(Online) ISSN 2303-4521
Periodicals of Engineering and Natural Sciences *Original Research*
Vol. 13, No. 2, June 2025, pp.417-424

Solubility of phytochemicals and challenges in *in vitro* studies: a literature review

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ABSTRACT

Poor solubility remains a critical barrier in the *in vitro* evaluation of phytochemicals, many of which are hydrophobic and difficult to dissolve in aqueous media. This review explores the physicochemical factors influencing phytochemical solubility, emphasizing the role of solvent properties such as polarity, proximity, and cytotoxicity. Commonly used solvents—including polar protic, polar aprotic, and non-polar solvents — are discussed concerning their solubilizing capacity and compatibility with biological systems. Solvent-induced changes in membrane dynamics and cytotoxic profiles are also examined, highlighting the need for cautious selection and optimization. Several advanced strategies to enhance solubility, such as co-solvent systems, pH modulation, nanocarrier encapsulation, surfactants, and deep eutectic solvents (DESs), are reviewed. A focused case study on curcumin illustrates how different solubilization methods can significantly improve *in vitro* performance. The review underscores the importance of standardized solvent reporting to ensure reproducibility and reliability in phytochemical research.

Keywords: Solubility, Phytochemicals, *In vitro*, Solvents, Curcumin

1. Introduction

An initial step in drug discovery and development involves using *in vitro* cell culture assays, which provide insights into the cytotoxicity of the compounds [1], [2]. Over 70% of compounds under investigation suffer from very low water solubility or are classified as insoluble [3]. Phytochemicals, despite their pharmacological potential, often belong to this category [4]. Phytochemicals are diverse plant based bioactive compounds that have gained considerable attention because of their anti-inflammatory, antioxidant, antimicrobial, and anticancer properties [5], [6]. However, their practical use is hindered by challenges such as poor solubility, instability, and low bioavailability [7]. The solubility of a phytochemical is primarily determined by its characteristics and the type of solvents employed [8]. Moreover, the composition of cell culture media can significantly influence both the structural integrity and physiological activity of the phytochemicals [9]. Therefore, it is essential to use an appropriate solvent as a vehicle to dissolve bioactive compounds in biological experiments [10], [11]. An important factor in solvent selection in *in vitro* assays is its compatibility with both the culture medium and cells, as the level of cytotoxicity varies according to the solvent and its concentration [12]. Moreover, the efficiency of a solvent is largely influenced by its physicochemical properties, such as polarity, proximity, and viscosity. These properties can affect how a solvent interacts with different classes of phytochemicals [13], [14].

Organic solvents, which are carbon-based, are commonly used in drug delivery research [15]. The characteristics of these solvents are defined by their volatility, boiling point, molecular weight, and color [16]. Based on some studies, organic solvents could be toxic to mammalian cells [17], [18]. Yet they have been utilized in experiments to solubilize hydrophobic compounds [17], [18]. Due to possible cytotoxicity, selecting an appropriate solvent concentration is crucial [15]. Commonly used organic solvents include dimethyl sulfoxide (DMSO), ethanol, and acetone [11], [12], [15].

A major limitation in the current literature is the inconsistent reporting of solvent use, concentration, and solubility status. Many studies fail to confirm whether a phytochemical is fully dissolved or present in

suspension. This oversight can compromise reproducibility and result in misleading conclusions regarding bioactivity. Best practices include documenting solvent type, volume, final concentration in culture medium, solubility confirmation (e.g., via visual inspection or UV-visible spectroscopy), and solvent-only control experiments [19].

This review critically examines the solubility challenges associated with phytochemicals in *in vitro* systems and explores solvent strategies, compound properties, compatibility considerations, and innovative solutions for improved solubilization.

1.1. Solvent properties and their role in compound solubility

Thermodynamic interaction between the solvent and the compound plays an important role in the solubility of the solute. Polar solvents are expected to dissolve polar compounds through hydrogen bonding interactions. In contrast, non-polar solvents are suitable for solubilizing non-polar compounds by providing a hydrophobic environment [20], [21]. Hence, it is beneficial to classify the selected solvent based on its polarity and proximity to be able to predict its solubilizing capacity for the compound of interest.

1.1.1. Polar protic solvents

Polar protic solvents have hydrogen-bond donating hydroxyl or amine groups. Widely used polar protic solvents include water, ethanol, methanol, and isopropanol [14]. The selection of polar protic solvents depends on two important factors: the solubility enhancement and biocompatibility [22]. For instance, even though ethanol and methanol exhibit low cytotoxicity compared to some other organic solvents such as chloroform, the utilization of these solvents should be within a controlled concentration range in *in vitro* assays [22]. According to Nguyen, T. T., et al. (2019), at a concentration ranging from 0.15% to 1.25%, ethanol and methanol were well tolerated by HepG2, MDA-MB-231, MCF-7, and VNBRCA1 cell lines [11]. Another study has shown that DMSO at a range of 0.5% to 5% exhibited a greater cytotoxicity with an IC50 value of 1.8%–1.9% (v/v) compared to ethanol with an IC50 value of >5% (v/v) in MCF-7, RAW.264.7, and HUVEC cell lines [15].

Facilitating the entry of the soluble compound into and through the lipid bilayer is another important element that should be noted. Dyrda et al. found that methanol, compared to DMSO and ethanol, caused a slight increase in the fluidity of the phospholipid system within the non-polar core of the membrane. Giovenco and Anwar investigated the effect of ethanol on the structure of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane in a concentration-dependent manner. Their findings reveal that ethanol at a concentration below approximately 12% (mol/mol) causes an expansion of the membrane, a reduction in its thickness, and also disordering and enhancement of the interdigitation of lipid acyl chains. [24]Despite these changes, the membrane's structure remained intact. Notably, when the concentration of ethanol exceeded 12% (mol/mol), more pronounced changes in the membrane structure were observed. The formation of multiple transient defects at the lipid-water interface is one of these changes[24]. Another study on the cell line from young stems of Taxus cuspidate demonstrated that a concentration of >1% (v/v) of DMSO did not negatively affect the cell membrane. However, ethanol, even at a low concentration of 0.4% (v/v), has disrupted cell membrane integrity, especially in the long term[25].Facilitation of entrance of the soluble compound into and through the lipid bilayer is another important element that should be noted. Dyrda et al. found that methanol compared to DMSO and ethanol caused a slight increase in the fluidity of the phospholipid system within the non-polar core of the membrane [23]. Giovenco and Anwar investigated the effect of ethanol on the structure of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane in a concentration dependent manner. Their findings reveal an expansion of the membrane, a reduction in the thickness of the membrane, and also disordering and enhancement of the interdigitation of lipid acyl chains are caused by ethanol at a concentration below approximately 12% (mol/mol) [24]. Despite these changes, the structure of the membrane remained intact. Notably, when the concentration of ethanol exceeded 12% (mol/mol), more apparent changes in the structure of the membrane were observed. Formation of multiple transient defects at the lipid-water interface is one of these changes [24]. Another study on the cell line from young stems of Taxus cuspidate has demonstrated that the concentration of >1% (v/v) of DMSO did not affect the cell membrane negatively. But, ethanol, even at a low concentration of 0.4% (v/v), has caused disruption in cell membrane integrity, especially in a long-term manner [25].

1.1.2. Polar aprotic solvents

Polar aprotic solvents do not donate hydrogen bonds; therefore, they are capable of solubilizing moderately polar to non-polar compounds [26]. One of the most commonly used solvents in biological research, dimethyl

sulfoxide (DMSO), acetone, and acetonitrile belong to this category [27]. DMSO is commonly employed as a solvent for hydrophobic compounds in biological research [28], [29], [30]. Despite its popularity due to the effectiveness in solubility of a wide range of compounds, the utilization of DMSO is highly dose-dependent in regard to the toxicity concerns [27], [31]. Based on a study performed by Galvao, J., et al. (2014), DMSO exhibited cytotoxicity at concentrations >1% (v/v) in a retinal neuronal cell line *in vitro*. In addition, they have demonstrated that 5 µl of the intravitreally dosed DMSO induced retinal apoptosis in rats [22]. There are several studies confirming the capacity of DMSO to enhance membrane permeability [32]. This solvent can facilitate the transportation of the soluble compound across the membrane by integrating into the lipid bilayer and causing thinning of the membrane and pore formation [32]. Even though this characteristic makes DMSO an effective and valuable solvent across cell culture studies, it is important to note that some studies have demonstrated that DMSO, even at low concentration, can significantly alter the structure and properties of the lipid bilayer due to membrane surface dehydration [33]. The impact of DMSO on the lipid membrane is similar in many ways to the observed effect of alcohols such as ethanol [34], [35], [36]. Low concentration of DMSO causes a lateral expansion of the membrane while leading to a reduction in its overall thickness [37]. However, a concentration higher than the critical threshold of DMSO could promote spontaneous pore formation in the membrane. Exceeding this threshold leads to severe destabilization of the bilayer structure. This effect is attributed to the preferential localization of DMSO below the membrane headgroup region. Hence, it can act as a spacer that increases lipid-lipid separation [36]. Consequently, if used at optimal concentration, DMSO can increase membrane fluidity, facilitating membrane fusion, lowering the energy barrier for molecular transport, and finally promoting pore formation [36].

Acetone has to be used at a lower concentration to avoid cytotoxic effects [12]. The least cytotoxic effects on MCF-7, RAW.264.7, and HUVEC cell lines were observed in concentrations ranging from 0.5% to 5% (v/v) [15]. The molecular mechanism of action of acetone on the membrane has been reported to be very similar to ethanol and DMSO [38]. Acetone, compared to methanol, has a stronger effect on the membrane dynamics and fluidization. However, this effect remains less apparent compared to DMSO. This observation indicates a comparatively lower potential for enhancing membrane permeability for acetone [23].

1.1.3. Non-polar solvents

Non-polar solvents are typically hydrocarbon-based and can solubilize lipophilic compounds. Chloroform is an example of a solvent that belongs to this category [16]. These solvents are utilized to extract lipophilic compounds [8], however, their use in cell-based assays is limited due to their cytotoxicity effects even at a lower range of concentrations [31].

1.1.4. Strategies to improve solubility in *in vitro* settings

Several strategies have been employed to address the solubility limitations of compounds *in vitro*. Using a cosolvent system is one of them. In this method, a combination of water-miscible solvents, such as ethanol-DMSO, is used to improve the solubility of the compound while reducing individual solvent toxicity. The polarity of this type of system can be adjusted following the characteristics of the compound to help their solubility without exceeding the toxic threshold [39].
Encapsulation is another method of enhancing the solubility of compounds and preserving them from photodegradation and hydrolysis. Nanocarriers such as liposomes, micelles, and solid lipid nanoparticles belong to this method [40].

Using surfactants such as Tween 80 could reduce the interfacial tension and help to obtain a homogenous solution in aqueous media. However, the utilized surfactant might have a dose and time-dependent cytotoxicity on cells. It is necessary to optimize the utilized surfactant concentration [12]. In addition, an optimal pH level could adjust the ionization state of the solvent and improve the solubility of the compound [41].

Ultrasonic treatment is another technique that can help with solubilization if used in a proper manner [42]. For instance, based on findings of a study, the solubility of piroxicam in water and gastric fluid was increased after the use of sonication during the homogenization phase. These results were obtained under a sufficiently high sonication power and time [43].

A recent and promising strategy in improving solubility of compounds is the use of deep eutectic solvents (DESs). DESs, formed by mixing a hydrogen bond donor and a hydrogen bond acceptor, have shown potential in solubilizing some phytochemicals [44], [45]. These solvents could increase the solubility of both polar and

non-polar compounds due to their unique hydrogen-bonding [45]. In some studies, a significant solubility of some bioactive compounds and biomolecules has been demonstrated [46], [47].

Incorporating these approaches into *in vitro* protocols could reduce solubility limitations, thereby enhancing the accuracy and reliability of an experiment.

1.1.5. Case study: Curcumin solubility in *in vitro* study

Curcumin, a hydrophobic polyphenol derived from the rhizome of Curcuma longa L., is a widely studied compound for its anticancer, anti-inflammatory, and antioxidant properties [48]. Its application in aqueous biological systems is severely hindered by its low solubility in water [49]. Novel delivery systems are being designed to address and overcome these challenges [50]. These approaches have shown promise in improving curcumin solubility and bioavailability *in vitro* [51], [52], [53]. Table 1 describes some of these strategies.

Table 1. Solvent systems used to enhance the solubility of curcumin.

Solvent/Method	Outcome measures	Solubility enhancement	Reference
Adjustment of the pH level	Solubility of turmeric in 5 different solutions was evaluated using colorimetry assay	Curcumin exhibited a solubility of 17.6 mg/mL in 0.2 M sodium hydroxide and 10.1 mg/mL in pure ethanol, whereas its solubility in hydrochloric acid was significantly lower at just 0.034 mg/mL	[54]
NADES[1]	The solubility of curcumin in seven different natural deep eutectic solvents was measured spectrophotometrically	Using a system with choline chloride and glycerol in equimolar proportions increased 12000 times the solubility of curcumin, compared to aqueous solution	[55]
Acetone	Solubility of curcumin in various solvents was evaluated experimentally using the shake flask method and in silico through COSMO-RS simulations	Curcumin demonstrated high solubility in DMSO[2], DMF[3], acetone, and PEG400[4], but is practically insoluble in glycerin and water	[56]
Co-solvent	Solubility of curcumin was experimentally determined using the saturation shake-flask method over a temperature range of 278.15 to 318.15 K at atmospheric pressure (101.1 kPa)	A mixture of n-propanol and water with a composition range of $0.20 < x_1 < 1$ enhanced curcumin solubility more effectively than equivalent compositions of ethanol or isopropanol	[57]
Encapsulation	A novel encapsulation technique was investigated by spray-drying a warm aqueous ethanol solution containing co-dissolved sodium caseinate and lipophilic food components, with curcumin used as the model compound. Solubility of curcumin was evaluated via spectrophotometry	At the same curcumin concentration, free curcumin appeared as insoluble particulates, whereas casein nanocapsules dispersed curcumin at concentrations over 4 decades times higher than its solubility limit while maintaining a transparent appearance.	[58]
Surfactant	A mixed double- and single-chained surfactant system at an equimolar ratio was analysed based on fluorescence and conductivity measurements	The solubility of curcumin in an aqueous medium, following the addition of a mixed double- and single-chained surfactant system at an equimolar ratio, increased to the order of 10^3 to 10^4, representing at least a tenfold enhancement compared to previously reported values	[59]
Nanofibers	Curcumin nanofibers were produced via electrospinning with a water-soluble polymer, while curcumin nanoparticles were prepared by	The water-soluble nanofiber formulation increased curcumin solubility by up to 38-fold comparison to free curcumin dissolved in the same medium, while water-insoluble	[60]

Solvent/Method	Outcome measures	Solubility enhancement	Reference
	nanoprecipitation using a water-insoluble polymer. Release and solubility of curcumin was measured using a modified dialysis diffusion method	nanoparticles enhanced cellular penetration of curcumin by 2-fold. Both formulations effectively lowered curcumin's IC_{50} and reduced cancer cell viability	

[1]NADES: Natural Deep Eutectic Solvents
[2]DMSO: Dimethyl Sulfoxide
[3]DMF: Dimethylformamide
[4]PEG400: Polyethylene Glycol 400

2. Conclusion

Solubility remains a significant challenge in the *in vitro* evaluation of phytochemicals. Careful selection of solvents and optimization of concentration are vital to ensure accurate and reproducible results. Emerging strategies, such as nanotechnology-based delivery systems, encapsulation systems, and DESs, offer promising solutions for improving solubility and bioavailability. Standardized guidelines for solvent use and reporting can further enhance the reliability of *in vitro* phytochemical research.

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