**Original Research Article**

**Effect Of Different Extraction Methods On the Concentration of Phenolics And Flavonoids in *Catharanthus Roseus* And Assessment Of Their Efficacy For Lifespan Extension In Model Organism *Caenorhabditis Elegans***

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**ABSTRACT**

**Objective:** In the preparation of plant extract, the extraction method plays a crucial role as it can have a significant impact on the quality of secondary metabolites extracted, as well as their stability and concentration. **Methodology:** The preliminary methodology in this research was to use two distinct methods of extraction on the leaves of Catharanthus roseus and then test the resulting extract for the quantitative and qualitative determination of phytoconstituents and further evaluating their biological efficiency on *Caenorhabditis elegans*. Thereafter, the resultant extracts were compared for total flavonoid concentration, total polyphenol content, total antioxidant activity, and DPPH free radical scavenging activity. The in vivo efficacy of these extracts was also evaluated for longevity of *Caenorhabditis elegans.* **Results:** Ultrasound-assisted extraction (UAE) yielded considerably more extracted chemicals (P < 0.05) than the conventional maceration process at 20°C. The free radical scavenging activity was found to be 60 % at 1 mg/ml concentration for the extract prepared by Ultrasonication, while for maceration, it was non-significant. The worms' lifespan was improved by 35 % using the ultrasound-based extraction approach, and in all instances, a dose-dependent effect was seen. **Conclusion:** The results also showed that the extraction process used has a vital impact on the extraction yield, the phytochemical content, and the tested biological activities. For Catharanthus roseus extract preparation, the ultrasonication technique yields better quality extraction of phytochemicals and should be given preference over other conventional methods. Catharanthus roseus can be promoted as an herb that improves the life span and is an adaptogen.

*Keywords: Catharanthus roseus, Different methods of extraction, Antioxidant activities, In vivo biological activities*

INTRODUCTION

Plant secondary metabolites can potentially prevent and treat many metabolic diseases and have been recommended as dietary supplements for ages (Dhanani et al., 2017).High-throughput extraction of secondary metabolites is based on various methods employed (Gligor et al., 2023)*.* Cold maceration is generally considered the classical extraction method. At the same time, Ultrasonication is regarded as the emerging, innovative and green chemistry-based method because it requires much lower quantities of the solvents and has a lesser requirement of the plant materials (Belwal et al., 2018).

Madagascar periwinkle, or *Catharanthus roseus*(L.), (*C. roseus*) is a well-known therapeutic plant that contains two advantageous anticancer terpenoid indole alkaloids (T.I.A.s), vincristine and vinblastine.(Nejat et al., 2015). The most significant components of *C. roseus* are its alkaloids and phenolics, but other phytochemicals have also been identified in other plant sections (Mustafa & Verpoorte, 2007). These include steroids, alkaloids, polyphenols, anthocyanins, flavonoid glycosides, & iridoid glucosides (Mustafa & Verpoorte, 2007). Diarrhoea skin infections, leukaemia,liqui dyspepsia, eye irritation, dysentery, toothaches, lung congestion and sore throats are among the conditions that can be treated with *C. roseus's* medicinal qualities (Fischhof et al., 1996; Hindmarch et al., 1991; Nayak & Pinto Pereira, 2006; Vega-Ávila et al., 2012). The identification of novel biomolecules for direct application by the pharmaceutical and agrochemical industries, or for use as lead compounds in the synthesis of more powerful molecules, requires the extraction of these chemicals from plants and the quantitative and qualitative assessment of those compounds (Altemimi et al., 2017).

An established model for studying the aging process is the short-lived nematode Caenorhabditis elegans (C. elegans)(Denzel et al., 2019). Aging research on *C. elegans* has proven helpful in exploring the role of phytochemicals in promoting the antiaging and adaptogenic potential of numerous plant extracts and isolated phytochemicals (Ernst et al., 2013; Kim & Park, 2013; Lima et al., 2014). Researchers have been able to uncover potential mechanisms of lifespan extension in *C. elegans* studies, including effects similar to dietary restriction, suppression of insulin-like growth factor signalling, stimulation of antioxidant defence systems, hormesis, and antimicrobial factors (Brown et al., 2006; Guha et al., 2013; Hartwig et al., 2009; Pietsch et al., 2009).

The current investigation involved preparing leaf extracts and quantitative analysis to assess the concentration of phenolics and flavonoids, free radical scavenging activity, and antioxidant activity. Further, in vivo studies were conducted on *C. elegans* wild-type organisms to evaluate the extracts' antiaging capabilities.

**MATERIALS AND METHODS**

**Collection of plant & preparation of plant extracts**

*Plants of C. roseus* were gathered from the greenhouse of the Loyola Center for Research and Development (LCRD). Plant extracts were prepared using the leaves of *C. roseus*. After thoroughly washing the sample under running water, it was left to dry for 10 days in the shade. Using a blender, 20 g dry material was ground into a powder, and 400 ml of ethanol:water (70:30) was used as the solvent to prepare the extracts using either sonication or maceration.The sonication was carried out in a bath sonicatorfor 60 minutes at room temperature for the three cycle. Extracts from each cycle were pooled and concentrated. The cold extraction was carried out at 20 oC in a B.O.D. incubator cum shaker in continuously agitated conditions at 100 RPM till the plant material turned colourless. The extracts were concentrated, and in both cases, a stock solution containing 100 mg of extract per millilitre was created by dissolving the extract in molecular biology-grade DMSO following solvent evaporation. The yield calculation formula was:

**Yield** = (Weight of crucible with extract- Weight of the empty crucible)/ weight of dried plant material used for extraction. The extracts were stored at 4 oC until further use.

**Quantification of phenolics and flavonoids**

The total phenolic and flavonoid content of each plant extract was determined using the Folin–Ciocalteu’s (F-C) method and an AlCl3 method, with modifications taken from Doshi S (Doshi & Braganza, 2018). To measure total phenolics, 25 µl of diluted F-C reagent (1:10 ratio) and 5µl of standard or sample were added to each well of a 96-well plate. Following a 6-minute incubation, 40 µl of 75 g/l Na2CO3 was added to each well. After 90 “minutes of dark incubation, the absorbance at 765 nm was measured on the plate. To measure flavonoids, 96-well plates were filled with 100 µl of distilled water, 10 µl of NaNO2 (50 g/l), and 5 µl of the sample or standard. Five minutes later, 15µl of 100 g/l AlCl3 was added, and six minutes were then allowed for the plate to incubate. Each well was then filled with 50µl of 1M NaOH and 50 µl of water. After 30 seconds of shaking the plate, the absorbance at 510nm was measured. Total phenolics were measured using gallic acid as a standard, and the outcomes were reported as gallic acid equivalent. Rutin was used as the flavonoid benchmark, and the outcomes were reported as rutin equivalent.

**Determination of nitric oxide free radical scavenging activity of plant extracts**

The extracts' ability to scavenge free radicals using Nitric Oxide was evaluated using the sodium Nitroprusside method using Griess reagent as per the method adopted by Ebrahimzadeh et al.(Ebrahimzadeh et al., n.d.). Absorbance was measured at 596nm and

The formula given below was used to calculate the percentage inhibition (Ali et al., 2020; Habu & Ibeh, 2015):

**% inhibition** = [(Absorbance of control- Absorbance of test)/Absorbance of control]\*100

**Lifespan analysis of *C. elegans***

The longevity experiment of *C. elegans* was conducted in 96-well plates, following the protocol described by Solis G (Solis & Petrascheck, 2011). *C. elegans* populations were synchronized through treatment with sodium hypochlorite. Each 96-well plate had approximately 10 L1 (L1) stage *C. elegans* together with 100 µl of S complete medium and OP50 as a food source. Adulthood was permitted for *C. elegans*. After that, on day 0 of adulthood, they received treatment with 5-fluoro-2'-deoxyuridine (FUDR) to stop the development of progeny. Subsequently, DMSO and plant extracts were applied to the corresponding wells as controls. To maintain effects, *C. elegans* was given weekly supplies of plant extracts and OP50. The experiment was conducted with *C. elegans* at 20 ºC. In each experiment, the average number of C. elegans (n) was between 50 and 55. Three of these trials were carried out. Every day, the animals' survival was observed under an inverted microscope. Kaplein Meyer statistical analysis was used to perform a lifespan study.

**RESULTS**

**Yields**

The higher yield was obtained through the Ultrasonication method (460 ± 24.60 mg/g), while a lower yield was obtained through the maceration method (344 ± 24.49 mg/g). There is a statistically significant difference in the yield obtained using different methods (Figure 1, Table 1).



Figure 1: The extraction procedure has an impact on the extract yield



Figure 2: The total phenolics and Flavonoids extract prepared by two different methods.

**Total phenolics and flavonoids**

The polyphenol content and flavonoid content are as mentioned below in Figure 2, Table 1.

**Table 1**: Yield of *Catharanthus roseus* extracts with various solvents*.*

|  |  |  |  |
| --- | --- | --- | --- |
| Method of extraction | Yield (mg/gm) | Total polyphenol content (mg/gm)a | Total flavonoids content (mg/gm)b |
| Sonication | 460 ± 24.60 | 24.43 ± 0.48 | 2.11 ± 0.01 |
| Maceration | 344 ± 24.49 | 10.72 ± 1.24 | 1.99 ± 0.065 |

Yield is expressed in mg of extract/g of dried material. Polyphenol content is expressed as a gallic acid equivalent. b The amount of flavonoids is represented as rutin equivalents. Every data point is represented as mean± S.D. with a one-way ANOVA. \*\*\* indicates a *P* value of less than .001.

**Nitric oxide scavenging assay**

The Nitric Oxide scavenging capacities of plant extracts were evaluated at 0.5 mg/ml, 1 mg/ml, 1.5mg/ml and 2 mg/ml concentrations. The percentage scavenging ability is as shown in Table 2. And Figure 3.



Fig. 3: Nitric Oxide scavenging activity of extracts prepared by the ultrasonication was significantly higher at all the four concentrations tested compared to Maceration.

Table No. 2: NO scavenging ability of plant extract prepared by two different methods in percentage.

|  |  |  |
| --- | --- | --- |
| Conncentration  (mg/ml) | Sonication | Maceration |
| 0.5 | 27.97 ± 2.28 | 10.93 ± 0.56 |
| 1 | 33.1 ± 0.6 | 24.03 ± 3.10 |
| 1.5 | 44.2 ± 4.49 | 35.54 ± 2.3 |
| 2 | 65.73 ± 2.42 | 39.2 ± 0.5 |

*Ethanol: Water extract made using a sonication-based technique showed better scavenging ability at all concentrations tested.*

**Lifespan analysis of *C. roseus***

The worms were subjected to three distinct doses of *C. roseus* extracts (0.01 mg/ ml,0.1 mg/ ml, & 1 mg/ ml in liquid S-complete medium along with the OP50 as a food source to ascertain the impact of *C. roseus* on the longevity of *C. elegans*. The control well was supplemented with DMSO as a solvent control. The impact of several extracts on *C. elegans* longevity is displayed below:

**Effect of *C. roseus* ethanol: water extract prepared by maceration method**

The wild type was found to have an average lifetime of 18.32 ± 0.98 days. At concentrations of 0.01mg/ ml,0.1mg/ ml, &1 mg/ ml respectively, the life spans of treated *C. elegans* rose by 11.24 %, 17.03 %, & 21.34 % (Figure 4 & Table 3).



**Fig 4**: **The survival curve depicts effect of ethanol: water extract of *C. roseus* prepared by maceration method.** Survival curve was plotted by Kaplan–Meieranalysis and analysed by Log-Rank (Mentel cox analysis). *C. roseus* treated *C. elegans* showed an increase in lifespan at 1 mg/ml concentration. Quantitative data and statistical analyses for the representative experiments are included in Table 3

Table 3: N2 Bristol *Caenorhabditis elegans* longevity as affected by *Catharanthus roseus* ethanol: water extract generated by Maceration technique.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **No. of *C. elegans*,n\*** | **Mean life span± S.E.(Days)** | ***P*-value v/s control** |
| Control | 144 | 18.32 ± 0.98 |  |
| *C. roseus* 0.01 mg/ml | 147 | 20.38 ± 1.5 | ≤.001 |
| *C. roseus* 0.1 mg/ml | 144 | 21.44 ± 0.90 | ≤.001 |
| *C. roseus* 1 mg/ml | 147 | 22.23 ± 1.2 | ≤.001 |

\*n represents a cumulative number of *C. elegans* studied in all experiment replicates.

**Effect of ethanol: water extract prepared using a sonication-based extraction method**

The wild type was found to have a mean lifespan of 20.04 ± 1.1 days. The lifespans of the treated worms rose by 19.86 %, 20.30 %, and 35.22 % for the treatments of 0.01 mg/ml, 0.1 mg/ml, & 1 mg/ml, respectively (Figure 5, Table 4).

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**Fig. 5**: The survival curve showed the effect of ethanol:water extract of *C.roseus* prepared by sonication based extraction method. The Kaplan-Meier technique was used to plot the survival curve, and Log-Rank (Mentel-Cox analysis) was used for analysis. *C.roseus* treated worms showed an increase in lifespan dose dependent manner at all concentrations tested. Table 4 contains quantitative information and statistical analyses for the typical studies.

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Table 4: Effect of *C. roseus* ethanol: water extract prepared by sonication-based extraction method on the lifespan of *C. elegans* N2(variety Bristol) worms.

|  |  |  |  |
| --- | --- | --- | --- |
|  | No. of worms,n\* | Mean life span ± S.E.(Days) | *P* value V/S control |
| Control | 144 | 20.04 ± 1.1 |  |
| *C.roseus* 0.01mg/ml | 147 | 23.92 ± 1.5 | ≤0.001 |
| *C.roseus* 0.1mg/ml | 144 | 24.04 ± 0.90 | ≤0.001 |
| *C.roseus* 1mg/ml | 147 | 27.10 ± 1.2 | ≤0.001 |

\*n represents the cumulative number of worms studied in all experiment replicates.

DISCUSSION

Numerous studies have shown the protective benefits of polyphenols, especially flavonoids, against age-related diseases, cancer, and cardiovascular disease using both *in vitro* & *in vivo* trials (Forni et al., 2019; Truong et al., 2018). Furthermore, these substances have the potential to function as potent antioxidants that could help prevent aging and other health issues in people (Salehi et al., 2020). This is because age-related diseases are closely linked to a steady rise in R.O.S. and a subsequent decrease in the organism's antioxidant defence system (Liguori et al., 2018). Numerous studies highlight the antioxidant capacity of phytochemicals in the prevention and therapy of age-associated decrease in functions to overcome this complex situation. (Chattopadhyay & Thirumurugan, 2018; Duangjan et al., 2019; Wiegant et al., 2009).

Traditional techniques, such as maceration, stirring-assisted maceration, and Soxhlet extraction, are most frequently employed to isolate the polyphenols and flavonoids. (Alara et al., 2021; Altemimi et al., 2017; Belwal et al., 2018). The basis of maceration extraction is the separation of solids from liquids, with water or an organic solvent serving as the liquid phase.

Another technique is ultrasound extraction, which combines pressure, temperature, and low-frequency vibrations to create cavitation and high-energy bubbles (Bitwell et al., 2023; Kapadia et al., 2022).

Two techniques were used in this work to prepare the crude extracts of *Catharanthus roseus*, resulting in varying levels of flavonoid and phenolic content. It is well known that the yield of extracted phenolics is influenced by several parameters, such as solvent polarity and the extraction procedure (Do et al., 2014). In line with other investigations, the current investigation discovered that sonication-prepared extracts had significant concentrations of phenolics and flavonoids. ((Dubey, 2014; Siddhuraju & Becker, 2003 ; Pham et al., 2020). The capacity of medicinal herbs to scavenge free radicals is an often used indicator of their antioxidant activity. The current investigations assessed the degree of Nitric Oxide free radical scavenging at various doses of leaf extracts of *C. roseus* made using two distinct techniques. Nitric oxide free radicals are generated when they encounter oxygen or superoxide. It acts as a pro-inflammatory agent, but if produced for a prolonged duration, it causes tissue damage and can induce chronic inflammation, which in turn reduces longevity (Mfotie Njoya et al., 2017; Sheikh, n.d.). The present study found that a higher concentration of plant extracts produced by the sonication method effectively scavenges the Nitric oxides.

To assess the effectiveness of the generated plant extracts, the *C. elegans* model—which is frequently employed to screen and assess the antiaging activity of various substances—was also employed (Xu et al., 2023). Three distinct doses of both extracts were utilized in the current investigation to slow down *C. elegans'* aging process. The life duration of *C .elegans* was shown to be greatly extended by *C. roseus* extracts at different concentrations. The greatest influence on life span extension was noted at a concentration of 1mg/ml of *C. roseus* extract made using the sonication method.

CONCLUSION

The present study evaluated the medicinally significant herb *C. roseus* for its total flavonoid content, phenolic content, free radical scavenging ability and antiaging potential. This approach has helped prove that the ultrasonication-based method is much more effective than the maceration method in optimising phytochemicals from plant material extraction. This study's findings support the notion that crucial factors in the production of crude extracts with phenolic components and antioxidant activity are the extraction conditions. *C. Roseus* additionally demonstrated possible antiaging properties. This implies that these plants may have future uses as agents that lengthen life. Further, it can be evaluated for its stress combating and anti-inflammatory potential.

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