope of St. curve } (\mu M^{-1})} \quad \text{Eq. (II)}$$

TAC: total antioxidant capacity

A_{570} : absorbance of light at 570 nm wavelength

Calculation for the TAC as mg Trolox equivalent per g of herb was then carried out according to Equation (III):

$$TAC\% = \frac{TAC(\mu M) \times MW\left(\frac{g}{mol}\right) \times V(L)}{\text{Weight of sample } (g)} \quad \text{Eq. (III)}$$

MW: molar weight of Trolox (250.3 g/mol)

V: volume of extraction solvent

2.4 Determination of Thermal Stability of *T. Stocksianum*

Thermogravimetric (TG) analysis of *T. stocksianum* plant material was conducted using a Thermogravimetric Analyzer (TGA Q50 V20.10, TA Instruments, New Castle, DE, USA) under a nitrogen atmosphere (40 mL/min balance and 60 mL/min sample) and Platinum crucibles. The mass of each sample was ~6.2 mg. The analysis was conducted thrice at 20–800°C with a constant heating rate of 10°C/min. Simultaneously, a thermal analyzer was used to identify the TG/difference TG (DTG) data.

2.5 Gas Chromatography-Mass Spectroscopy Analysis

Gas Chromatography-Mass Spectroscopy (GC-MS) single quadrupole was carried out to identify the chemical compounds in extracted samples. Agilent GC (model 7890B) was used, equipped with Agilent MS (model 5977B) and Agilent Autosampler (model 7693). The analytical column was Agilent HP-5MS (30 m x 0.25 mm ID, 0.25 μ m film thickness (Part #19091S-433). Carrier Gas was Helium with column Flow Rate 14 mL/min. Injector Temperature was 300°C with split ratio 10% and split flow 10 mL/min. Initial temperature was 50°C held for 2 min then increased up to 116°C with a rate of 17°C/min, then increased up to 143°C with a rate of 15°C/min, then to 220°C with 30°C/min and finally to 300°C with 60°C/min and held at constant temperature for 5 min. Total Run time was 24.0 min. For mass spectroscopy, acquisition delay was 4 min and transfer line temperature was 300°C. Furthermore, MS quad temperature was 150°C and source was 230°C.

Chapter 3: Results

3.1 Extraction Yields

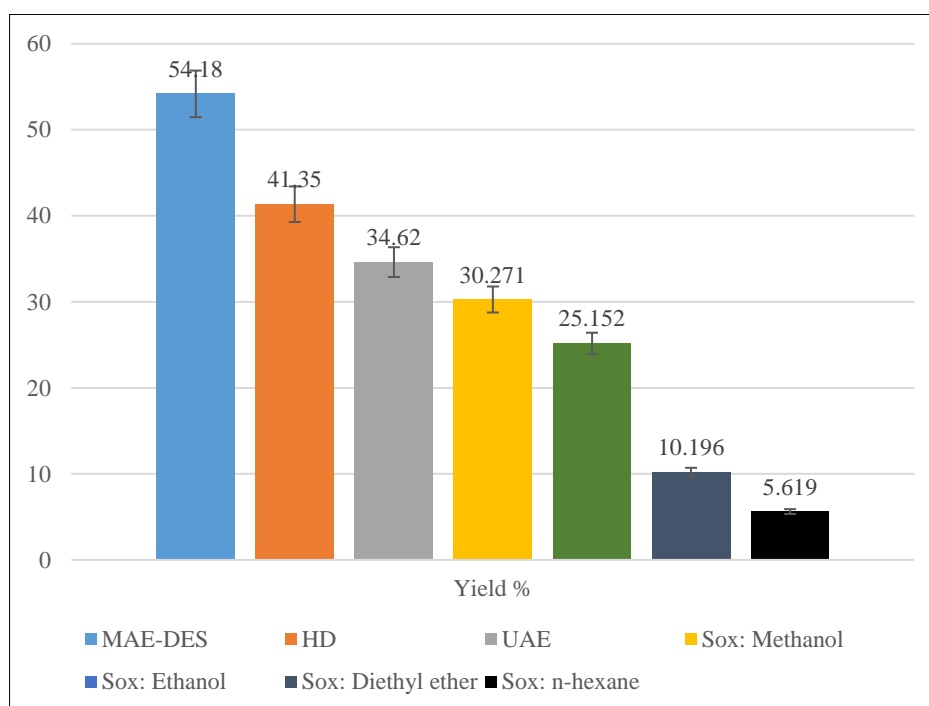
The results of extraction yields were calculated for each extraction method. The yields are compared, and the behaviors are explained. For conventional methods, HD with water showed the highest extraction yield (41.35%) and the lowest yield was achieved using Soxhlet with n-hexane (5.62%). Thus, water was selected to be used in ultrasound-assisted extraction, which achieved (34.62%). All of the above methods were carried out to eventually compare it with MAE-DES to validate the effectiveness of the new suggested method. The highest extraction yield using MAE-DES was achieved with the following parameters (MW time: 180 s, MW power: 400 W, DES concentration: 50% DES, solid-liquid ration: 1:50) producing a yield of (54.18%).

3.1.1 Conventional Methods

In this study, three different methods were used to be compared with the MAE-DES method. The methods are Soxhlet extraction with four different solvents, reflux HD and ultrasound assisted extraction using water. Soxhlet extraction was the first method to be conducted, and it showed that the yield of extraction increased with the increment of polarity of the extraction solvent in directly proportional behavior. This result indicated that water could be the best solvent for ultrasound assisted extraction and HD extractions. The results of each method are presented in Table 3. Figure 6 shows the yields with comparison to the optimized conditions of MAE-DES.

Table 3: Extraction yield of *T. stocksianum* using conventional methods

Method	Extraction solvent	Color of Extract	Yield %
Soxhlet Extraction	Methanol	Darker Green	30.271 + 0.421
	Ethanol	Dark Green	25.152 + 0.157
	Diethyl ether	Green	10.196 + 1.108
	n-hexane	Yellowish Green	5.619 + 0.137
Reflux HD	Water	Brown	41.35 + 0.192
Ultrasound assisted extraction	Water	Reddish Brown	34.62 + 0.256

Figure 6: Extraction yield of *T. stocksianum* (MAE-DES and Conventional methods)

The results presented in Figure 6 indicates the high polarity of the chemical compounds found in *T. stocksianum*. MAE-DES scored the highest extraction yield which proves the effectiveness of this method. The MWs increase the kinetic energy of the bioactive compounds dramatically which enhances the mass transfer and the

DES damages the cell wall of the herb, hence less diffusion limitations. Both factors enhance the diffusion of the bioactive compounds from the inside of the herb to the surrounding solvent.

Fluctuation was noticed when using Diethyl ether as an extraction solvent which resulted in a larger standard deviation. This could be a result of the high volatility of diethyl ether that affected the solvent amount. It is observed that while Soxhlet is carried out, Diethyl ether amount is reducing in uncontrollable way because of vaporization. This could be fixed by using better coolant in the condenser or using a specially designed condenser. This disadvantage is not present in other solvents where tap water is enough for the condensation.

3.1.2 Microwave-Assisted Extraction with Deep Eutectic Solvent

MAE with DES was carried out to test the effect of four different parameters on the yield of extraction and the TAC. The parameters are solid-liquid ratio, MW power, MW time and DES concentration. Each parameter was tested for three different values. A single run would test for a single variable only while the rest of the parameters are fixed at the selected default values (Default values: 400 W, 60 s, 1:30, 50%).

3.1.2.1 Effect of Microwave Time on Extraction Yield

The effect of microwave time on the yield of extraction was measured. The results were compatible with what is expected; as the time increases, the yield of extraction increased until it reached a limit where temperature started to affect the bioactivity of the samples due to thermal decomposition. The graph in Figure 7 shows

clearly the direct proportional relationship between the extraction yield and microwave time.

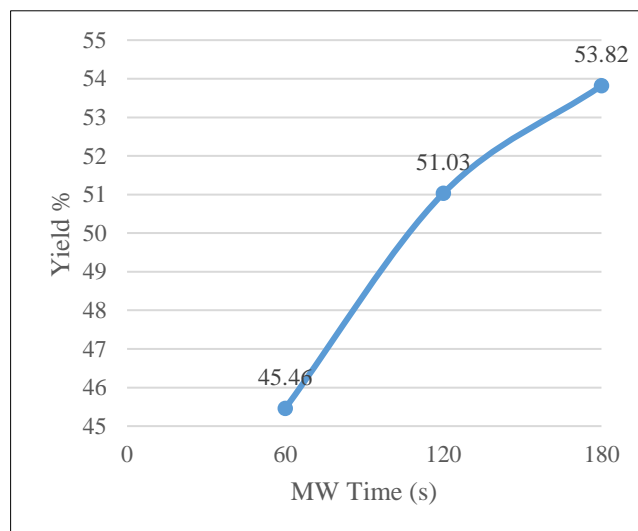


Figure 7: Effect of microwave time on extraction yield

3.1.2.2 Effect of Microwave Power on Yield of Extraction

The microwave power is one of the main parameters in MAE-DES method. The expected effect was that the more the power, the higher the yield of extraction. This assumption is based on the fact that, higher power will reach higher extraction temperature which will enhance the diffusion of NBACs. However, MW power and yield were inversely related. Figure 8 shows the trend of the extraction yield with the MW power.

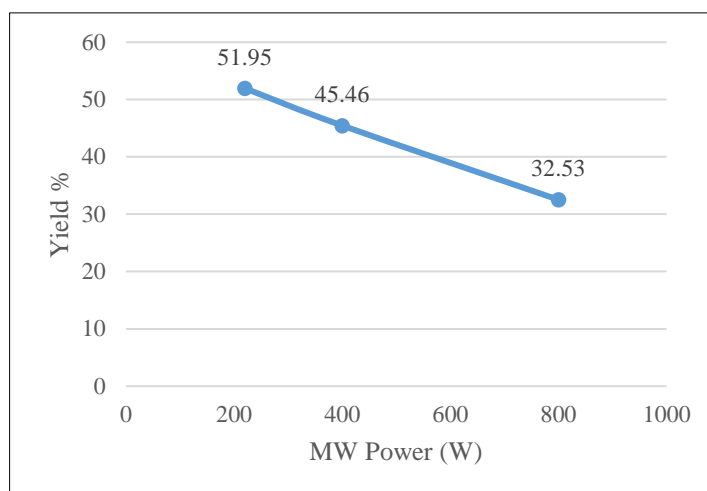


Figure 8: Effect of microwave power on extraction yield

3.1.2.3 Effect of Solid-Liquid Ratio on Yield of Extraction

Decreasing the solid-liquid ratio (g of herb/mL of solvent) increased the yield of extraction. This result is expected where the more solvent is available to solvate and extract the bioactive compounds the better the extraction becomes. Figure 9 shows a plot of the amount of solvent used for 1 gram of herb versus the yield of extraction.

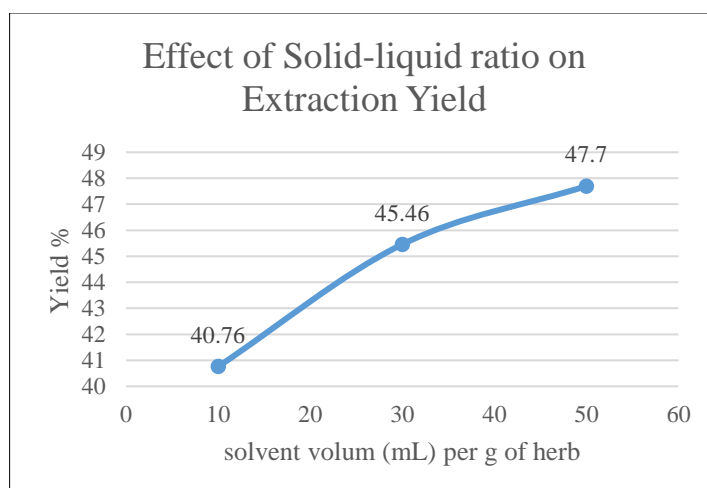


Figure 9: Effect of solid-liquid ratio on extraction yield

3.1.2.4 Effect of DES Concentration on Yield of Extraction

The yield of extraction was thought to be most dependent on the DES concentration, where the high viscosity of pure Choline Chloride:Urea DES was a hard-to-handle characteristic. Regardless, the DES has the ability to solvate the cellulose in plant structures, which led to assuming direct proportionality between DES concentration and the yield. The results showed a more complex relation. The yield of extraction increased with the increase of DES concentration in the beginning, then it started to fall with the continuous increment of the concentration. Figure 10 shows the graph for the relationship between the yield and DES concentration.

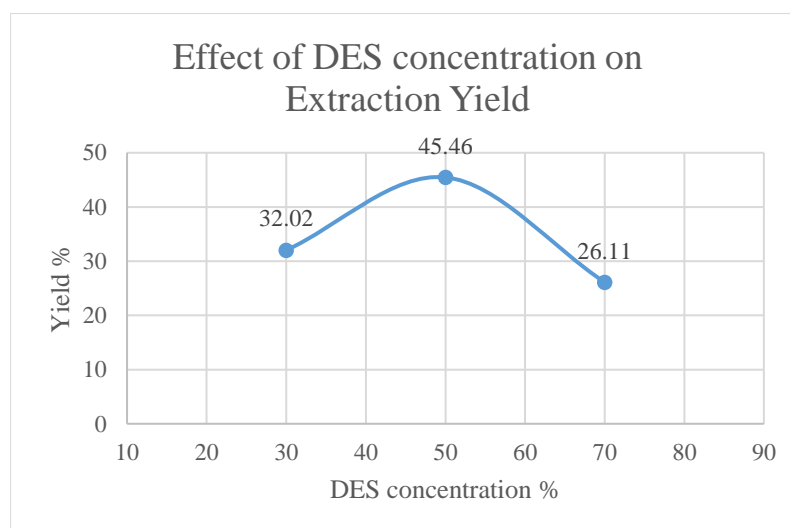


Figure 10: Effect of DES concentration on extraction yield

3.2 Antioxidant Bioactivity

T. Stocksianum has many potential biological activities that could be researched. In this thesis, the antioxidant bioactivity was selected to be examined using Total antioxidant assay kit with reference to a standard Trolox curve. The bioactivity was expressed as mg of Trolox Equivalent (TE) per g of Dry Weight (DW) of the herb

feed stock. Using this unit relates between the raw material and the bioactivity directly which is better than expressing the percentage yield as a weight difference.

3.2.1 Antioxidant Bioactivity of Conventional Extraction Methods

The antioxidant bioactivity of *T. stocksianum* extracts prepared by three conventional methods were measured. The nonpolar solvents showed no antioxidant activity at all which could confirm the high polarity of antioxidant compounds. Figure 11 shows the results of prepared extracts. It is obvious that water was the best solvent out of the five solvents used. MAE-DES optimized sample was also added to the figure to show the difference between methods.

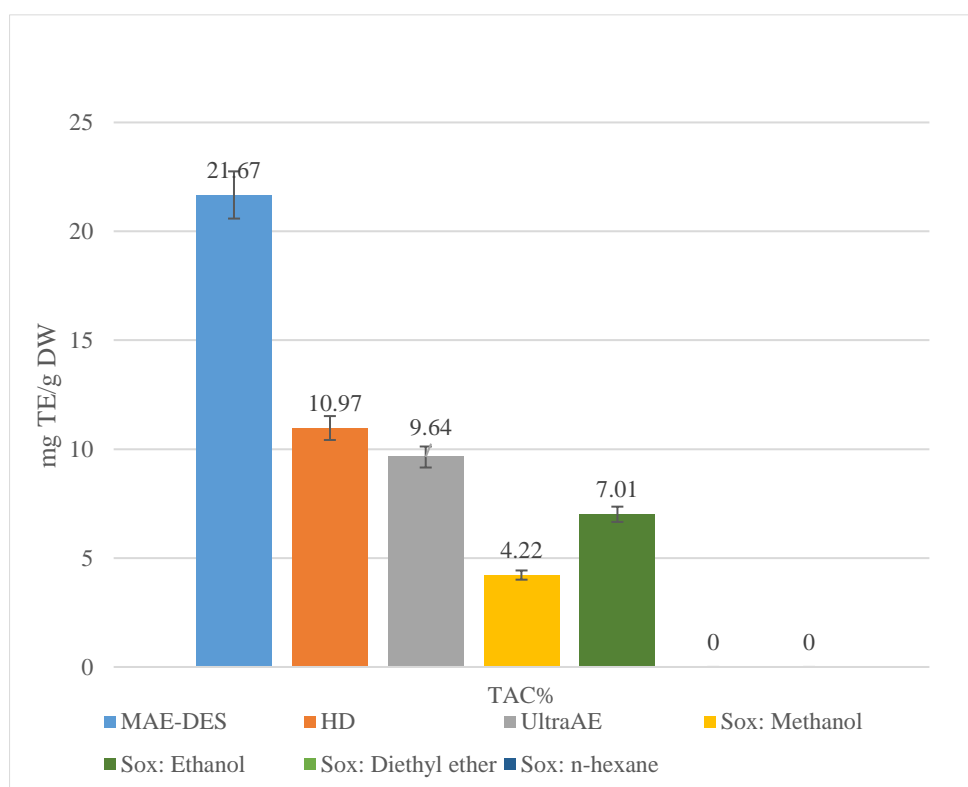


Figure 11: Total antioxidant capacity of conventional methods and MAE-DES

3.2.2 Antioxidant Bioactivity of MAE-DES

MAE with DES was carried out to test the effect of four different parameters on the yield of extraction and the TAC. The parameters are solid-liquid ratio, MW power, MW time and DES concentration. Each parameter was tested for three different values. A single run would test for a single variable only, while the rest of the parameters are fixed at the selected default values (Default values: 400 W, 60 s, 1:30, 50%). Each result was triplicated to ensure the accuracy of the experiment.

3.2.2.1 Effect of Microwave Time on Antioxidant Activity

The antioxidant activity was expected to be directly proportional with the MW time because the extraction yield increased with the increase of MW time. The higher the time exposure to MW the higher the temperature will be which will enhance the extraction of bioactive compounds. Nonetheless, in case of excess radiation time, the relationship could become inversed where MW could destroy the bioactive compounds. Figure 12 presents the change in antioxidant activity with increasing time.

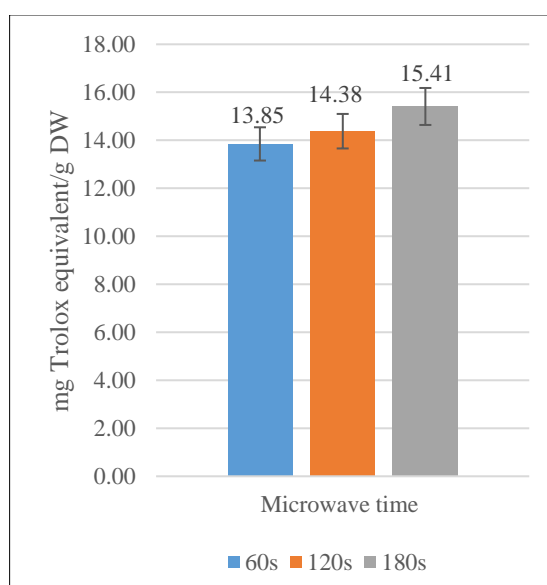


Figure 12: Microwave time effect on total antioxidant capacity

3.2.2.2 Effect of Microwave Power on Antioxidant Activity

The antioxidant activity had a complex relationship with the MW power. The bioactivity was expected to behave like the behavior of the extraction yield (i.e., decreased with the increase of MW power). However, the bioactivity increased in the beginning then it started to fall with higher MW powers. Figure 13 shows the effect of MW power on the antioxidant activity.

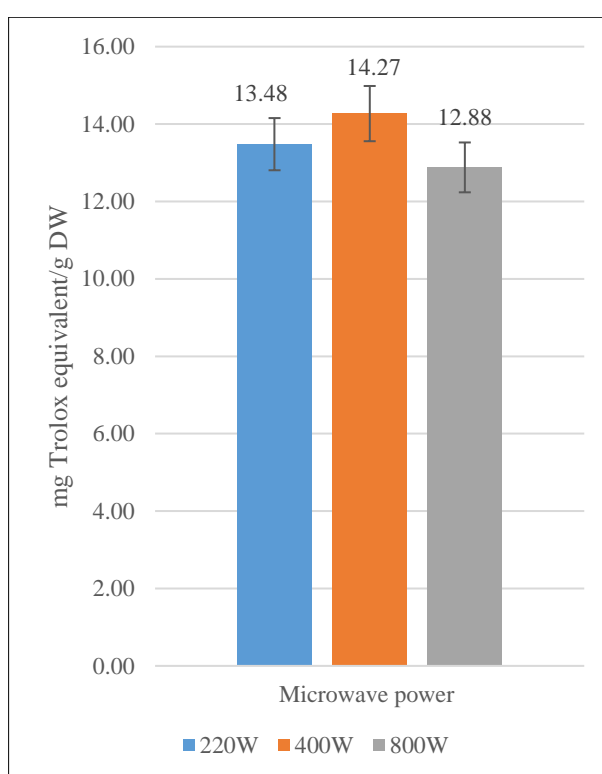


Figure 13: Microwave power effect on total antioxidant capacity

3.2.2.3 Effect of Solid-Liquid Ratio on Antioxidant Activity

The third tested parameter was solid-liquid ratio. The antioxidant activity was, as expected, inversely proportional with solid-liquid ratio. With lower ratios (x grams of biomass/ y mL of solvents), more solvent is available to solvate and extract the bioactive compounds. Figure 14 represents the effect of solid-liquid ratio of the DES with the herb on the TAC.

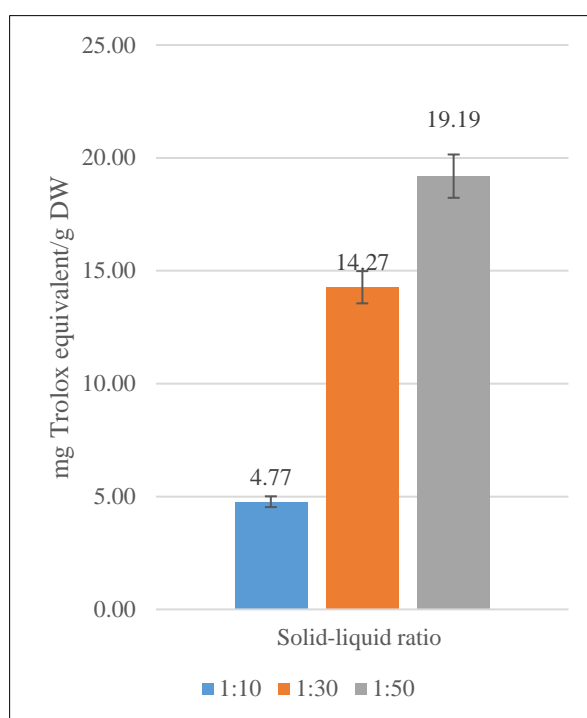


Figure 14: Solid-liquid ratio effect on total antioxidant capacity

3.2.2.4 Effect of DES Concentration on Antioxidant Activity

The DES has the ability to dissolve the cellulose in the plant cell wall. Which is why it was expected to increase diffusion of the NBACs. However, the antioxidant activity decreased with the increment of DES concentration. Figure 15 shows the effect of the DES concentration on the TAC.

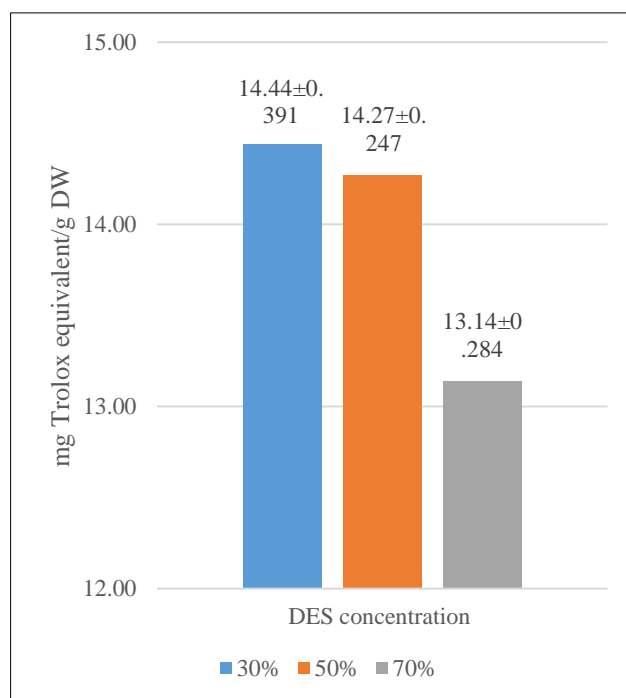


Figure 15: DES concentration effect on total antioxidant capacity

3.3 Thermogravimetric Analysis of *T. Stocksianum* Herb

Analysis of the thermal properties of *T. stocksianum* was carried out by studying the thermograms of TGA. The TG curves in Figure 16, showed two decomposition stages. In the initial stage, more than 55% of the mass was decomposed. This stage is associated with the Volatile Organic Compounds (VOC) starting from 150°C to 450°C. The second stage is around 27% of the weight loss and is associated with fixed carbon decomposition, which starts at 625°C and ends at 700°C. The beginning and ends of each stage are easier shown on the derivative of the graph (Figure 17). The results of the TG analysis show that the possibility of degradation during the extraction methods are not likely to happen since the temperature in extraction methods does not exceed 100°C in most cases. It is noticed that there is a loss in weight (around 5%) from 50°C to 100°C, this could be attributed to moisture content in the sample, but it does not eliminate the possibility of degrading bioactive compounds responsible for

other bioactivities than antioxidant activity, since it was not affected severely by heat exposure.

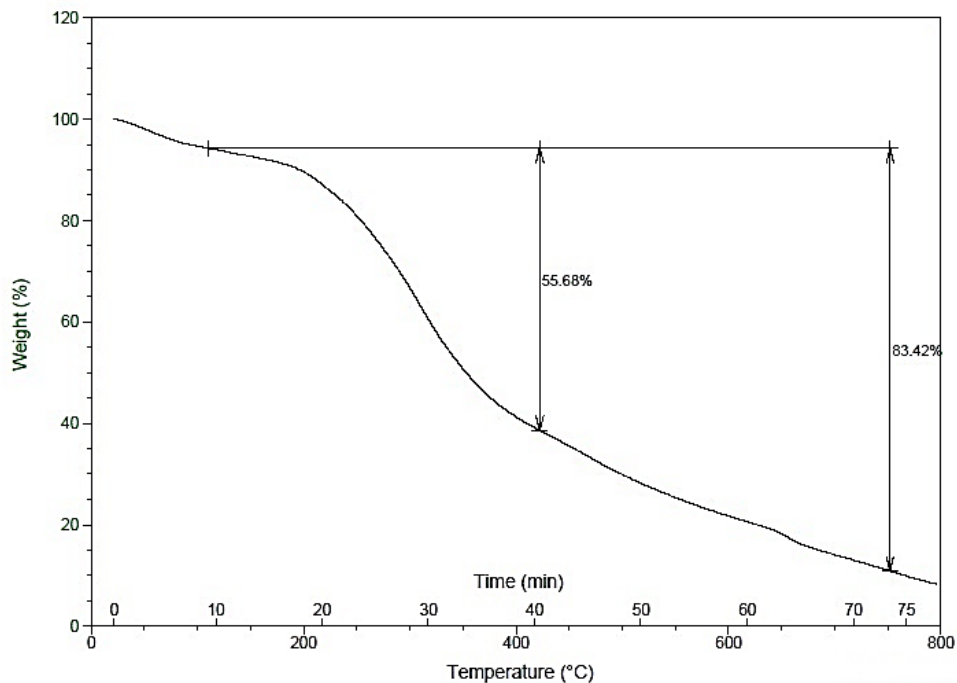


Figure 16: TGA of *T. stocksianum* and percentage of each decomposition stage

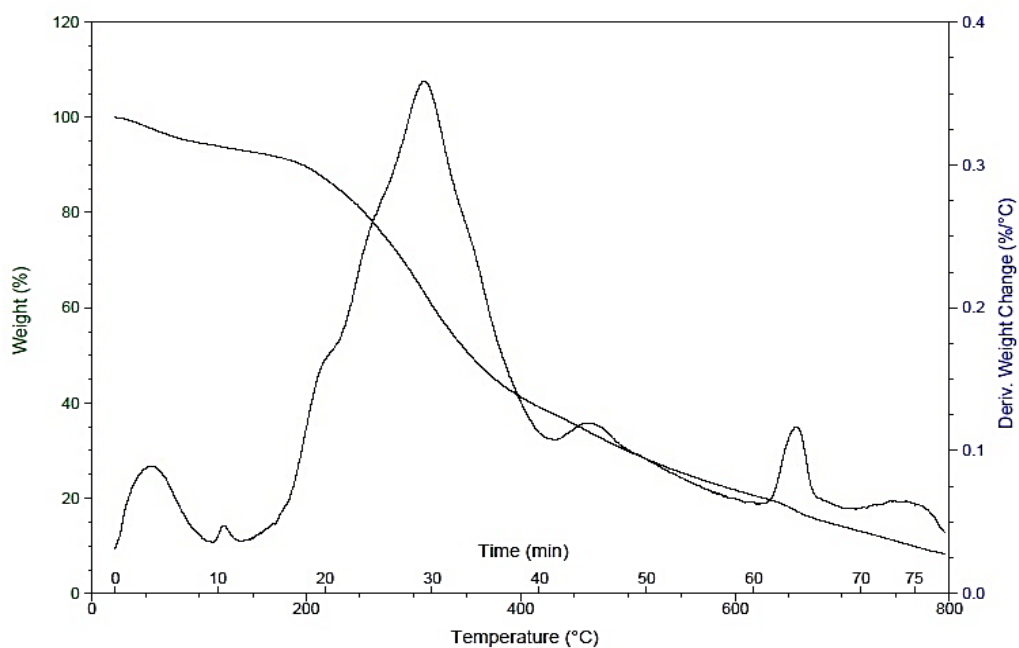


Figure 17: TGA of *T. stocksianum* with derivative of the graph (DTG)

3.4 Identification and Quantification Using GC-MS

The chemical compounds in the Soxhlet extracts of *T. stocksianum* were analyzed using GCMS. The identification was based on the MassHunet MS library and GC standards when available. Table 4 presents the results of methanol, ethanol, diethyl ether and n-hexane extracts, respectively. The table shows the most dominant compounds for each extract while more detailed tables are presented in the Appendix. The majority of the organic families are from Sesquiterpenes, Monoterpenes and the oxygenated forms of those families (Terpenoids).

Table 4: Major identified bioactive compounds using GCMS from Soxhlet extraction

#	Compounds	Meth%	Ethn%	Dee%	Hex%	Organic Family and Effect
1	Hydroxylamine	7.27	1.4			Inorganic compound: antitumor
2	Caryophyllene	2.34	2.71	2.10	2.57	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
3	Alloaromadendrene	2.16	2.51	1.72	2.08	Sesquiterpenoid: antibacterial antifungal
4	Germacrene D	0.65	0.92	0.69	0.96	Sesquiterpene: antibacterial, antifungal and antioxidant activities
5	δ -Cadinene	-	6.34	-	-	Sesquiterpene: antibacterial and antioxidant activities
6	camphene	-	-	5.18	5.02	Monoterpenes: anti-fungal antibacterial sedative, antioxidant
7	Elemene	-	-	2.48	2.06	Sesquiterpenes: antitumor, antioxidant and anti-inflammatory
8	γ -muurolene	4.96	-	-	-	Sesquiterpene: antimicrobial and antioxidant

Table 4: Major identified bioactive compounds using GCMS from Soxhlet extraction (continued)

#	Compounds	Meth%	Ethn%	Dee%	Hex%	Organic Family and Effect
9	Caryophyllene oxide	2.80	2.42	2.58	0.32	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
10	Isomycorene	4.24	3.94	-	-	Monoterpenoids: Antibacterial activity
11	Fenchol	2.82	-	6.01	2.22	Terpene: antibacterial, antimicrobial, and antioxidant
12	flexibilide	0.24	7.68	-	-	Diterpenes: Antimicrobial activity
13	Phytol	2.67	1.98	-	0.61	Terpene: antitumor, anti-inflammation
14	Octadecane	-	-	5.72	0.52	Alkane hydrocarbon: antibacterial activity
15	9-Eicosyne	-	-	7.11	0.75	Antimicrobial and antiviral
16	Eicosane	-	-	-	4.26	Alkanes: antibacterial
17	pseudodiosgenin	3.10	-	-	-	steroidal saponin
18	Tetracosane	-	-	10.08	7.36	n-Alkanes: antioxidant
19	Vitamin-E	0.89	1.05	-	0.72	Tocopherols: antioxidant
20	Stigmasterol	2.72	-	1.59	-	Anti-cardiovascular diseases

Meth: methanol, ETHN: ethanol, DEE: diethyl ether, HEX: n-hexane

Chapter 4: Discussion

4.1 Effect of Microwave Time and Microwave Power on Antioxidant Activity

With the increase of MW time, the temperature of extraction increased. Diffusion of bioactive compounds is expected to be faster with the high temperature which is why the extraction yield and antioxidant activity was found to be better. On the other hand, the MW power did not have a positive impact on the extraction yield, and had a complex effect on the bioactivity, even though MW increased the temperature too. The antioxidant bioactivity increased by 5% with the increase of MW power from 220 W to 400 W. This increase happened because the diffusion of bioactive compounds from the biomass to the extraction solvent is expected to increase with higher temperatures. However, when increasing the MW power from 500 W to 800 W, the bioactivity decreased. This behavior is believed to be caused by the degradation of antioxidant compounds at higher MW power, and similar trend was observed by other researchers. Pan et al. [98] extracted antioxidant compounds from *Osmanthus fragrans* flower with MAE and four different DESs. The antioxidant bioactivity increased with the increase of MW power from 300 to 500 W, then it decreased when MW power was set to 700 W. Their results show the same behavior as this research. Nie et al. [99] carried out MAE-DES of Tobacco coupled with headspace solid-phase microextraction and the same results were observed too. The extraction reached a maximum at 500 W and decreased afterward. Nie et al. [99] claimed that some of the volatile compounds are evaporating with higher MW powers. Chen et al. [97] observed the same behavior when extracting *Salviae miltiorrhizae*. The bioactivity increased initially with the increase of MW power then started declining with further increment.

4.2 Effect of Solid-liquid Ratio on Antioxidant Activity

When the solid-liquid ratio decreases, it means that there is more solvent available to extract the antioxidant compounds. This is why when the solvent used for 1 gram of herb was increased from 10 mL to 30 mL, the extraction yield increased from 40.76% to 45.46%, and bioactivity was almost tripled. This huge change makes Solid- liquid ratio extremely important in MAE-DES for *T. stocksianum*. When the ratio was further decreased from 1 g/30 mL to 1 g/50 mL, bioactivity increased by 25%. This indicates that after a certain point, increasing the amount of solvent will stop affecting the extraction positively and it will be a waste of solvent only. Pan et al. [98] found that the maximum bioactivity was achieved when the ratio was increased from 1:10 to 1:30 solid-liquid ratio and did not increase beyond that which is similar with the findings of this research, while Wang et al. [96] stated that the effect of solid-liquid ratio was positive on the extraction of Baicalin but was not as significant as the effect of other factors. Chen et al. [97] found similar results where solid-liquid ratio had little effect but, regardless, the bioactivity increased with the increase of amount of solvent. The differences between this research and others could be because of using different types of DESs or the type of herb.

4.3 Effect of DES Concentration on Antioxidant Activity

The DES concentration had a complex effect on the yield of extraction, with the increase of DES concentration from 30% to 50%, the yield of extraction increased from 32% to 45.5% while the total antioxidant capacity did not change. This could mean that the increase of DES concentration to 50% improved the extraction of un-active compounds because DES can solvate the cell wall structure of the herb which generally allows diffusion of internal compounds. When DES concentration was

increased to 70%, the yield dropped to 26% and the bioactivity decreased by 8%. This behavior is understandable because of the high viscosity of DES which hinders diffusion of the bioactive compounds to the solvent. It is true that higher DES concentrations will have better ability of dissolving the wall of plant cell, but it will increase the viscosity too, which has a negative effect on the diffusion. Balancing between the two will give the optimum DES concentration. Pan et al. [98] selected 20% DES concentration because the bioactivity decreased with higher concentrations. Wang et al. [96] expressed the DES concentration as water content, and it was observed that the yield is increasing with the increase of water content. Chen et al. [97] measured the extraction yields of five bioactive compounds and found that some compounds had the maximum yield at low DES concentration (higher water content) and other compounds had better yields at high DES concentration. Chen et al. [97] related the behavior to the hydrophobicity of the bioactive compounds. Hydrophilic compounds concentrations increased in the beginning with the increase of water content and then started to decrease with further increment. While the hydrophobic compounds concentrations were highest with pure DES.

4.4 Comparison of Extraction Yields for *T. Stocksianum* Herb

There is very limited research about the extraction of *T. stocksianum*. According to the knowledge of the author, all conducted research focused on the biological aspect of the herb or the bioactive compounds family with minimum attention given to the yield or extraction optimization. Nonetheless, some published articles mentioned the extraction yield which could be used for relative comparison.

Rahim et al. [100] used maceration with continuous shaking for 24 hours to extract *T. stocksianum* using nine solvents (acetone, butanol, chloroform, ethyl acetate,

ethanol, methanol, n-hexane, petroleum ether and water). The yield of extraction was determined and the antioxidant bioactivity using DPPH assay was carried out. Rahim et al. [100] also evaluated the chemical constituents of the extracts to identify the compounds families (i.e., alkaloids, flavonoids, saponins, terpenoids, etc.). The highest yield was achieved using n-hexane (21.6%) but the methanolic extracts contained all compounds families which is why it was used for antioxidant assessment. In this research, Soxhlet extraction using n-hexane achieved a yield of (5.6%) even though Soxhlet should, in theory, reach higher yields than the method followed by Rahim et al. [100]. This difference could be attributed to the difference in raw material. Rahim et al. [100] used the flowers of the herb while in this research the whole plant was used. Flowers are known to contain more of the volatile compounds which could be more soluble in nonpolar solvents.

Shah et al. [101] used *T. stocksianum* to extract saponins. The herb was soaked for one week in methanol (80%) giving a total yield of 6.2%, while the crude saponins were further fractionated producing a yield of 5% (1 g of crude saponins per 20 g of plant material). The samples were tested for cytotoxic bioactivity which is different than the bioactivity tested in this thesis. Soxhlet extraction using methanol in this thesis resulted in a yield of 30.27% which is much higher than what Shah et al. reported [101]. This could be attributed to the difference in extraction methods, where Soxhlet is known to give higher yields, and the difference in the content of the plant material. Even though Shah et al. [101] used the whole plant, which is done in this research too, the geographical location affects the content of the herb significantly, Shah et al. [101] collected the herb from District Swat in Pakistan while in this research the herb was collected from Khor Fakkan city in UAE.

It is worth mentioning that, Hisham et al. [102] and Shah et al. [103] used HD with Clevenger apparatus to extract *T. stocksianum* and both research studies gave a yield of 0.4%. In this thesis, reflux HD was used and it gave a much higher yield (41.35%) because the mechanism of extraction is different from what Hisham et al. [102] and Shah et al. [103] did. Reflux HD will extract any water-soluble compounds in the herb while the Clevenger apparatus does the opposite. The Clevenger apparatus is usually used to extract the hydrophobic constituents which is called Essential oils. Comparison between reflux HD and HD with Clevenger apparatus is not suitable because they differ in everything, beside the similar names and solvent.

Chapter 5: Conclusion

Industries based on Natural Bioactive Compounds (NBACs) are significantly rising due to the various favorable biological bioactivities. One of the traditionally used herbs in the UAE as a remedy was studied in this thesis. *T. stocksianum* was extracted using different extraction methods and the antioxidant bioactivity was measured.

5.1 Managerial Implications

The objectives of this research were to efficiently extract bioactive compounds for a traditional home remedy herb in the UAE using an innovative extraction method. Microwave-assisted extraction using deep eutectic solvent was used to extract antioxidant compounds from *T. stocksianum* and initial optimization of four different extraction parameters was also conducted. When comparing antioxidant bioactivity results with conventional extraction methods, the MAE-DES showed higher results (54.18% yield, 21.67 mg TE/g DW). Initial optimization of four different extraction parameters was carried out. The Microwave power and time, DES concentration and the solid-liquid ratio were optimized initially.

5.2 Research Implications

The extraction of *T. stocksianum* using MAE-DES was carried out in this research for the first time, according to the knowledge of author. The results proved the efficiency of this method in laboratory scale. However, some obstacles were faced in the current research that should be avoided in future research.

After MAE-DES was finished, drying the biomass sample was needed to measure the weight change in order to calculate the yield of extraction. Drying up DES was challenging; the solvent is hard to evaporate even when prolonged periods with high temperatures were used (150°C, 24 h). The best solution for this problem is to wash the biomass residue and filter it after finishing the extraction with cold water. The washed sample should be free from DES and the cold water will not extract the biomass anymore, thus not affecting the actual yield.

In addition, the recyclability of DES is a favorable characteristic, but it was not studied in this research. Future research should attempt to separate the extracts from the DES in order to recycle the solvent and test the pure extracts in *in-vivo* analysis.

Finally, the goal of herbal extraction is preparation of medical substances, which is why a method of encapsulation should be combined with MAE-DES if possible. Also, temperature and temperature rate could be better parameters than MW power and time in optimizing MAE-DES.

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Appendix

Table 5: Details of the major identified bioactive compounds using GCMS from Soxhlet extraction (Methanol)

#	Compounds	rt	%	Details
1	Hydroxylamine	1.422	7.27	Inorganic compound: antitumor
2	Syringol	11.737	2.51	Phenols: antimicrobial
3	Caryophyllene	12.307	2.34	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
4	humulene	12.527	1.06	Terpene: anti-inflammatory, antibacterial
5	Alloaromadendrene	12.574	2.16	Sesquiterpenoid: antibacterial antifungal
6	γ -muurolene	12.901	4.96	Sesquiterpene: antimicrobial and antioxidant
7	Spathulenol	13.221	1.38	Sesquiterpene: antibacterial and antioxidant
8	Caryophyllene oxide	13.257	2.80	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
9	Ledol	13.352	1.17	Sesquiterpenes: antifungal
10	Bisabolol	13.482	1.26	Sesquiterpenes: anti-inflammatory, and antimicrobial
11	neophytadiene	14.082	2.8	antibacterial antioxidant
12	Ledene Oxide	14.331	1.99	Sesquiterpene: Anticancer and antioxidant
13	Isomycorene	14.480	4.24	Monoterpenoids: Antibacterial activity
14	Fenchol	14.592	2.82	Terpene: antibacterial, antimicrobial, and antioxidant
15	Phytol	14.836	2.67	Terpene: antitumor, anti-inflammation
16	pseudodiosgenin	14.948	3.10	steroidal saponin
17	Vitamin-E	20.334	0.89	Tocopherols: antioxidant
18	Stigmasterol	22.943	2.72	Anti-cardiovascular diseases

Table 6: Details of the major identified bioactive compounds using GCMS from Soxhlet extraction (Ethanol)

#	Compounds	rt	%	Details
1	Hydroxylamine, O-methyl-	4.312	1.4	Hydroxylamines: Antitumor
2	2-methoxy-4-vinylphenol	11.441	1.44	Phenols: antimicrobial
3	Caryophyllene	12.307	2.71	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
4	humulene	12.527	1.80	Terpene: anti-inflammatory, antibacterial
5	Alloaromadendrene	12.574	2.51	Sesquiterpenoid: antibacterial antifungal
6	Germacrene D	12.687	0.92	Sesquiterpene: antibacterial, antifungal and antioxidant activities
7	δ -Cadinene	12.901	6.34	Sesquiterpene: antibacterial and antioxidant activities
8	Duvatriene-1,3-diol	12.936	1.75	antimicrobial
9	Spathulenol	13.221	1.47	Sesquiterpene: Anti-inflammatory and antioxidant
10	Caryophyllene oxide	13.263	2.42	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
11	Ledol	13.352	1.28	Sesquiterpenes: antifungal
12	3-Methylenecycloheptene	13.548	1.32	Cytotoxic activity
13	Epoxycholesterol	13.738	1.80	Sterols: cholesterol homeostasis
14	Alloaromadendrene oxide	14.005	1.55	Sesquiterpenoid: antibacterial antifungal
15	Neophytadiene	14.082	3.27	acyclic olefin: analgesic, antipyretic, anti-inflammatory, antimicrobial, and antioxidant
16	flexibilide	14.135	7.68	Diterpenes: Antimicrobial activity
17	Isomycorene	14.480	3.94	Monoterpenoids: Antibacterial activity
18	1-Terpineol	14.586	2.78	Monoterpene: anticancer
19	Phytol	14.830	1.98	Terpene: antitumor, anti-inflammation
20	Longiborneol	14.943	3.74	Sesquiterpene: antifungal activity antitumor activity
21	Vitamin-E	20.332	1.05	Tocopherols: antioxidant

Table 7: Details of the major identified bioactive compounds using GCMS from Soxhlet extraction (Diethyl ether)

#	Compounds	rt	%	Details
1	β -Bourbonene	12.058	0.63	Sesquiterpene: antitumor and antioxidant
2	Caryophyllene	12.313	2.10	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
3	Alloaromadendrene	12.580	1.72	Sesquiterpenoid: antibacterial antifungal
4	Germacrene D	12.693	0.96	Sesquiterpene: antibacterial, antifungal and antioxidant activities
5	Butylated hydroxytoluene	12.818	1.03	Phenols: antioxidant
6	camphene	12.913	5.18	Monoterpenes: anti-fungal antibacterial sedative, antioxidant
7	Elemene	12.942	2.48	Sesquiterpenes: antitumor, antioxidant and anti-inflammatory
8	Spathulenol	13.227	1.88	Sesquiterpene: antibacterial and antioxidant
9	Caryophyllene oxide	13.269	2.58	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
10	Ledol	13.358	1.22	Sesquiterpenes: antifungal
11	β -gurjunene	13.488	1.26	Sesquiterpene: antioxidant
12	Alloisolongifolene	13.548	1.48	Sesquiterpene: antioxidant and antimicrobial
13	Longifolenealdehyde	13.750	1.90	Antioxidant
14	9-Eicosyne	14.165	7.11	Antimicrobial and antiviral
15	Fenchol	14.497	6.01	Terpene: antibacterial, antimicrobial, and antioxidant
16	Citronellyl acetate	14.955	2.51	Monoterpenes: antinociceptive activity
17	Octadecane	19.673	5.72	Alkane hydrocarbon: antibacterial activity
18	Tetracosane	22.356	10.08	n-Alkanes: antioxidant
19	Stigmasterol	22.967	1.59	Anti-cardiovascular diseases

Table 8: Details of the major identified bioactive compounds using GCMS from Soxhlet extraction (n-hexane)

#	Compounds	rt	%	Details
1	β -Bourbonene	12.058	0.72	Sesquiterpene: antitumor and antioxidant
2	Caryophyllene	12.313	2.57	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
3	humulene	12.533	0.91	Terpene: anti-inflammatory, antibacterial
4	β -Caryophyllene	12.580	2.08	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
5	Germacrene D	12.693	0.96	Sesquiterpene: antibacterial, antifungal and antioxidant activities
6	Calarene	12.764	0.97	Monoterpenes: Inflammatory, sedative
7	camphene	12.913	5.02	Monoterpenes: anti-fungal antibacterial sedative, antioxidant
8	Elemene	12.94	2.06	Sesquiterpenes: antitumor, antioxidant and anti-inflammatory
9	Nerolidol	13.061	0.77	Sesquiterpenes: antioxidant and anti-inflammatory
10	β -germacrenol	13.210	1.02	Sesquiterpene: antifungal and antioxidant activities
11	Caryophyllene oxide	13.269	2.65	Sesquiterpene: Anticancer, Antioxidant and Antimicrobial
12	Ledol	13.352	1.18	Sesquiterpenes: antifungal
13	Germacrene B	13.488	1.90	Sesquiterpene: antibacterial, antifungal and antioxidant activities
14	Epoxycholesterol	13.744	1.09	Sterols: cholesterol homeostasis
15	Oplopanone	13.868	0.73	Sesquiterpenoids: Anti-inflammatory
16	Thunbergol	14.017	1.35	Diterpenes: antibacterial
17	Bicyclooctane, 2-methyl-	14.159	8.90	
18	Alloaromadendrene oxide	14.284	1.17	Sesquiterpenoid: antibacterial antifungal
19	Germacatrienol	14.337	1.88	Sesquiterpene: antibacterial, antifungal and antioxidant activities

Table 8: Details of the major identified bioactive compounds using GCMS from Soxhlet extraction (n-hexane) (continued)

#	Compounds	rt	%	Details
20	Duvatriene-1,3-diol	14.414	0.81	antimicrobial
21	2,4-decadienal	14.492	3.86	Cytotoxic effects, good scent
22	Fenchol	14.598	2.22	Terpene: antibacterial, antimicrobial, and antioxidant
23	Phytol	14.83	0.61	Terpene: antitumor, anti-inflammation
24	Butyldocosane	17.898	1.36	Alkanes: antibacterial
25	Eicosane	19.655	4.26	Alkanes: antibacterial
26	Vitamin-E	20.338	0.72	Tocopherols: antioxidant
27	Tetracosane	22.320	7.36	n-Alkanes: antioxidant