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Extraction methods for *Escherichia coli* antibacterial assay

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ABSTRACT

The recent increased interest in plant-based medication and dietary supplements has resulted in researchers from various fields of ethnopharmacology, botany, microbiology, and natural product chemistry scouring the planet for phytochemicals and "leads" that might be used to treat infectious diseases. However, even though about 25 to 50% of today's medications come from plants, none of them is employed as antimicrobials. Western medicine is attempting to replicate the effectiveness of traditional healers who have employed plants for a long time to prevent or treat infectious diseases. Secondary metabolites that have been shown to have antimicrobial activities in vitro include tannins, terpenoids, alkaloids, and flavonoids, which are abundant in plants.

Plants comprise a complex variety of metabolites and bioactive compounds. Since extraction is the first step in obtaining herbal plant components, many factors must be considered while choosing the best extraction techniques. The correct extraction techniques employed will ensure that the maximal plant compounds are produced sufficiently for the required antibacterial assays. This review discusses several traditional and more recently developed plant extraction methods specifically used for antibacterial assay and includes an overview of the general idea, benefits, and drawbacks of common extraction techniques.

Keywords: extraction, antibacterial assay, *Escherichia coli*

INTRODUCTION

The World Health Organization 2020 report on global deaths stated that infectious diseases from respiratory illnesses ranked as the 4th leading cause of global death while the WHO 2022 report unsurprisingly positioned infectious disease as the most predominant cause of global mortality with 18 million excess deaths in 2021 (World Health Organization). Infectious diseases are defined as proven illnesses caused by pathogenic microorganisms such as viruses and bacteria (Yeo et al., 2014; Situmeang et al., 2019).

Escherichia coli is a Gram-negative bacterium that is commonly found in the lower intestines of humans and animals. While some are harmless and help maintain the balance of normal intestinal flora (bacteria) against harmful bacteria and synthesize or produce some vitamins, some *Escherichia coli* strains have been implicated in serious poisoning (Mueller, 2022).

An example of this is the Shiga toxin-producing *E. coli* (STEC), which can cause severe foodborne diseases. It is transmitted to humans primarily through the consumption of contaminated foods, such as raw or undercooked ground meat products, raw milk, and contaminated raw vegetables and sprouts. STEC produces toxins, known as Shiga-toxins because of their similarity to the toxins produced by *Shigella dysenteriae*. STEC can grow in temperatures ranging from 7°C to 50°C, with an optimum temperature of 37°C. Some STEC can grow in acidic foods, down to a pH of 4.4, and in foods with a minimum water activity (a_W) of 0.95 (WHO, n.d.).

Another strain of interest is *E. coli* O157:H7 which causes severe intestinal infection in humans. It is the most common strain to cause illness in people. It can be differentiated from other *E. coli* by the production of a potent toxin that damages the lining of the intestinal wall causing bloody diarrhoea (Lim et al., 2010). It is also known as an enterohemorrhagic *E. coli* infection. The Centers for Disease Control and Prevention (CDC) reports about 70,000 cases of this type of *E. coli* infection occur in the United States each year (Center for Disease Control, 2022)

Additionally, *E. coli* strains have been found to confer resistance against many antibiotics, especially in Extended Spectrum Beta Lactamase *E. coli* (ESBL). In a study by Wu et al. (2021), Minimum Inhibitory Concentration assays carried out in 100 *E. coli* strains from a neonatal ward, it was found that 26% of strains were multidrug resistant while the rates of resistance to amoxicillin, cefuroxime and sulfonamides were 65, 60 and 47%, respectively.-

Antimicrobial agents are substances that can kill or constrain the growth of pathogenic microorganisms (Yeo et al., 2014; Abas et al., 2018). Antibiotics are an example and are the most common treatment of infectious diseases. Nonetheless, WHO has found that most conventional antimicrobial agents like ampicillin and ciprofloxacin are incompetent against bacterial pathogens (Nayak et al., 2015). Thus, many researchers redirect their efforts to search for natural plants that have medicinal values as potential antimicrobial agents.

Extraction is the crucial first step in the analysis of medicinal plants because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. Plant extraction reveals a relatively complex mixture of metabolites and bioactive molecules like alkaloids and flavonoids (Wang & Weller, 2006). The selection of the most appropriate extraction method assures that potential active constituent are not lost, distorted, or destroyed during the preparation of the extract from plant samples.

Plant extraction methods comprise the selection of various solvents depending on the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol, or ethyl acetate. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in the ratio of 1:1 is used. In some instances, extraction with hexane is used to remove chlorophyll (Cos et al., 2006).

As the target compounds of the plants vary in their polarity (non-polar to polar) and thermal lability, the importance of a suitable extraction method cannot be undermined. Methods commonly used are maceration, sonification and Soxhlet extraction (United States Pharmacopeia and National Formulary, 2002). This review discusses the principle, advantages, and disadvantages of frequently used and newer extraction approaches with some examples of studies.

Extraction Methods

The 'extraction' term used in pharmaceutical studies is defined as the separation of soluble medicinally active plants or animal metabolites using selective solvents through standard procedures, leaving behind the insoluble cellular tissue (Wang & Weller, 2006). Various conventional extraction methods have been utilized since years ago namely maceration and Soxhlet system (Naviglio et al., 2019, Sasidharan et al., 2011, Abubakar & Haque, 2020). Naviglio (2019) added that new advanced technologies have been invented to overcome the drawbacks of conventional extraction techniques which are termed as non-conventional such as microwave-assisted and ultrasound-assisted extraction. These more recently developed techniques place a strong emphasis on being 'green', i.e safer on the environment in those lesser harmful solvents that are

corrosive and harmful to the environment are used and replaced with other cleaner technologies. In this review, 5 methods both conventional and recently developed will be discussed. The two conventional methods are maceration and Soxhlet extraction methods while the more recently developed are Microwave-Assisted Extraction (MAE), Ultrasound-Assisted Extraction (UAE) and Supercritical Fluid Extraction (SFE) techniques.

Maceration and Percolation Processes

A maceration process involves coarsely powdered plant material being placed in a closed container with selected solvents and sets aside at room temperature for at least three days along with regular agitation to ensure complete dissolution of the soluble matter (Cunha et al., 2004). The final crude extract is then further separated by filtration. A percolation process is a form of maceration where the plant material is moistened with a suitable amount of boiling water and left to stand for approximately four hours using a percolator (Handa et al., 2008). The valve of the percolator is later opened, and the liquid is allowed to drip slowly until the extraction is completed.

The advantages of these extraction methods are that they are traditional techniques which are easy to perform and do not require complicated or expensive equipment (Ngaha Njila et al., 2017). More importantly, this technique can be applied in deeper parts of a forest or jungle where the bioresources are most likely to be (Cunha et al., 2004). However, the drawbacks of these processes are the use of a huge amount of solvents and a longer time for extraction is needed where they are not only time-consuming, the extended periods of extraction can potentially cause undesirable chemical changes in the plant extracts (Yeo et al., 2014, Phrompittayarat et al., 2007).

Soxhlet Extraction

The Soxhlet extraction method involves finely ground plant material being added into a thimble made of strong filter paper or cellulose. The thimble is then placed in the thimble chamber of a Soxhlet apparatus (Huie, 2002). A bottom flask is then filled with extracting solvent and heat. Subsequently, the vapours will condense into a condenser which drips back into a thimble containing plant material and extracts it by contact. When the solution level reaches the siphon arm and overflows, the solution is unloaded back into the bottom flask and the cycle is repeated (Handa et al., 2008).

Handa et al. (2008) also listed that the strength of this method is that it does not need any extra filtration steps and it can maintain a quite high temperature of extraction with heat from the distillation flask. The Soxhlet method is faster than maceration and applicable to extracting high boiling substances (Naviglio et al., 2019). Another advantage of this method is that is useful for the extraction of partially soluble plants (Zygmunt & Namiesnik, 2003). The limitations are that a large volume of solvents is needed and there is a possibility for the thermal decomposition of target compounds

to happen because the extraction process happens at the boiling point of the solvent for a long time (Handa et al., 2008)

Microwave-Assisted Extraction (MAE)

The use of microwave technology for organic compound extraction was first reported in 1986 by Ganzler and colleagues. MAE is a process where heat from the electromagnetic wave is directly transmitted to the solid plant material with no absorption by the microwave-transparent solvent. Intense heating causes the heated moisture to evaporate and breaks the cell walls by generating high vapour pressure. This method is commonly used for essential oil extraction.

Being selective and quick in compound extraction as well as using low energy and solvent usage are the privileges of the MAE method (Abdennabi et al., 2017). Moreover, the extraction time can be reduced by increasing the microwave power (Rezvanpanah et al., 2011) Other applications of this technology include the detection of organic contaminants in food (Moret, 2019). Apart from these advantages, MAE has several other advantages including a higher extraction rate and lower cost, over the traditional method of extraction of compounds from various matrices, especially natural products (Delazar et al., 2012). Contrarily, Wang and Weller (2006) revealed that this method requires an additional step of filtration or centrifugation to remove the solid residue and it does not apply to non-polar and volatile solvents or targeted compounds.

Ultrasound-Assisted Extraction (UAE)

This technique utilizes ultrasound with frequencies ranging from 20 kHz to 2,000 kHz, where its mechanical effect will induce better penetration of solvent into plant cellular material and at the same time improve the mass transfer (Wang & Weller, 2006; Handa et al., 2008). In addition, extraction effectiveness can be influenced by cell disruption and mass transfer factors (Wang & Weller, 2006).

The UAE process is cheap, simple, and efficient as an alternative to conventional extraction methods (Garcia-Castello et al., 2015). Moreover, it uses low operating temperatures and is thus suitable to extract thermolabile constituents as well as various types of solvents can be used for extraction (Chemat et al., 2017). Conversely, the use of high ultrasound energy can cause the degradation of certain active phytochemicals and the solid plant material is completely crushed in the process making it difficult to separate into its components (Naviglio et al., 2019). Additionally, the common use of ultrasound bath and probe as the most used UAE equipment has drawbacks is variation in the effect of ultrasound waves which is dependent on container positioning as well as a lack of efficiency in the energy transfer within the vessel containing the extract (Carreira-Casais et al., 2021)

Supercritical Fluid Extraction (SFE)

The use of Supercritical Fluid Extraction Systems is a promising method for drug discovery from natural sources. These methods offer advantages such as methods relatively short processing times, producing extracts with little

or no organic co-solvent and can extract bioactive molecules whilst minimising degradation (Khaw et al., 2017). Supercritical fluid extraction (SFE) provides a range of benefits, as well as offers routes to overcome some of the limitations that exist with the conventional methods of extraction.

A supercritical state is achieved when the temperature and pressure of a material are elevated over its critical value. SFE system is equipped with temperature and pressure controllers to maintain the desired condition throughout the extraction process (De Melo et al., 2014). The raw plant material is loaded into an extraction vessel before being pressurized with the fluid by a pump. The fluid salvation power is reduced when both fluid and dissolved elements are transported to separators. The product is then collected via a valve located in the lower part of the separators before the fluid is recycled.

The presence of controllers in the SFE system makes it possible to manipulate the temperature and pressure based on the solubility of a substance. Furthermore, the targeted compound can be separated from the supercritical solvent without a loss of volatiles (Wang & Weller, 2006). Yet, SFE requires high operating costs and is not universally relevant because of the water interference contained in plant material (Naviglio et al., 2019; Gwiazdowska et al., 2022; Capuzzo, 2013).

Table 1 outlines the principles of applications, advantages, and disadvantages of each of the five extraction methods discussed above.

Table 1 Principles of applications, advantages, and disadvantages of different types of extraction methods

Method	Principle of application	Advantages	Disadvantages
Soxhlet extraction	Finely ground plant material is solvent extracted via heated vapour using soxhlet apparatus.	• A very simple and inexpensive method. • The temperature in the extraction system can be maintained. • It can extract substances that are partially soluble in a solvent.	• Requires excessive extraction times using large amounts of extractants (solvent) and no agitation that can accelerate the process of thermal decomposition of the heat-sensitive compound. • A prolonged period is required to obtain the products. • A disadvantage is that it is not an efficient method if the substance is fully soluble.

Maceration and percolation	Powdered plant material is placed in a closed container with selected solvents and sets aside at room temperature for at least three days along with regular agitation and filtering.	• Shorter extraction time with a high percentage of oil recovery. • The simplicity of the process and cost-effectiveness.	• The lower yield produced and solvent use. • A large quantity of inert material (ballast) that has no therapeutic value is extracted.
Microwave-assisted extraction (MAE)	The heat from the electromagnetic wave is directly transmitted to the solid plant material with no absorption by the microwave-transparent solvent	• Rapid extraction; a small amount of solvent; relatively low additional costs. • Applicable for both industrial and laboratory scales less time-consuming than conventional methods can provide high returns on capital investment. • Solvent recycling can be achieved through all methods. • Pure extraction yield can be attained.	• Use of high pressure and temperature; a limited amount of sample; non-selective (a large number of compounds extracted). • The efficiency of microwaves is very poor for nonpolar target compounds or solvents or extremely viscous solvents not appropriate for heat-sensitive compounds expensive equipment and difficult to operate.
Ultrasound-assisted extraction (UAE)	This technique utilizes ultrasound with frequencies ranging from 20 kHz to 2,000 kHz.	Rapid extraction; a small amount of solvent; relatively low additional cost.	Non-selective
Supercritical fluid extraction (SFE)	Process of separating one component (the extractant) from another (the matrix) using supercritical fluids as the extracting solvent.	• Rapid extraction, small amount of organic solvent or no solvent; no solvent residue. • Preserves thermally labile compounds; tunable solvent (SCF) density, selective extraction (small number of compounds extracted). • Inexpensive to operate/ run. • Solvent (CO_2) is inexpensive.	• High setup cost, technical knowledge of SCF properties required (e.g. phase behaviour, cross-over region). • Specialized equipment is required. • Loss of desired compounds with improper solvent selection.

DISCUSSION

The richest bioresource of drugs for modern pharmaceuticals, nutraceuticals, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs is comprised of medicinal plants. Chemical terpenes, perfumes, flavours, cosmeceuticals, and health beverages are all derived from medicinal and aromatic plants (MAPs) (Mathur & Hoskins, 2017).

The creation of herbal medicine preparations (i.e., extracts), utilising a variety of methods from basic traditional technologies to modern extraction techniques, is the first stage in the value addition of MAP bioresources. The word "extraction" refers to the typical process of using selected solvents to separate the medicinally active components of plant (and animal) tissues (Handa, 2008). These extraction methods separate the plant's soluble compounds from the insoluble cellular components. The products are mixtures of metabolites in liquid, semisolid, or dry powder form after the solvent has been removed. To obtain therapeutically effective amounts of crude medicines (medical plant components), standardised extraction processes are used (Sasidharan et al., 2011).

Standardized extraction techniques are applied to obtain the therapeutically required components of crude pharmaceuticals (medical plant parts) and remove undesirable substances through treatment with selective solvents. All these items contain a complex mixture of several plant metabolites that have therapeutic properties, including lignans, alkaloids, glycosides, terpenoids, and flavonoids (Abubakar & Haque, 2020). An extract may undergo additional processing using various fractionation methods to separate specific chemical components, such as vincristine, vinblastine, hyoscyamine, hyoscine, pilocarpine, forskolin, and codeine.

Conventional and non-conventional extraction methods of medicinal plants have been described by several authors and these include decoction and accelerated solvent extraction (Azwanida, 2015) as well as Abubakar and Haque (2020) who also described thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), paper chromatography (PC), and gas chromatography (GC) as methods used in separation and purification of the secondary metabolites. However, some differences exist between this review and others. This is the first known review on extraction methods specifically for antibacterial assays where high yields and variable polarity are of essence against pathogenic bacteria. This review also only focuses specifically on extraction methods while others include pre-extraction steps such as drying (Azwanida, 2015).

In this review, five extraction methods for antibacterial assay using medicinal plants are discussed: Soxhlet extraction, maceration and percolation, Microwave Assisted Extraction, SPE and UAE. While each method possesses advantages and drawbacks as summarised in Table 1, the eventual selection of methods primarily depends on experimental condition requirements as well as availability or equipment. Furthermore, the efficiency of each method is influenced by factors such as solvent types, agitation and temperature which eventually affect the amount of yield extracted and compound contents.

In general, while conventional extraction techniques such as maceration and Soxhlet extraction require simple and cheaper devices they are more time-consuming. In addition, most of the conventional methods require a huge amount of solvent which is detrimental to the environment. More recently developed extraction methods involve 'greener' processes which require little or no solvent for acquiring high yields of extracts. These include using ultrasound, microwave, and supercritical fluid extraction methods. A major drawback of these greener methods will be the high cost of the instrument required as well as expertise.

Several studies describe the variable use of the above-described extraction methods for medicinal plants. For instance, studies from Hassim et al. (2015) research illustrated different solvents used during extraction possessed numerous percentage yields of *Polygonum minus* (kesum) leaves extracts. Methanol extract produced the highest amount with 31.17% and showed moderate antibacterial activity against *Escherichia coli* than distilled water extract. In short, the extraction yield is depending on the solvent chemical structure and polarity used. Besides, a different compound present in different solvent extracts possibly affects the differences in bacterial inhibition ability.

Another study showed that methanol extract of *Aloe barbadensis* (aloe vera) leaves through 48 hours' maceration on a shaking incubator was the most effective when tested in an antibacterial assay against *E. coli* strain (Irshad et al., 2011). The final quantity of extracts can also be maximized by choosing the appropriate extraction technique. Research done by Xainhiaxang et al. (2018) reported that A*llium sativum* (garlic), *Alpinia galanga* (galangal) and *Azadirachta indica* (Neem) produced higher yields when extracted using the SFE method compared to hydrodistillation technique. However, when tested against *E. coli* only galangal SFE extract exhibits a lower MIC value and is capable to inhibit the bacteria growth.

On the other hand, the percolation method has been suggested to give better extract compared to maceration and Soxhlet methods because the former technique increased the contact time of *Hamelia patens* crude with ethanol producing higher yield and antimicrobial activity against *E. coli* (Paz et al., 2018). From both cases, it was revealed that a high amount of products do not always indicate the high antimicrobial activity of the extracts.

Although maceration is a simple method, it requires a longer extraction time. Hence, a method such as ultrasound-assisted extraction which has a shorter time of extraction is preferable. It has been demonstrated that *P. minus* ethanolic extract yield from UAE is significantly higher than the maceration technique and the extraction time is reduced to about 98.61% (Imelda et al., 2014). To add, the extract exhibited the greatest inhibition zones when tested against *E. coli*.

Certain factors like temperature may also need to be evaluated before extracting thermolabile compounds. For instance, *Hibiscus sabdariffa* (Roselle) calyx extracted using microwave power 10 contains high phenolic contents, however, only extract from power 50 exhibits the highest antibacterial activity against *E. coli* (Alam et al., 2019). It is likely the phenolic compounds are oxidized by heating, thus reducing the efficiency of the extract.

CONCLUSION AND WAY FORWARD

It can thus be concluded that each plant extraction method has its strength and limitation. Therefore, more research needs to be done to evaluate the optimization setting of respective methods regarding their influential parameters as each extraction process is unique to different plants. Factors such as plant species, research objectives and types of bioactive compounds targeted can also be considered when choosing the most applicable technique. However, more efforts need to be placed in developing techniques that are less harmful to the environment i.e usage of a lesser degree of solvents as well as cheaper to run and simple set up. As many potential bioresources for biomedicines are located in many Third World Countries of the world such as India and other parts of Southeast Asia, the use of simpler and cheaper equipment that can be used in mobility and remote areas will be a great advancing factor of drug discovery in these parts of the world.

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