OPTIMISATION OF PHENOLIC COMPOUNDS EXTRACTION FROM MISAI KUCING USING RSM

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ABSTRACT

Misai kucing or the scientific name is Orthosiphon stamineus contains lots of phenolic compounds such as phenolic acid, flavanoids and antioxidants. These compounds have various benefits likes antifungal, antimicrobial, antitumor and antibacterial. Besides that, people also used it as remedy to treat the diseases. In order to get components from plant materials, normally they used extraction method. The yield of phenolic compounds extraction is dependent on the size of particles, solvent used, extraction method, power irradiation and condition. The method used for this work is microwave assisted extraction was studied by using response surface methodology (RSM) design for optimization. The 2 level factorial and Box behnken design have been used to determine the optimum extraction and to develop a quadratic polynomial. The highest peak for rosmarinic acid from the extraction of misai kucing is 2.203 at 2.00 to 2.30 minutes while the standard peak of rosmarinic acid is at 2.215 at 2.00 o 2.30 minutes. The highest yield of rosmarinic acid (RA) of 24.7971 mg RA/g DW was obtained at 66.6% of aqueous ethanol concentration with 112W microwave power for 3.75 minutes. The yield for total phenolic content (TPC) and total flavonoid content (TFC) are 48.51 mg GAE/g DW and 233.243 mg QE/gDW respectively. The optimum yields were obtained at 54.68% of ethanol at 170W of microwave power for 4.34 minutes. The lower total solid (TS) was produced higher rosmarinic acid per DW of material but higher TS contain more rosmarinic acid and hence it is affect by reduced its solubility in solvent and lower extraction yield. Longer extraction time increased rosmarinic acid extraction yield at lower TS (0.1 g/ml) because longer time needed for rosmarinic acid to diffuse in the solvent. Since solvent is not saturated at lower TS so the extraction process continues and become higher at longer time. The microwave assisted extraction provides rapid extraction of phenolic compounds without significantly compromising the extraction yield.

ABSTRAK

Misai kucing atau nama saintifiknya adalah Orthosiphon stamineus mengandungi banyak sebatian fenolik seperti asid fenolik, flavanoids dan antioksidan. Sebatian-sebatian ini mempunyai pelbagai manfaat suka antikulat, antimikrob, antitumor dan anti-bakteria. Selain itu, rakyat juga menggunakannya sebagai ubat untuk merawat penyakit. Dalam usaha untuk mendapatkan komponen daripada bahan-bahan tumbuhan, biasanya mereka menggunakan kaedah pengekstrakan. Hasil pengekstrakan sebatian fenolik bergantung kepada saiz zarah, pelarut yang digunakan, kaedah pengekstrakan, kuasa sinaran dan keadaan. Kaedah yang digunakan untuk kerja-kerja ini adalah gelombang mikro pengekstrakan dibantu dikaji dengan menggunakan kaedah permukaan respons (RSM) reka bentuk untuk pengoptimuman. Tahap 2 faktorial dan Box reka bentuk behnken telah digunakan untuk menentukan pengeluaran yang optimum dan untuk membangunkan polinomial kuadratik. Puncak tertinggi asid rosmarinic daripada pengekstrakan misai kucing adalah 2,203 di 2,00-2,30 minit sambil puncak taraf asid rosmarinic adalah pada 2,215 pada 2.00 o 2.30 minit. Hasil tertinggi asid rosmarinic (RA) daripada 24,7971 mg RA / g DW telah diperolehi pada 66.6% daripada kepekatan etanol akueus dengan 112W kuasa gelombang mikro untuk 3.75 minit. Hasil bagi jumlah kandungan fenolik (TPC) dan jumlah kandungan flavonoid (TFC) adalah 48,51 mg GAE / g DW masing-masing dan 233,243 mg QE / GDW. Hasil optimum diperolehi di 54,68% etanol pada 170W kuasa gelombang mikro untuk 4.34 minit. Semakin rendah jumlah pepejal (TS) telah dihasilkan asid rosmarinic lebih tinggi bagi setiap DW bahan tetapi TS tinggi mengandungi lebih banyak asid rosmarinic dan dengan itu ia memberi kesan dengan mengurangkan kelarutan dalam hasil pengekstrakan pelarut dan atas. Masa pengeluaran yang lebih panjang meningkat rosmarinic hasil pengekstrakan asid di TS lebih rendah (0.1 g / ml) kerana masa yang lebih lama diperlukan untuk asid rosmarinic untuk meresap dalam pelarut. Sejak pelarut tidak tepu di TS rendah supaya proses pengekstrakan itu berterusan dan menjadi lebih tinggi pada masa yang lebih lama. Microwave dibantu pengekstrakan menyediakan pengekstrakan pesat sebatian fenolik tanpa menjejaskan dengan ketara hasil pengekstrakan.

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LIST OF SYMBOLS

%	Percent
ºC	Degree Celcius
hr	Hour
etc	Etcetera
λ	Wavelength
ε	Dielectric constant
δ	Dissipator factor
wt	Weight
cm	Centimeter
mm	Milimeter
m	Meter
µm	Micrometer
nm	Nanometer
kg	Kilogram
g	Gram
mg	Miligram
ml	Mililiter
W	Watt
Min	Minute

LIST OF ABBREVIATIONS

BHA	Butylated hydroxyanisole
DW	Dry weight
Eup	Eupatorin
Sin	Sinensetin
RA	Rosmarinic acid
H_20	Water
HPLC	High Performance Liquid Chromatography
UPLC	Ultra Performance Liquid Chromatography
MAE	Microwave-assisted extraction
UAE	Ultrasound-assisted extraction
ME	Maceration
FDA	Food and Drug Administration
EtOH	Ethanol
Sec	Second
TPC	Total phenolic content
TFC	Total flavonoid content

CHAPTER 1

1 INTRODUCTION

1.1 Motivation and statement of problem

Misai Kucing is commonly known as cat's whiskers and the scientific name is 'Orthosiphon stamineus Benth'. In Java they called misai kucing as Java tea. Misai Kucing is belonging to the Lamiaceae family and can be found in Southeast Asian such as Malaysia, Indonesia and Thailand. Misai kucing has attracted the interest of researchers and peoples concern about the compound content and the benefits of it. In Malaysia, Misai kucing is help in treats various ailments because it is easy to access and consumed (Khamsah et al., 2006). Misai kucing also used as remedy for kidney stone and nephritis. The leaves of Misai kucing commonly used in Southeast Asia to treat diuresis, diabetes, rheumatism, oedema, hepatitis, eruptive fever, influenza and hypertension (Sumaryono et al., 1991). In Malaysia, they have formed the leaves of Misai kucing into the product in form of tablets, drinks, raw herbs, dried leaves and tea sachet herbs as a health drink (Ibrahim et al., 2010). Herbal tea of Misai kucing will help to improve health and treatment of kidney disease, bladder inflammation, gout and diabetes (Arafat et al., 2008). Misai kucing is popular as herbal tea in Southeast Asian.

In Malaysia, they use traditional method more than modern method to cure diseases. So that most of people have taken herbal drinks to take care of their health because they have lots of benefit. In 2002, there are four lead chronic illnesses that caused 29 million deaths which are cardiovascular disease, cancer, chronic lung diseases and diabetes mellitus (Organization, 2003). In 18 years period (1975 to 2005), the populations of Malaysia have been increased from 12.3 to 26.7 million peoples and have been increase about 8.3% to 28.96 million in between 2005 to 2010 (N. M. Amal et al., 2006). On 2006, the third National Health Morbidity Survey had conducted a survey about the chronic illness. Out of 57000 respondents, only 56710 (98.6%) had participated. Based on the survey, females got higher ranking having chronic illness which is 16.8% (16.3 to 17.3). The common illness was hypertension which is 7.9%

(7.6 to 8.2), followed by diabetes mellitus which is 4.0% (3.8 to 4.2) (N. M. Amal, et al., 2006). Lot of peoples suffer in handle stress. In Misai kucing leaves, the phenolic compounds to reduce the oxidative stress have been found by retard the lipid oxidation in biological systems. The extraction of Misai kucing contains many valuable bioactive compounds such as antibacterial, antifungal, antimicrobial and antitumour, and previous work have approved it (Saravanan et al., 2006).

The bioactive compounds contains in Misai kucing usually can get by performed solvent extraction method. The yields of bioactive compounds are depending on the extraction methods, type of solvent, pH, solid-to-liquid ratio and size of particles Misai kucing leaves (Wang et al., 2004), (Kosar et al., 2005) , (Durling, et al., 2007). Based on the previous work, extraction yield of flavonoid compounds is depend on the extraction method, temperature and solvent polarity (Sultana et al., 2009), (Lapornik et al., 2005). There are several types of methods that can be used to perform extraction of Misai kucing leaves. Previous studies have used the conventional methods such as soxhlet extraction and maceration extraction. These methods used high temperatures and perform for a longer time. After few years forward, there are better extraction methods such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and supercritical extraction. These methods have been developed based on the extraction time, yield and quality of extraction.

The extraction of bioactive compound from plant material is depending on the type of solvent used. Previous work have been studied the polarities of the phenolic range from polar to non-polar and also the range of solvents which are methanol, ethanol and water as their mixture (Cuvelier et al., 1996), (Kosar et al., 2005). Wang et al. (2004) have been explored the influence of the different solvents such as methanol, ethanol, water and acetonitrile on the amount of the extracted phenolic acids. The use of water as the extraction solvent gives 20% less rosmarinic acid compared to other solvents. The best extraction yields of caffeic acid and rosmarinic acid were obtained at 30 to 60% of aqueous ethanol solution. The most suitable solvents uses for extraction are water and ethanol mixture (Maja et.al, 2013).

Optimization is refer to the process of a system have improve to obtain the maximum benefit of it. In this case, to get the maximum amount of phenolic compounds

extraction in Misai kucing leaves, the optimal variables or parameters are required. In the present study, the response surface methodology (RSM) was examined for optimization variables such as extraction time, temperature, ethanol concentration and solid-to-liquid ratio to get the maximum yield of total phenolic compounds. The 2 level factorial designs with 2 replicate were used to determine the most significant effect of extraction. The RSM design is used to determine the optimum extraction condition.

1.2 Objectives

The following are the objectives of this research:

- To optimize the phenolic compounds extraction from misai kucing leaves via RSM.
- To develop a fast analysis for phenolic compounds from misai kucing
- To develop innovative extraction method which is fast efficient extraction of phenolic compounds from misai kucing

1.3 Scope of this research

The following are the scope of this research:

i. Extraction of the phenolic compounds from misai kucing.

The work will focus on the method use to extract the phenolic compounds from misai kucing. There are many types of extraction method such as microwave assisted, ultrasound assisted, maceration, soxhlet, supercritical and so on. Therefore, microwave assisted has been chosen to extract the compounds.

ii. Quantification or analysis of total phenolic content (TPC), total flavonoid content (TFC) and ultra-performance liquid chromatography (UPLC) of the plant leaves extract.

The work will focus on the method use to determine the total phenolic content, total flavonoid content and UPLC analysis from the plant extract.

iii. Optimization study via RSM

The RSM have been used to optimize the extraction. 2 level factorial designs with 2 replicate have been used to determine the most significant effect of the extraction and Box-behnken design model have been used to develop the quadratic polynomial. The methods help in produce the higher yield of extraction.

1.4 Main contribution of this work

Firstly, this work aim to optimize the phenolic compounds from Misai kucing leaves by using extraction methods such as microwave-assisted extraction, ultrasound-assisted extraction and maceration method. Response surface methodology (RSM) was used to examine the optimization variables or parameters to produce higher yield of extraction.

UPLC method has been used in this work to separate and identify the major active components such as rosmarinin acid, sinensetin and eupatorin. UPLC method is the best analytical method because it is take a shorter time to detect the peak or the particles. It is produce accurate qualification and quantification analysis of the Misai kucing extract.

Lastly, the total phenolic content (TPC) and total flavonoids content (TFC) are determined by using their method. TPC was determined using Follin-Ciocalteu reagent (Trabelsi, et al., 2010). The absorbance was measured at λ = 760 nm using a calibrated ultraviolet-visible spectrometer (Hitachi U-1800, Japan). TFC was determined by aluminium chloride colometric assay (Abouzid & Elsherbeiny, 2008) was measured at λ = 414 nm using a calibrated ultraviolet-visible spectrometer (Hitachi U-1800, Japan).

1.5 Organisation of this thesis

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 is review the previous work by the researchers on Misai kucing leaves from characteristics, extraction, analytical method and compound contains in the Misai kucing leaves.

Chapter 3 presents the experimental method to extract the phenolic compounds. The methods to extract the active compounds have been discussed in this part such as microwave assisted extraction, ultrasound assisted extraction and maceration method. Qualification and quantification analysis (determination of total phenolic and total flavonoid) and ultra-performance liquid chromatography was presented in this chapter.

Chapter 4 shows the results and discussion on the yield of extraction from misai kucing leaves. The optimum parameters to get better extraction yield also have been discussed in this chapter. The influences of various percentages of solvents, time and power of extractor on the phenolic compounds are presented in detail.

Chapter 5 is a conclusion and summary of the thesis and outlines of future work which might be derived from the technique developed for this work.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Overview

This chapter have reviews the previous work on Misai kucing. The famous active compounds in Misai kucing and their benefits had been discussed. Based on the previous journal, they are various methods to extract the active compounds such as microwave-assisted extraction, ultrasound-assisted extraction and maceration method. The literatures present the method of extraction that can be used to optimize the phenolic compounds from Misai kucing using response surface methodology (RSM). The best method will help to get the best result of extraction yield.

2.2 Introduction

The extraction of Misai kucing leaves have a lot of useful bioactive compounds such as terpenoids, phenolic and sterol that expose diuretic (Arafat et al., 2008), antidiabetic (Mohamed et al., 2013), antiangiogenic and antiproliferative properties (Doleckova et al., 2012). There are twenty phenolic compounds found from misai kucing. There are nine lipophilic flavones, two flavonol glycoside and nine caffeic acid derivatives such as rosmarinic acid, sinensetin, eupatorin and 2,3,-dicaffeoyltartaric acid (Akowuah et al., 2004). In aqueous methanol extracts, caffeic acid derivatives have become the most abundant polyphenol. They also will be appearing in the polymethoxylated flavones predominate. The special part found in Misai kucing is polymethoxylated flavones, because of the rare structural features which is the methoxy group at C-5. The other groups of chemically active constituents also are found in Misai kucing likes terpenoids such as diterpenes and triterpenes and sterols.

Misai kucing contain phenolic compounds that have great interest due to their health-benefit antioxidant properties. The examples of antioxidants are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Spigno & de Faveri,

2007). The presences of phenolic compounds which are flavonoids have made the Misai Kucing popular in search engine. The researchers believe that the leaves of misai kucing have antiallergenic, antihypertensive and anti-inflammatory properties. Phytochemicals are popular to have several health-benefit properties, reduce the risks of cancer, cardiovascular, heart and neurodegenerative diseases (Dahmoune et al., 2015).

2.2.1 Misai kucing plant characteristic

Table 2. 1: The characteristics of Misai kucing plant

CHARACTERISTICS	DESCRIPTION
Height	• It can grow up to 1.5 metres.
Stem	• Rectangular and it easy to break
Leaves	• Opposite, oval-shaped, acuminate and grossly dentate, elongated and pointed.
Flowers	• Lax and grow in terminal pseudo spikes. • Pale violet to white colour with remarkably long stamens extending far beyond the flower.

Based on Table 2.1, the characteristic of the plant have shown about its height which can grow up to 1.5 metres. The stem of the Misai kucing plant is in rectangular shape and it is easy to break. There are two types of Misai kucing plant which are Orthosiphon stamineus (white flower) and Orthosiphon aristatus (purple flower).

2.3 Previous work on Misai kucing

Table 2. 2: Previous study on Misai kucing

Author	**Study**	**Remarks**
Arafat et al. (2008)	Biological activity	Analyze the diuretic impact of a methanol extract of O. stamineus in regular rat.
Akowuah et al. (2004)	HPLC method development and biological activity	Separation was developed from HPLC method to determine the methoxylate flavones (sinensetin, eupatorin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone and rosmarinic acid) and estimate the antioxidative properties.
Anna et al. (2008)	Physiological and biological activity	Carry out the physiological studies by using gamma irradiation and evaluate the concentration of rosmarinic acid.
Olah et al. (2003)	Biological activity and HPLC method development	Higher diuretic and uricosuric action have been found in 50% ethanol extract compared to 70% ethanol extract. HPLC method was used for quantification analysis.
Maheswari et al. (2008)	Biological activity	O. stamineus extract has decreased the level of lipid peroxidation and extraction of O. stamineus leaves contain hepatoprotective activity.
Adam et al. (2009)	Biological activity	O. stamineus have shown contain diuretic activity but it was less effective than hydrochlorothiazide and furosemide.
Yasuhiro et al. (2000)	Biological activity	Examine the diterpene compound from methanol extraction.
Amzad et al. (2008)	Biological activity	O. stamineus extract possess the anti-fungal properties of the essential oil and crude extraction.

Table 2. 3: Size of sieve tray based on previous study

Author	Title	Plants	Size of sieve tray
Chung et al. (2013)	Modelling and prediction of extraction profile for microwave assisted extraction based on absorbed microwave energy.	Theobroma cacao L. leaves (cocoa)	0.25 – 0.60 mm
Dahmoune et al. (2015)	Optimization of microwave-assisted extraction of polyphenols from Myrtus communis L. leaves.	Myrtus communis L. leaves	125 µm
Zhizhe et al. (2014)	Comparison of four kinds of extraction techniques and kinetics of microwaves-assisted extraction of vanilla planifolia Andrews.	Vanilla planifolia Andrews	0.630 mm
Ma et al. (2013)	Microwave-assisted aqueous two-phase extraction of isoflavonoids from Dalbergia odorifera T. Chen leaves.	Dalbergia odorifera leaves	30-80 mesh
Ghasemzadeh et al. (2014)	Optimization of ultrasound-assisted extraction of flavonoid compounds and their pharmaceutic activity from curry leaf (Murraya koenigii L.) using RSM.	Murraya koenigii L. (curry leaf)	80 mesh
Majid et al. (2014)	Optimization of ultrasonic-assisted extraction of phenolic compounds from bovine pennyroyal (Phlomidoschema parviflorum) leaves using RSM.	Phlomidoschema parviflorum leaves	149 µm
Vetal et al. (2014)	Microwave-assisted extraction of urolic acid and oleanolic acid from ocimum sanctum.	Ocimum sanctum leaves	0.50-1.0 mm
Zhang et al. (2014)	Ultrasound-assisted extraction of bergenin from Astilbe chinensis.	Astilbe chinensis leaves	250 µm

2.4 Analysis methods for bioactive compounds from Misai kucing leaves

The leaves of Misai kucing have lots of benefits to human to treat various diseases such as diuresis, diabetes, rheumatism, oedema, hepatitis, eruptive fever, influenza and hypertension. There are lots of compound that will be good to human like antioxidant itself can help in treat various ailment. The one of most important step is extraction of compound from plant material. Extraction is a mass transfer process. The previous studies have used conventional extraction methods to extract phenolic compounds from Misai kucing leaves because they have found out that active compounds are more able to extract by using lower polarity solvent. The old version of solvent extraction such as heating, boiling or refluxing are associated with longer extraction times and lower production yield. It also used large quantity of organic solvents and bad extraction efficiency (Shirsath et al., 2012), (Zhang et al., 2011).

The extraction of phenolic compound from misai kucing leaves usually used conventional extraction method because it is provide more area for improvement such as to reduce the extraction time without affect the quality of extraction. Maceration is one of the conventional extraction method had been found by researcher Akowuah et al. (2004) that sinensetin and eupatorin are more able to extract by using lower polarity solvent. Maceration method also able to extract active component of O. stamineus such as toxicity and biological activity test but Olah et al. (2003) and Mohamed et al. (2011) have approve that maceration method take a longer time to extract the active compound. Olah et al. (2003) able to get the extract of active component after 5 days by using 50% and 70% ethanol while Mohamed et al. (2011) have used 50% ethanol and able to extract the toxicity in 24 hours.

Many researchers have studies how to improve the extraction method of phenolic compound due to lack of previous method. Recently, they have been develop better extraction methods of phenolic compound such as microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), accelerated solvent extraction (ASE) and supercritical extraction (Zhang et al., 2011). Microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE) are accepted because the function of this method can cut down the working time, increase yield and able to keep the quality of extract.

2.4.1 Microwave assisted extraction method

Microwave extraction method is the process of heating solvents in contact with sample with microwave energy to partition compound. Microwave is an electromagnetic radiation that can transmit as a wave. The two principle of heating using microwave energy are ionic conduction and dipole rotation. Heat and mass gradient must work in the same way to produce higher extraction yield (Chemat et al., 2009). Heat is dissipated volumetrically inside the irradiated medium in MAE. The most important factor is solvent to select microwave physical constants. It is important to select a solvent that have high extracting power and strong interaction. As a new method extraction, MAE is known as a more environmental-friendly process with economic advantages than the traditional extraction methods. Microwave-assisted extraction is the simplest and the inexpensive technique for the extraction of nutraceuticals (Hemwimon et al., 2007). Recently, this technique has been commonly used for sample preparation (Chen et al., 2008). In the present study, the extraction of polyphenols was under microwave dry process without adding any organic solvent or water, which is different from conventional solvent extraction techniques. The benefits of MAE are the extensive reduction in time and solvent consumption with better extraction yield (Eskilsson & Bjorklund., 2000) (Wang & Weller, 2006). Besides, it is also produce higher extraction rate and keep the quality of the extraction.

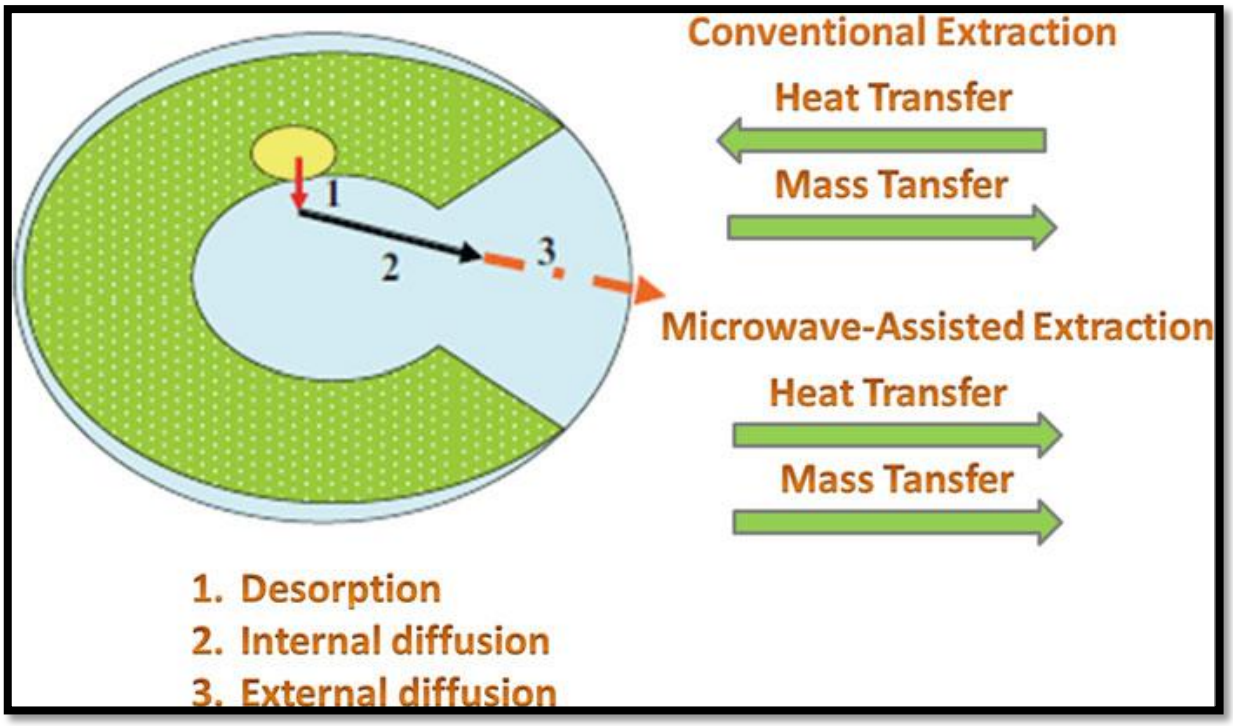

Figure 2. 1: Basic heat and mass transfer mechanisms in microwave and conventional extraction (Perino-Issartier et al., 2011)