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EVALUATION OF THE OPTIMAL EXTRACTION METHOD BETWEEN HOT AND COLD EXTRACTION FOR *PLUMERIA PUDICA* JACQ.

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ABSTRACT

The leaf extracts of *Plumeria pudica* Jacq. were obtained using isopropyl alcohol, distilled water, petroleum ether, and hydroalcoholic solvents via two extraction methods: hot extraction (Soxhlet) and cold extraction (Maceration). For clarity, extracts obtained by the Soxhlet method will be denoted as hot extracts, while those from maceration will be denoted as cold extracts. To determine the superior extraction method, yield of extracts, qualitative phytochemical analysis, and antioxidant activity evaluation were considered. The results revealed that hot extracts demonstrated a significantly higher yield and a greater presence of phytochemicals in the preliminary analysis. Furthermore, both the reducing power assay and the DPPH assay showed that hot extracts displayed higher antioxidant activity. Consequently, the hot extraction method was considered more effective for this specific plant.

Key words : Phytochemicals, Anti-oxidants, Maceration, Soxhlet, Extraction, Radical-scavenging.

Introduction

The study of plant-based biochemicals to assess primary and secondary metabolites and their various activities such as antimicrobial, antioxidant, pharmacological and bio medicinal properties heavily relies on the initial step of extracting plant extracts. The qualitative and quantitative analysis of bioactive compounds from plant materials largely hinges upon selecting an appropriate extraction method. Extraction, being the foremost stage in any medicinal plant investigation, plays a pivotal role in determining the outcomes. Often referred to as 'sample preparation techniques,' extraction methods are crucial yet sometimes overlooked, performed by personnel lacking specialized training, despite comprising a significant portion of the efforts in analytical chemistry (Hennion *et al*., 1996). Research by Majors (1999) underscored the consensus among researchers regarding the criticality of sample preparation in analytical studies. While advancements in chromatographic and spectrometric techniques have simplified the analysis of bioactive compounds, the efficacy still heavily depends on the extraction methods employed, input parameters and the specific characteristics of plant components (Poole *et al*., 1990). Key factors influencing extraction processes include the matrix properties of plant parts, choice of solvent, temperature, pressure and duration of extraction. Enhanced understanding of the dynamic chemical composition of diverse bioactive molecules has driven significant progress in bioactive compound analysis over the past decade. These technological and technical advancements have piqued the interest of pharmaceutical, food additive, and natural pesticide sectors in bioactive molecules sourced from natural origins (Ambrosino *et al*., 1990). Typically, bioactive compounds coexist with other plant constituents and can be identified and characterized from various plant components such as leaves, stems, flowers and fruits. Extraction of plant

Abbreviations : IPA: Isopropyl alcohol, AQ: Aqueous, PE: Petroleum ether HYA: Hydro-alcohol, DPPH: 2,2-diphenyl-1-picrylhydrazyl.

materials can be achieved through a variety of extraction procedures. Over the last 50 years, non-conventional methods have emerged that are more environmentally friendly due to reduced use of synthetic chemicals, shorter processing times, and improvements in yield and extract quality. In our research, we have selected four solvents based on their polarity for the extraction process, namely IPA, Aqueous, Petroleum ether, and HYA solvents. For more efficient communication, we have categorized the extracts prepared using the maceration technique as cold extracts and those prepared using the Soxhlet technique as hot extracts. The results of our evaluations, encompassing % yield of crude, qualitative phytochemical analysis, and the reducing power assay and DPPH assay, consistently indicated the superior performance of hot extracts. These findings substantiate our initial hypothesis that Soxhlet extraction represents the optimal method for further comprehensive studies and investigations into our plant.

Materials and Methods

Collection of plant material

The fresh leaves of *P. pudica*. were collected from various locations across the Gandhinagar district of Gujarat state in January and March 2024 (23°14' N latitude and 72°38' longitude). Dr. B.A. Jadeja identified the plant, and the voucher specimens of the collected roots (KS15X and KS15Y) were deposited at the Department of Botany, M.D. Science College in Porbandar, Gujarat, India. The leaves were thoroughly cleaned with distilled water, air-dried for eight days, and then carefully ground into a coarse powder for storage in glass bottles for future use.

Extraction methods

Cold extraction (Maceration method):

The maceration process is an established technique utilized in the preparation of homemade tonics, recognized for its effectiveness and cost-efficiency in extracting essential oils and bioactive compounds. At a small scale, maceration typically entails a series of sequential steps. Initially, plant materials are finely ground to increase the surface area, which enhances their interaction with the solvent. Subsequently, an appropriate solvent, referred to as a menstruum, is introduced into a sealed vessel containing the ground plant materials. The resulting liquid is then separated from the solid residue, known as marc, which contains a substantial number of occluded solutions and is further processed through pressing. The extracted liquid is subsequently filtered to eliminate impurities. Intermittent agitation during the maceration process is pivotal in expediting extraction through two primary mechanisms: enhancing diffusion and removing the concentrated solution from the sample surface, thus allowing fresh solvent to further facilitate extraction. In this specific extraction procedure, cold maceration was employed. The plant leaves underwent a sun-drying process for a duration of 5 to 6 days, after which they were pulverized into a fine powder using an electric mixer, yielding 50 grams of powder. Subsequently, 500 milliliters of the designated solvent were combined with the 50 grams of leaf powder, achieving a plant material-solvent ratio of 1:10. This method yielded extracts in isopropyl alcohol, aqueous, petroleum ether and hydroalcoholic (water and methanol 50:50) forms. The solutions were left to stand at room temperature for approximately 2 days before being filtered through muslin cloth. The filtered supernatant was then transferred to pre-weighed Petri dishes and allowed to stand for 4 to 5 days, during which time complete evaporation of the solvent occurred.

Hot Extraction (Soxhlet method)

The Soxhlet extractor, created by German chemist Franz Ritter Von Soxhlet in 1879, was originally intended for extracting lipids but has since become widely utilized for extracting bioactive compounds from natural sources. The process involves placing a dry sample into a thimble, which is then positioned in a distillation flask containing a solvent. As the solvent level rises, it is siphoned upwards, carrying extracted solutes into the main liquid. The solutes accumulate in the distillation flask while the solvent returns to interact further with the solid plant material. This cycle is repeated until the extraction process is deemed complete (Azmir *et al*., 2013). The method serves as a standard for assessing the effectiveness of new extraction techniques. Solvent extraction (SE) offers several advantages, including simplicity, applicability at elevated temperatures to enhance process kinetics, minimal start-up expenses, absence of the need for filtration, and the continuous presence of both solvent and sample throughout the extraction process (Grigonis *et al*., 2005). The powdered leaf, enclosed in a muslin cloth thimble, was introduced into a glass chamber. A solvent was added in a 1:10 g/mL ratio and subjected to extraction at specific temperatures: 82.3°C for IPA, 100°C for Aqueous, 60°C for petroleum ether, and 66°C for the hydroalcoholic extract, with the volume of hydroalcoholic extract adhering to a 1:1 DW to Methanol ratio. The resulting supernatant underwent filtration using Whatman filter paper, followed by air-drying, enabling the determination of the yield of the extract through measurement of its dry weight. This method serves to provide insight into the productivity of the extraction process.

Yield of plant extract =

$$\frac{\text{Weight of crude extract obtained (gram)}}{\text{Total weight of plant powder (gram)}} \times 100$$

Bioassays

Preliminary phytochemical analysis

Individual stock solutions, each with a concentration of 1 mg/ml, were prepared for the plant extracts. Subsequently, the efficacy of various bioactive compounds, including alkaloids, carbohydrates, terpenoids, flavonoids, phenols, tannins, quinones, saponins, amino acids, and proteins, was assessed using these stock solutions. The qualitative phytochemical screening was conducted following the methodologies detailed in the references (Arya *et al*., 2012 and Patel *et al*., 2014).

Carbohydrates

Benedict's test: Mix 2 ml of plant extract with 1 ml of Benedict's reagent and heat in a water bath. Colored precipitates indicate the presence of sugars.

Alkaloids

Mayer's test: Add 2-3 drops of Mayer's reagent to 2 ml of leaf extracts. Look for white creamy precipitates to show the presence of alkaloids.

Terpenoids

Salkowski's test: Take 2 ml of leaf extract and add 2 ml of chloroform and 3 ml of concentrated sulfuric acid. Look for the formation of a red-brown color.

Flavonoids

Lead acetate test: Test 2 ml of leaf extract with a few drops of 10% lead acetate. Look for yellow-colored precipitates to indicate the presence of flavonoids.

Phenols

Ferric chloride test: Take 2 ml of leaf extract and add 2 drops of 5% ferric chloride. Look for a dark green color to reveal the presence of phenolic compounds.

Tannins

Folin Ciocalteau test: Mix 2 ml of leaf extract with 1 ml of Folin Ciocalteau reagent. Look for a blue-green color.

Quinones

Hydrochloric acid test: Add a few drops of concentrated HCl to 2 mL of plant extracts. Look for a yellow precipitate or coloration to indicate the presence of quinones.

Amino acids and proteins

Biuret test: Add a drop of 2% copper sulphate and a pallet of potassium hydroxide to 2 ml of leaf extract in a test tube. Look for a pink color in the ether layer to reveal the presence of proteins and amino acids.

Saponins

Foam test: Add 20 ml of distilled water to 2 ml of leaf extract and shake well. Look for the formation of a layer of foam to indicate the presence of saponins.

Antioxidant activities

Due to the intricate reactive properties of phytochemicals, the assessment of antioxidant activities necessitates the use of at least two test systems to verify authenticity.

Reducing power assay

An increase in absorbance may indicate the antioxidant capacity of antioxidants or their extracts. Compounds possessing antioxidant capabilities react with potassium ferricyanide ($K_3[Fe(CN)_6]$), yielding potassium ferrocyanide ($K_4[Fe(CN)_6]$). This resultant compound further reacts with ferric trichloride, leading to the formation of ferric ferrocyanide, a blue-colored complex that exhibits its peak absorbance at 700 nm (Mokrani *et al*., 2016).

Preparation of the sample solution

The plant extracts were taken at concentrations of 100, 200, 300 to 1000 µg/ml and the overall solution was made in 1 ml quantity by adding distilled water. Now to these solutions phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%) were added. The mixtures were then incubated at 50 °C for 20 min. Aliquots (2.5 ml) of trichloroacetic acid (10%) were added to each mixture, which was then centrifuged for 10 min at 1036 x g. The upper layer of the solutions (2.5 ml) was mixed separately with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance levels were measured at 700 nm using a spectrophotometer. Methanol was used as a control. Ascorbic acid was used as positive control and reducing power was expressed as mg ascorbic acid equivalent per g of dry weight extract (mg AA/g). All tests were run in triplicates (n=3) and average values were calculated.

DPPH radical scavenging assay

Free radicals are implicated in over one hundred health disorders in humans, including atherosclerosis, arthritis, ischemia and reperfusion injury in various tissues, central nervous system injury, gastritis, cancer, and AIDS (Kumpulainen and Salonen, 1999; Cook and Samman, 1996). The antioxidant properties of the extracts were assessed using the DPPH free radical scavenging assay,

following the method described by Nithianantham *et al.* (2011) with necessary modifications. This well-established method allows for a quick and efficient analysis of the antioxidants' ability to neutralize free radicals. DPPH, when in its oxidized state, exhibits a deep violet color in methanol. As the antioxidant compounds transfer electrons to DPPH, it undergoes reduction and changes from violet to yellow-blue. The absorbance of DPPH solutions is measured at 517 nm. Evaluating the extracts' capacity to scavenge DPPH free radicals helps us understand their antioxidant potential and their role in mitigating injury within biological systems.

Preparation of DPPH solution (0.1 M)

A solution of DPPH was prepared by dissolving 0.39 mg of the reagent in methanol within a volumetric flask, and subsequently adjusting the volume to approximately 100 ml. The resultant purple-colored DPPH solution was then stored at -15°C in a freezer for future use.

Preparation of Extract solutions

Stock solutions of extracts at a concentration of 1 mg/ml were prepared by dissolving the specified quantity of each extract in the necessary volume of methanol. Subsequently, solutions of 20, 40, 60, 80, and 100 µg/ml for each extract were derived from the initial sample.

Evaluation of antioxidant potential

To the sample solutions of different concentrations, 1 ml DPPH solution was added and incubated at room temperature for 30 min in the dark. A control preparation was done by mixing 1 ml of methanol and 1 ml of DPPH solution. At last, the absorbance of the solutions was measured using a spectrophotometer at 517 nm. Ascorbic acid was used as standard. 50% inhibitory concentrations (IC_{50} values) of the extracts were calculated from the graph as with concentration versus percentage inhibition.

Inhibition of DPPH free radical in percentage was calculated by the formula:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Where, $A_{control}$ is the absorbance of the control (DPPH solution without the addition of plant extract) and A_{test} is the absorbance of reaction mixture samples (in the presence of sample). All tests were run in triplicates (n=3) and average values were calculated.

IC_{50} value

The IC_{50} parameter was employed for the analysis of results obtained from the DPPH method (Stankovic, 2011). The IC_{50} value, indicative of the sample quantity required to diminish the absorbance of DPPH by 50%, was derived by correlating the discoloration of the sample with its concentration.

Results

Yield of extracts

The extraction process affects the overall quantity and quality of the crude that we get. As observed in Table 1, it is understandable that there is parity between yields of hot and cold extraction methods. The highest parity of % yield was found between HYA cold and HYA hot extracts.

Table 1 : The % yield of all the leaf extracts with the characterization of the extracts.

Extracts	% Yield	Color of extract	pH
IPA Cold	9.07	Parrot green	9.3
IPA Hot	**13.69**	Clay like	6.5
Aqueous Cold	41.5	Green	7.1
Aqueous Hot	**62.83**	Black	6.9
PE Cold	6.45	Light green	8.5
PE Hot	**8.45**	Light brown	6.8
HYA Cold	26.63	Dark green	6.2
HYA Hot	**52.89**	Dark brown	7.5

Qualitative phytochemical analysis

The results of the preliminary qualitative phytochemical analysis indicated that the hot extracts were more effective in detecting various phytochemicals. As mentioned in Table 2 the IPA extracts exhibited the most comprehensive results, with the IPA Hot extract showing the presence of all 9 phytochemicals and the IPA Cold extract revealing the presence of seven phytochemicals. The Aqueous Hot extract demonstrated the presence of 8 phytochemicals, while the Aqueous Cold extract displayed 6 phytochemicals. In contrast, both hot and cold petroleum ether extracts exhibited the lowest number of phytochemicals relative to the other extracts, with the Petroleum ether hot extract showing the presence of 5 phytochemicals and the petroleum ether cold extract showing the presence of only three phytochemicals. The hydroalcoholic extracts illustrated the highest consistency in content, as evidenced by the HYA Hot extract revealing the presence of 7 phytochemicals, while the HYA Cold extract indicated the presence of only three phytochemicals.

Antioxidant tests

Reducing power assay

In the course of this assay, the dark green and light green extracts exhibited a range of blue shades, indicative of the distinct reducing capabilities of each compound. The presence of reducing agents instigated the conversion

Table 2 : Results of preliminary phytochemical analysis of the hot extracts and cold extracts.

Phytochemicals	IPA cold	IPA hot	Aq. cold	Aq. hot	PE. cold	PE. hot	HYA. cold	HYA. hot
Carbohydrates	+	+	+	+	-	+	-	+
Alkaloids	+	+	-	-	-	-	-	-
Terpenoids	+	+	+	+	+	+	-	+
Flavonoids	+	+	+	+	-	+	+	+
Phenols	+	+	-	+	+	+	+	+
Tannins	-	+	+	+	-	+	-	+
Quinones	+	+	+	+	-	-	-	+
Amino acids and proteins	-	+	-	+	+	-	-	+
Saponins	+	+	+	+	-	-	+	-
Phytochemicals present	7/9	9/9	6/9	8/9	3/9	5/9	3/9	7/9

Where, IPA: Isopropyl alcohol, Aq: Aqueous, PE: Petroleum ether, and HYA: Hydroalcoholic extracts.

Table 3 : IC_{50} values of all the extracts.

Plant extracts	IC_{50} values
Ascorbic acid	35.199
IPA Cold extract	69.782
IPA Hot extract	**33.509**
Aqueous Cold extract	231.383
Aqueous Hot extract	**162.043**
Petroleum Ether Cold extract	169.759
Petroleum Ether Hot extract	**61.43**
Hydroalcoholic Cold extract	86.67
Hydroalcoholic Hot extract	**71.94**

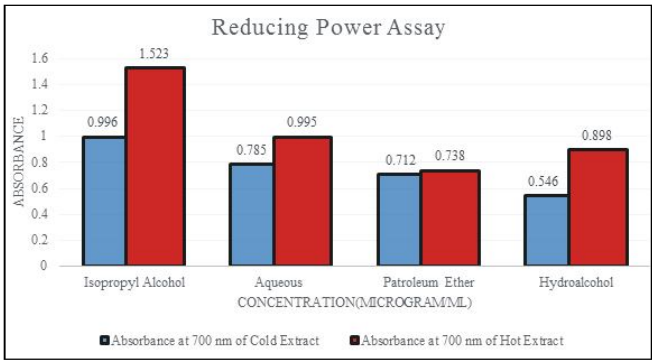

Fig. 1 : Results of the reducing power assay.

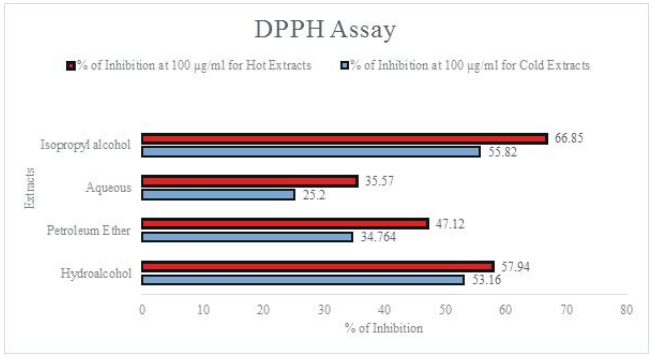

Fig. 2 : Results of DPPH assay.

of the Fe_{3+}/ferricyanide complex to its ferrous state. The quantification of Prussian blue formation at 700 nm facilitated the assessment of ferrous ion concentration. Notably, at the maximum concentration (1000 µg/ml), Ascorbic acid demonstrated an absorbance of 1.394, while the IPA Hot extract and the IPA Cold extract exhibited absorbances of 1.523 and 0.996, respectively (Fig. 1) Particularly noteworthy, the IPA Hot and Cold extracts yielded closely aligned results. Furthermore, the Aqueous Hot extract yielded an absorbance of 0.995, whereas the Aqueous Cold extract showed an absorbance of 0.785. Conversely, the Petroleum ether extracts evidenced the least concordance in results, with the PE Hot and PE Cold extracts registering absorbances of 0.778 and 0.769, respectively. Moreover, the HYA Hot extract displayed an absorbance of 0.898, while the HYA Cold extract exhibited an absorbance of 0.546.

DPPH assay

The DPPH stable free radical method offers a rapid and sensitive means to assess the antioxidant potential of a specific compound or plant extract. The efficacy of the extract in scavenging DPPH radicals is contingent not only upon the plant species but also on the extraction method employed. The oxidized form of DPPH is quantifiable at 517 nm (Jadid *et al.*, 2017). The percentage of inhibition serves as a metric to gauge the antioxidant activity of the extract, reflecting its capability to mitigate free radicals in this assessment. At the maximum concentration (100 µg/ml), the standard ascorbic acid demonstrated a % inhibition of 63.37, whereas the PE Hot extract exhibited the highest % inhibition at 47.12 and the PE Cold extract at 34.764. This discrepancy was most pronounced between these two extracts. The IPA Hot extract demonstrated a % inhibition of 66.85 and the IPA Cold 55.82. The Aqueous extract exhibited a % inhibition of 35.67, while the

Aqueous Cold displayed 25.20 denoting the least activity. The HYA Hot extract demonstrated a % inhibition of 57.94, and the HYA Cold of 53.16 (Fig. 2).

IC_{50} calculation

If the IC_{50} value is low, it indicates a high antioxidant activity (Brand-Williams *Et al.*,1995). As mentioned in Table 3 the highest parity of the values was observed in the petroleum ether extracts for the antioxidant activity. The IC_{50} value of ascorbic acid is 35.199.

Discussion

The first step in the isolation and purification of bioactive compounds from plant material is extraction. Extraction of secondary metabolites such as phenolic acids and flavonoids is difficult due to their insoluble nature. An appropriate extraction technique that balances product quality, process efficiency, production costs and environmentally acceptable methods should be used for the extraction of bioactive compounds from plant tissues. Our study demonstrated the efficacy of the hot extraction method, which resulted in superior yields of extracts. Furthermore, the hot extracts exhibited a greater diversity of phytochemicals and displayed enhanced antioxidant activities in the DPPH and reducing power assays, comparable to those of ascorbic acid. A study conducted by Murugesu *et al*. (2013) focused on the red Pitaya, also known as *Hylocereus polyrhizus*, which is renowned for its delicious pulp that is attached to sticky and mucilage-coated seeds. The properties of the seed oils are greatly influenced by the seed extraction techniques. These techniques are commonly known as the hot and cold methods for extracting clear mucilage-free red Pitaya seeds. HPLC analysis of the pitaya seed oil showed that Linoleic: Linoleic: Linoleic (LLL) and Oleic: Linoleic: Linoleic (OLL) dominated the TAG composition of the oil with an average percentage of 14.065% and 13.990% for the cold-extracted seed oil, and 15.620% and 15.795% for the hot-extracted seed oil. The results show that different seed extraction techniques influence the oil yield and the properties of the seed oils. The study has shown that different seed extraction techniques may influence the seed oil composition and quality. They concluded that the application of high temperature during the seed extraction does not affect the oil quality of the seed. The fatty acid composition of hot and cold extracted seed oil showed no significant differences. Moreover, a higher seed yield was observed in the hot procedure compared to the cold method, as seed separation using depulper contributed to greater seed loss and was time-consuming. In another study, the antioxidant activities of coconut oil extracted under hot and cold conditions were compared. The coconut oil extracted under hot conditions (HECO) is found to contain a higher concentration of phenolic substances compared to the coconut oil extracted under cold conditions (CECO). Analyses using DPPH assay, deoxyribose assay, and in vivo assay of serum antioxidant capacity indicate that the antioxidant potential of HECO surpasses that of CECO. While, it is commonly believed that virgin coconut oil extracted under cold conditions retains several thermally unstable antioxidants, thereby exhibiting superior beneficial qualities, the hot extraction process Favors the inclusion of more thermally stable phenolic antioxidants in coconut oil. Consequently, the consumption of HECO may result in greater improvement in antioxidant-related health benefits compared to the consumption of CECO (Seneviratne *et al.*, 2009). The aforementioned studies establish the credibility and significance of our research that the hot extraction method is superior both in terms of extraction quality and quantity.

Conclusion

The selection of extraction methods significantly impacts the assessment of research efficacy, particularly considering the rising economic significance of bioactive compounds and their sources. This trend may prompt the exploration of more sophisticated extraction techniques in the future. The escalating demand for plant bioactive compound extraction encourages an ongoing quest for practical extraction methods. Our investigation has validated the exceptional performance of the Soxhlet extraction method, surpassing the maceration method in all parameters for the *P. pudica* plant. Comparable outcomes are anticipated for numerous plants within the Apocynaceae family. Ultimately, both the Soxhlet and maceration methods exemplify commendable usability and applicability due to their cost-effectiveness and ease of implementation. These methods are commonly employed at postgraduate and Ph.D. levels, as well as within various industries.

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