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Optimization of Chlorophyll Extraction from Hibiscus Leaves; A Comparative Study of Solvents, Methods, and Antimicrobial Activity

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Abstract

Chlorophyll is a natural pigment, which exhibits strong antioxidant and antimicrobial activities, but has not been widely used in industrial applications due to low extraction efficiency and instability. Therefore, this work aimed to improve chlorophyll extraction from Hibiscus mutabilis leaves through ultrasonic-assisted extraction with different polarity solvent systems. The polarity of the solvent system affected the phytochemical characteristics and bioactivities of the extract. Five solvent systems (acetone/hexane (1:1), acetone/water (1:1), ethanol/water (1:1), acetone, and hexane) were compared for extraction yield, phytochemical content, and biological activities. The acetone/hexane (1:1) solvent system gave the highest chlorophyll content (22.48 mg/L) and pheophytin content (19.32 mg/L), leading to the highest extraction yield of 23.57%. This extract also showed the maximum radical scavenging effect with an IC_{50} value of 68.1 µg/mL and the highest antimicrobial activity, with inhibition zones of 35 mm against Escherichia coli and 32 mm against Staphylococcus aureus. These results show that the solvent polarity during extraction plays a key role in enhancing both extraction efficiency and bioactivity. The results also demonstrate the potential of Hibiscus mutabilis leaf extract as a natural and sustainable preservative for use in food, cosmetic, and pharmaceutical products.

Keywords

Acetone/Hexane, Antimicrobial Activity, Chlorophyll Extraction, Hibiscus Leaves, Pheophytin, Solvents

INTRODUCTION

Plants are not only the essential source of nutrition but also act as traditional medicine due to its bioactive compounds which has various therapeutic properties. Among these, a major pigment is chlorophyll, which is a natural pigment that has been the subject of research for its antioxidant, antimicrobial, and detoxifying properties [1]. Furthermore, chlorophyll is the main pigment that gives them their green colour and is a cellular protection molecule that plays the important role of keeping the cells healthy and is the reason for the process called cellular rejuvenation [2].

Hibiscus mutabilis belongs to the Malvaceae family and is widely used in traditional medicine, as well as a herbal beverage because it is packed with phenolics, flavonoids, anthocyanins, and chlorophyll [3, 4]. As many studies have focused for a long time on the flowers and calyces of Hibiscus, the leaves remain largely unexplored although they are a valuable source of chlorophyll which can be used in food and pharmaceutical industries.However, the commercial application of chlorophyll remains limited primarily due to its inherent instability.

It is because chlorophyll is completely broken down into pheophytin in adverse environments such as heat, light, or acidic conditions [5].

The efficiency of extractable chlorophyll and the pigment's stability are significantly affected by the solvent polarity, the extraction conditions applied, and the pigment's solubility [6]. Although Soxhlet extraction and maceration, the conventional extraction techniques are commonly used, they have multiple constraints like prolonged extraction time, high solvent utilization, and the risk of thermal destruction of heat-sensitive compounds [7].

Ultrasonic-assisted extraction (UAE) is new technique that's been found to be a very effective alternative because it also helps in cell disruption, increases the solvent penetration, reduces the extraction time, and keeps the thermolabile bioactive compounds intact [8, 9]. Currently, limited research has addressed the optimization of ultrasound-assisted extraction (UAE) for chlorophyll recovery from *Hibiscus mutabilis* leaves, especially with regard to the role of solvent polarity in achieving an optimal balance among extraction efficiency, pigment stability, and biological activity.

The current study thus intended to evaluate the impact of various solvent systems in a standardized way on the ultrasonic-assisted extraction of chlorophyll from Hibiscus mutabilis leaves. The study also included the extraction yield, the amount of chlorophyll and pheophytin, antioxidant, and the antimicrobial activity against Escherichia coli and Staphylococcus aureus. The analysis values of this study is helpful for the optimization of extraction conditions, besides emphasizing the potential of bioactive-derived chlorophyll as green and innovative chemical agents for the food, cosmetic, and pharmaceuticals sectors.

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METHODS AND MATERIALS

Plant Material and Chemicals

Fresh Hibiscus mutabilis leaves were collected from Green Farm Agri and Garden Nursery, Mannuthy, Thrissur, Kerala, India. All the chemicals and reagents used in the present investigation were of analytical reagent grade and obtained from Sigma-Aldrich (USA).

Preparation of Dried Hibiscus Leaves

The leaves were cleaned with double-distilled water and dried in a vacuum oven at 75°C to a constant weight. The dried leaves were flaked using a laboratory flaker. The powdered samples were stored in airtight containers at room temperature until further use.

Solvent Systems

To study the solvent polarity effect on chlorophyll extraction, five solvent systems were evaluated, namely acetone/hexane (1:1, v/v), acetone/water (1:1, v/v), ethanol/water (1:1, v/v), acetone, and hexane.

Ultrasonic-Assisted Extraction (UAE)

Ultrasound-assisted extraction was carried out according to a previously described procedure [10], with slight modifications.The dried Hibiscus leaf powder (5.0 g) was mixed with 50 mL of solvent (solid: liquid ratio, 1:10 w/v) in a 100 mL glass container. Extraction was performed using a Sartorius ultrasonic homogenizer (Germany) with a 14 mm titanium probe operating at a frequency of 30 kHz and 50% amplitude in the pulsed mode for three cycles of 10 min each. To avoid pigment degradation, the extraction vessel was kept in an ice bath. The extract was filtered using Whatman No. 1 filter paper after each cycle and the residue was re-extracted twice under the same conditions. Finally, the combined extracts were concentrated on a rotary evaporator (Buchi, Switzerland) at 40°C under reduced pressure and transferred to amber vials and stored at −20°C until further analysis.

Calculation of Yield

The dry extract which remained after evaporation of the solvent was weighed. The yield of extraction was calculated as mentioned below:

Yield (%) = (Weight of dry extract/Weight of dry sample) × 100

All the measurements were carried out three times and reported as mean ± standard deviation (SD).

Quantifying Chlorophyll and Pheophytin

To obtain absorbance values, the extracts were diluted with acetone. Absorbance was determined at 409 nm and 405 nm for chlorophyll and pheophytin, respectively, by using a UV–Vis spectrophotometer (Shimadzu, Japan). Extinction coefficients given by Lichtenthaler [11] were used to calculate the concentrations.

Chlorophyll (mg/L) = Absorbance at 409 nm × conversion factor

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Pheophytin (mg/L) = Absorbance at 405 nm × conversion factor

Calibration was conducted using blank samples with only solvent. The measurements were made in triplicate for all samples.

Antioxidant Activity (DPPH Assay)

The determination of the antioxidant activity was done using DPPH radical scavenging method. A 2 mL solution of 0.1 mM DPPH was mixed with 2 mL of extract at different concentrations (25–150 µg/mL). The obtained mixture was kept in a dark place under the room temperature for 30 minutes, and then the extract was measured at 517 nm for the absorbance value. The percentage of inhibition was calculated with the following equation: % Inhibition = [(Absorbance of control – Absorbance of Sample) / Absorbance of control] × 100. IC_{50} value, is the concentration required to inhibit 50% of DPPH radicals, which is calculated from the graph of inhibition percentage vs. extract concentration.

Antimicrobial Activity

The antimicrobial activity was evaluated through the disc diffusion method with the help of both *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) strains. Bacterial suspensions were set at the 0.5 McFarland standard; (~1.5×10^8 CFU/mL) and spread on Mueller–Hinton agar plates. Sterile 6 mm discs were treated with 60 µL of extract and placed on the inoculated agar. Discs only containing solvent served as negative controls, and gentamicin (10 µg/disc) was used as a positive control. The incubation of the plates took place at a temperature of 37°C for 24 hours, and the dimensions of the inhibition zones were taken in millimetres.

Statistical Analysis

The statistical breakdown of the data being analysed was represented as the means and standard deviations of all the triplicate data obtained from the experiments. The statistical analysis was determined through one-way analysis of variance (ANOVA). Further statistics were made using Tukey's HSD post hoc test from the SPSS software (version 25.0, IBM, USA). A p-value that is below 0.05 is considered as the level of statistical significance.

RESULTS AND DISCUSSION

Yield

A broad variation in quantitative extraction efficiency was observed among the different solvent systems, as shown in Table 1. Among the tested systems, the highest extraction efficiency was obtained with the acetone/hexane system (1:1, v/v), with a yield of 23.57%; the next was acetone/water (21.7%). On the other hand the pure acetone (8.6%) and hexane (5.1%) solvents showed medium yields, while the ethanol/water system was the least productive (7.8%). The dominant role of the mixed solvent systems may be due to the

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presence of intermediate polarity, which in turn, facilitates the solubilization of both hydrophilic and lipophilic parts present in plant matrices. This synergistic action enhances the transfer and extraction efficiency. Similar findings have been reported in the literature, where the mixed solvent systems performed better than the single solvents in chlorophyll and another bioactive polymer extraction [11]. The disparities were significant statistically ($p < 0.05$).

Chlorophyll and Pheophytin Content

The quantitative analyses of pigments highlighted that the acetone/hexane extract was enriched in chlorophyll (22.48 mg/L) and pheophytin (19.32 mg/L) whereas other extracts like acetone and hexane were slightly lower as can be seen from Table 1. However, ethanol/water extracts exhibited a significantly lower yield of pigments.The high pigment extraction efficiency of the acetone/hexane system can be attributed to its optimized solvent polarity, which facilitates effective solubilization of chlorophyll while minimizing its degradation.

In contrast, aqueous solvent systems may promote hydrolysis and create acidic conditions that facilitate the conversion of chlorophyll to pheophytin, thereby reducing pigment stability.This observation aligns with earlier literature reports highlighting the sensitivity of chlorophyll to variations in solvent polarity and environmental conditions [12].The data exclusively shows that the choice of solvent is an essential factor in the equilibrium between pigment extraction efficiency and pigment stability.

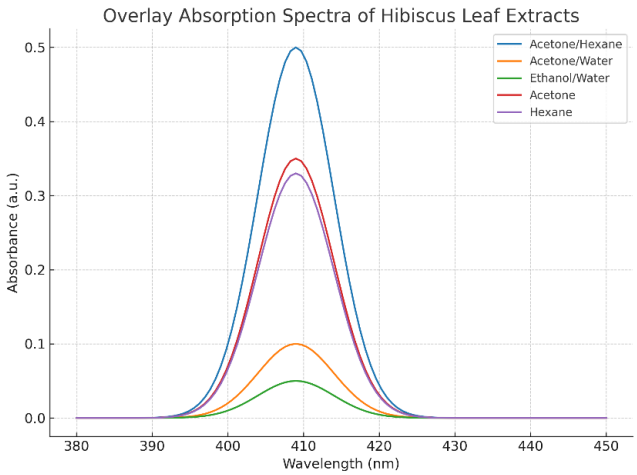

Figure 1. Overlay absorption spectrum of Hibiscus leaf extract.

Antioxidant Activity

The DPPH radical scavenging assay was utilized to examine the antioxidant activity of Hibiscus leaf extracts, the results of which are presented in Table 1. Acetone/hexane extract had the antioxidant activity, of IC_{50} value of 68.1 µg/mL, then it was acetone extract (72.5 µg/mL) followed by hexane extract (75.6 µg/mL). Aqueous alkali extract, on the other hand, showed least active, while the ethanol/water extracts were virtually inactive (IC_{50} = 125.4 µg/mL).

The high antioxidant activity of the acetone/hexane extract is primarily attributed to high chlorophyll content, because of chlorophyll and its derivatives having high capacity to neutralize the free radicals by donating the electrons and hydrogen. Therefore, this pigment concentration, which is found in the extract, is associated with the antioxidant capacity of the extract, indicating that the solvent polarity is a factor in preserving the bioactive molecules in the extraction process. The data collected in the research confirm previous results published in other studies related to the fact that chlorophyll-containing extracts have a strong free radical scavenging power which is obtained from redox properties [13, 14]. The identified changes were statistically significant ($p < 0.05$).

Antimicrobial Activity

The extracts were targeted against gram-negative bacteria and gram-positive bacteria using the disc diffusion method, and the experiment outcomes were gathered in Table 1. The acetone/hexane ones were the extracts that had the best results in terms of the antimicrobial activity exhibiting inhibition zones of 35 mm against *E. coli* and 32 mm against *S. aureus*, respectively, which were quite similar to the values achieved with the standard antibiotic gentamicin. Also, Acetone and hexane extracts significantly added to the antimicrobial activity but the inhibition zones they had were 28-30 mm range. Unlike them, aqueous solvent systems were far less effective in inhibiting microorganisms with the inhibition zones not exceeding15 mm. The acetone/hexane extracts were observed with the highest activity may be due to conferring chlorophyll and pheophytin in their higher concentration, molecules that are able to interfere with the integrity of microbial cell membranes and cause oxidative stress via the generation of reactive oxygen species upon photoactivation. The findings are correlating with earlier studies that has been reported the antimicrobial activity of natural pigments and their derivatives [15, 16]. The presented differences showed definiteness in the statistics ($p < 0.05$).

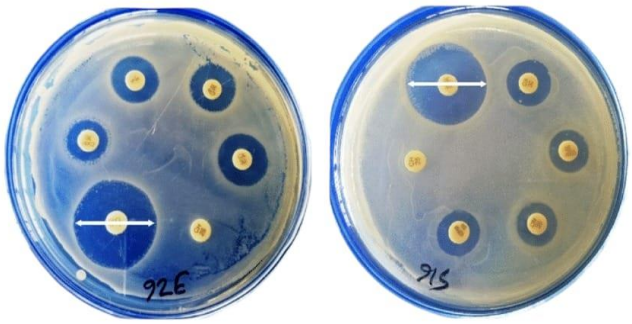

Figure 2. Zones of inhibition against *E. coli* and *S. aureus.*

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Table 1. Hibiscus leaf extracts processed in different solvent systems were evaluated for their yield, pigment concentrations, and antioxidant activity (IC50) as well as antimicrobial activity.

Solvent System	Yield (%)	Chlorophyll (mg/L)	Pheophytin (mg/L)	IC50 (µg/mL)	E. coli (mm)	S. aureus (mm)
Acetone/Hexane	23.57	22.48	19.32	68.1	35	32
Acetone/Water	21.7	4.2	3.6	102.3	15	12
Ethanol/Water	7.8	1.52	1.3	125.4	10	8
Acetone	8.6	17.91	15.29	72.5	30	28
Hexane	5.1	18.45	16.1	75.6	30	28

DISCUSSION

The present study gives clear evidence of the prominent effect of solvent polarity on chlorophyll extraction and bioactivity from *Hibiscus mutabilis* leaves. Among the solvent systems analysed, the combination of acetone/hexane remained the most effective in terms of extraction yield, chlorophyll and pheophytin concentrations, antioxidant activity, and antimicrobial efficiency. The predominance of this method can be explained as the mixed solvent occurs with intermediate polarity, thus, both hydrophilic and lipophilic compounds are solubilized at the same time while the pigment degradation remains at a minimum level. Meanwhile, aqueous solvent systems turned out to be inefficient since they may be responsible for the hydrolysis of chlorophyll into pheophytin, which consequently had a negative effect on pigment stability and bioactivity.

These findings agree with the reports that refer to stability of chlorophyll which is mainly connected with the polarity and the extraction conditions [17, 18]. The reliability of the current evidence is additionally supported by the comparison with previous studies. Past research works have proved that the use of binary solvent systems instead of single solvent ones is a decisive factor to increase chlorophyll extraction efficiency and antioxidant ability considerably [19]. Another point is a widely recognized association between the concentration of chlorophyll and the presence of free radicals, which states that pigment-rich extracts are featuring higher free radical scavenging capacity because of their redox-active properties [20].

The strong antimicrobial activity against acetone/hexane extracts is also matches with the results of earlier research that reported on the capability of chlorophyll and its derivatives to make microorganisms disrupt their membranes and inflict damage through the formation of reactive oxygen species, thereby, leading to microbial inhibition [21,22]. The use of chlorophyll from Hibiscus as a natural and multifunctional ingredient in industries related to food, cosmetics, and pharmaceuticals has a great potential. Its effective antioxidant action makes it possible to use in food preservation, and nutraceutical formulations, and, its antimicrobial activity broadens its application as a natural preservative, or potentially as a therapeutic agent in topical and medical uses [23, 24].

CONCLUSION

Solvent polarity played an essential role in the ultrasonic-assisted extraction of chlorophyll from Hibiscus mutabilis leaves. Acetone/hexane (1:1, v/v) exhibited the highest extraction yield (23.57%), as well as the maximum chlorophyll (22.48 mg/L) and pheophytin contents (19.32 mg/L). It also showed the strongest antioxidant activity (IC50 = 68.1 µg/mL) and the significant antimicrobial activity against Escherichia coli (35 mm) and Staphylococcus aureus (32 mm). These results provide evidence to the fact that ultrasonic-assisted extraction is efficient pigments' recovery method while maintaining the activity compared to conventional extraction methods.

The combination of solvent system with intermediate polarity and optimization was the main factor to reach maximum extraction efficiency and desirable functional properties. All in all, the chlorophyll from Hibiscus demonstrates the potential of sustainable and multi-functional natural ingredients in various fields such as food, cosmetics, and pharmaceuticals. It is advised to conduct further investigations on the process scalability, stability testing, and formulation development for the industrial application of this material.

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