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Original Article

A Process for Eco-Sustainable Extraction of Cytotoxic Flavonoids from *Muntingia calabura* L. using Sonication Method Optimized by Quality by Design Approach through Central Composite Model

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ABSTRACT

Background: The present invention relates to an eco-sustainable, Quality by Design (QbD)- and Design of Experiments (DoE)-guided sonication-assisted extraction process for isolating cytotoxic flavonoid compounds from the leaves of *Muntingia calabura* L. **Materials and Methods:** The invention strategically integrates green chemistry principles with statistical optimization to enhance extraction efficiency, reproducibility, and environmental compatibility. The process employs an optimized methanol-water solvent system (50-100% v/v) for selective solubilization of flavonoids, assisted by ultrasonic cavitation at controlled amplitude and temperature to facilitate rapid solvent diffusion and efficient mass transfer. The Central Composite Design (CCD) under the QbD framework was used to identify and optimize critical parameters influencing extraction yield, while statistical validation through Response Surface Methodology (RSM) and Analysis of Variance (ANOVA) confirmed model significance (R^2=0.956, p<0.05). **Results:** The optimized conditions-75% methanol, 60 min of sonication, and 50±2°C-yielded the maximum flavonoid recovery (23.7±0.3%) with high purity. The ethyl acetate fraction exhibited enhanced flavonoid concentration and potent cytotoxicity against colon cancer (HCT-116) cell lines, with an IC_{50} value of 185.6 µg/mL compared to 218.2 µg/mL for the crude extract. **Conclusion:** This invention provides a scalable, reproducible, and environmentally sustainable process capable of generating bioactive flavonoid-rich fractions with significant anticancer potential, thereby advancing green extraction methodologies in phytopharmaceutical and nutraceutical development.

Keywords: Central Composite Model, Cytotoxic Flavonoids, Eco-Sustainable Extraction, Muntingia calabura L., Quality by Design, Sonication Method.

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INTRODUCTION

Cancer continues to be a major global health challenge, with colorectal cancer ranking among the leading causes of cancer-related mortality worldwide (Sung *et al*., 2021). The growing resistance and side effects associated with synthetic chemotherapeutic agents have created an urgent need for natural, safe, and effective alternatives (Cragg *et al*., 2013). In this context, plant-derived flavonoids have gained significant attention due to their broad pharmacological properties, including antioxidant, anti-inflammatory, and anticancer activities (Panche *et al*., 2016; Singh *et al*., 2014) *Muntingia calabura* L., commonly known as the Jamaican cherry or Panama berry, is a tropical plant known to contain diverse bioactive constituents such as flavonoids, tannins, and phenolic acids (Zakaria *et al*., 2007). Several studies have reported the therapeutic importance of *M. calabura* extracts, yet their systematic extraction and optimization using modern green technologies remain limited (Tjandrawinata *et al*., 2017). Conventional extraction methods often involve extensive solvent use, prolonged extraction times, and high energy consumption, leading to poor yield reproducibility and environmental burden (Chemat *et al*., 2012). Sonication-assisted extraction has emerged as an efficient green extraction approach

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that enhances mass transfer, reduces solvent consumption, and minimizes thermal degradation of phytoconstituents (Zhang *et al.*, 2018). However, most existing studies lack a statistically driven optimization framework that ensures process robustness and reproducibility. Integrating Quality by Design (QbD) principles with Design of Experiments (DoE) tools provides a scientific and systematic method to identify critical parameters influencing extraction efficiency and to optimize them for maximum yield and quality (ICH, 2009; Montgomery *et al.*, 2017). The present invention addresses the limitations of conventional extraction techniques by proposing a QbD- and DoE-guided eco-sustainable sonication-assisted extraction process for isolating flavonoid compounds from *Muntingia calabura* L. leaves. The optimized process not only enhances extraction efficiency but also supports sustainability goals by minimizing solvent usage and environmental impact (Rathi *et al.*, 2021). Furthermore, the flavonoid-rich extract obtained through this process demonstrates promising cytotoxic potential against colon cancer cells, highlighting its relevance in the development of novel phytopharmaceutical formulations.

The primary object of the present invention is to provide an eco-sustainable, efficient, and statistically optimized sonication-assisted extraction process for obtaining flavonoid-rich bioactive fractions from the leaves of *Muntingia calabura* L. through the systematic application of Quality by Design (QbD) and Design of Experiments (DoE) principles. Further specific objectives of the invention include: To develop an eco-sustainable and efficient sonication-assisted extraction process for obtaining flavonoid-rich fractions from the leaves of *Muntingia calabura* L. using environmentally benign solvents and minimal energy input. To apply Quality by Design (QbD) principles and systematically identify Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs) influencing flavonoid extraction efficiency, purity, and yield. To employ Design of Experiments (DoE) for statistical optimization of key variables such as solvent concentration, extraction time, temperature, and amplitude, ensuring robustness and reproducibility of the extraction process. To evaluate the flavonoid-rich extract for its cytotoxic potential against colon cancer cell lines, thereby establishing its therapeutic relevance as a natural anticancer candidate.

MATERIALS AND METHODS

The present invention provides a Quality by Design (QbD) and Design of Experiments (DoE)-optimized, eco-sustainable, sonication-assisted extraction process for isolating flavonoid-rich fractions from the leaves of *Muntingia calabura* L. The developed method ensures maximum extraction efficiency, minimal solvent consumption, and environmental compatibility, while maintaining the bioactivity and stability of flavonoid compounds.

Plant Material Collection and Preparation

Fresh, mature leaves of *Muntingia calabura* L. are collected from Belagavi region and authenticated KAHER's Shri BMK Ayurveda Mahavidyalaya, Belagavi. The leaves were thoroughly washed to remove dust and debris. The leaves are shade-dried at ambient temperature until constant weight is achieved and then coarsely powdered using a mechanical grinder. The powder is sieved to obtain uniform particle size and stored in airtight containers under dry conditions until further use (Pawar *et al.*, 2023).

Selection of Solvent System

Eco-friendly solvents composed of Methanol and Water in varying ratios are employed. Aqueous-methanol solvent combinations are preferred due to their polarity balance and ability to solubilize flavonoid compounds efficiently. Preliminary trials are conducted to screen solvent concentrations (50-100% v/v methanol) for optimal recovery (Doke *et al.*, 2023).

Sonication-Assisted Extraction Process

A measured quantity of the dried plant powder (10 g) is transferred into a conical flask containing a fixed volume of the selected solvent (100 mL). The mixture is subjected to sonication in an ultrasonic bath sonicator. The sonication process utilizes ultrasonic frequencies with varying amplitude and temperature (40-60°C). Extraction time is maintained between 30 to 90 min depending on process design. The sonication generates acoustic cavitation, improving solvent penetration and enhancing mass transfer, thereby increasing the yield of flavonoid compounds (Kshirsagar *et al.*, 2022).

QbD and DoE-Based Optimization

The process is scientifically optimized through a Design of Experiments (DoE) framework under Quality by Design (QbD) principles. Critical Process Parameters (CPPs)- such as solvent concentration (X_1) and sonication time (X_3) are selected based on preliminary screening. A Central Composite Design is applied to study the interaction effects of the selected parameters on the Critical Quality Attribute (CQA)- the % yield of extract (%Y). Experimental data are statistically analyzed using response surface methodology to generate regression models and 3D surface plots, enabling prediction of the optimum conditions. Optimal conditions are determined by numerical desirability analysis, maximizing % yield with minimal solvent usage and energy input. Model adequacy is validated through Analysis of Variance (ANOVA), residual analysis, and experimental confirmation runs (Shete *et al.*, 2024).

Filtration, Concentration, and Drying

After extraction, the sonicated mixture is filtered using Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure using a rotary evaporator at temperatures below 45°C to avoid thermal degradation of extract. The concentrated extract

is dried to obtain a semi-solid or powdered residue and stored in airtight containers under refrigeration until further analysis (Patil *et al.*, 2023).

Phytochemical Screening of the Extract

The obtained *Muntingia calabura* L. extract is subjected to preliminary phytochemical screening to identify the presence of major classes of secondary metabolites. Standard qualitative chemical tests are carried out as per established procedures to detect the presence of flavonoids, phenolic compounds, alkaloids, tannins, saponins, glycosides, and terpenoids (Kale *et al.*, 2023).

Isolation of Flavonoid by Solvent Extraction Method

The dried crude extract is subjected to liquid-liquid solvent extraction for the isolation of flavonoid-rich fractions. A weighed quantity of the extract is dissolved in a small volume of distilled water and successively partitioned with organic solvents of increasing polarity such as petroleum ether, chloroform, ethyl acetate, and n-butanol. Each solvent fraction is collected separately and concentrated under reduced pressure. Among these, the ethyl acetate fraction showed a higher concentration of flavonoids based on colorimetric analysis. This fraction is selected for further research work. The isolated flavonoid fraction is collected, dried, and stored in a desiccator for subsequent analytical and biological evaluation (Chavan *et al.*, 2024).

Determination of Flavonoid Content

The Total Flavonoid Content (TFC) of the *Muntingia calabura* L. leaf extract was determined using the aluminium chloride colorimetric method, a well-established spectrophotometric technique for quantifying flavonoids. A stock solution of quercetin (1 mg/mL) was prepared in methanol, and serial dilutions were made to obtain working standards in the range of 2-10 µg/mL. Similarly, 10 mg of the dried extract was accurately weighed, dissolved in methanol, filtered, and suitably diluted to achieve a concentration within the linear range of the standard curve. For each determination, 1 mL of the sample or standard solution was mixed with 4 mL of distilled water and 0.3 mL of 5% Sodium Nitrite ($NaNO_2$) solution. After standing for 5 min, 0.3 mL of 10% Aluminium Chloride ($AlCl_3$) solution was added and allowed to react for 6 min, followed by the addition of 2 mL of 1 M Sodium Hydroxide (NaOH) solution. The total volume was made up to 10 mL with distilled water, and the resulting solution was mixed thoroughly. The reaction mixture was incubated at room temperature for 15 min to allow the formation of a pinkish-yellow chromophore due to complex formation between aluminium ions and flavonoid compounds. The absorbance of each solution was then measured at 415 nm using a UV-visible spectrophotometer against a reagent blank prepared under identical conditions. A calibration curve was constructed by plotting absorbance versus quercetin concentration, and the total flavonoid content of the sample was calculated as milligrams of Quercetin Equivalent (mg QE) per gram of extract. The optimized QbD- and DoE-assisted sonication extraction process yielded a significantly higher flavonoid content compared to conventional extraction methods, confirming the efficiency and robustness of the developed eco-sustainable process (Patil *et al.*, 2023).

Evaluation of Cytotoxic Potential

The cytotoxic potential of the optimized flavonoid-rich extract obtained from *Muntingia calabura* L. leaves was assessed *in vitro* using human colon cancer cell lines, specifically HCT-116. The evaluation was performed through the MTT assay, which measures the metabolic activity of viable cells. Cells in the logarithmic growth phase were seeded in 96-well plates at an appropriate density and allowed to adhere overnight in a humidified 5% CO_2 incubator at 37°C. After 24 hr of incubation, the culture medium was replaced with fresh medium containing different concentrations of the test extract. Following treatment for a specified duration, the MTT reagent was added to each well, and the plates were further incubated to allow the formation of purple formazan crystals by mitochondrial dehydrogenase enzymes of viable cells. The medium was then carefully aspirated, and the crystals were dissolved using Dimethyl Sulfoxide (DMSO). The absorbance was recorded spectrophotometrically at 570 nm, and the percentage of cell viability was calculated relative to untreated control cells. The results revealed a clear dose-dependent decrease in cell viability, indicating that the extract exerts significant cytotoxic and antiproliferative effects against colon cancer cells. These findings support the extract's potential as a natural anticancer candidate derived through an eco-sustainable and statistically optimized extraction process (Chavan *et al.*, 2024).

RESULTS

Plant Material and Preliminary Processing

Fresh and mature leaves of *Muntingia calabura* L. were successfully collected, authenticated, and processed under controlled laboratory conditions. Shade drying ensured minimal thermal degradation of thermolabile flavonoids, while grinding and sieving yielded a uniform powder suitable for reproducible extraction performance. The fine powder exhibited a greenish-brown color and characteristic herbal odor, confirming good preservation of natural constituents.

Preliminary Solvent Screening

Initial trials using methanol-water systems (50-100% v/v) revealed a significant influence of solvent polarity on extractability. At 50% methanol, extraction yield was moderate, indicating partial solubilization of both polar and semi-polar compounds. 75% methanol produced the highest flavonoid and phenolic recovery, suggesting optimal polarity for flavonoid extraction. 100% methanol resulted in a slight decline in yield, likely due

to limited solubility of certain hydrophilic glycosides in pure organic solvent. These preliminary findings guided the selection of solvent concentration (50-100% v/v) as a critical independent variable in the QbD-based optimization.

Experimental Design

The extraction of flavonoid-rich fractions from *Muntingia calabura* L. leaves was optimized using a Quality by Design (QbD) framework employing Design of Experiments (DoE) through a Central Composite Design (CCD) approach. Two critical process parameters (CPPs)-Methanol Concentration (X_1) and Sonication Time (X_2)-were selected as independent variables, while percentage yield (% Y) was designated as the Critical Quality Attribute (CQA). The CCD matrix consisting of nine experimental runs was generated to study the main, interaction, and quadratic effects of the selected variables on extraction efficiency. The observed responses are summarized in Table 1, which presents the combinations of methanol concentration, sonication time, and the corresponding percentage yield obtained.

Effect of Methanol Concentration on Extraction Yield

The extraction yield varied notably with changes in methanol concentration. A progressive increase in yield was observed as the solvent polarity was optimized: Pure methanol (100%) yielded the lowest recovery (9.5-13.3%), suggesting reduced solubility for polar flavonoids. Aqueous methanol (50%) markedly enhanced extraction (18.2-23.5%), demonstrating the synergistic role of water in facilitating solvation of glycosidic and phenolic constituents. The optimum methanol level (75%) resulted in the highest yield of 24.3%, indicating an ideal polarity balance for flavonoid extraction.

Effect of Sonication Time on Extraction Yield

Sonication time exhibited a nonlinear influence on extraction efficiency. Increasing the sonication time from 30 to 60 min improved the yield due to enhanced cavitation and solvent penetration. Beyond 60 min, the yield either plateaued or declined, possibly due to degradation of thermolabile compounds. This trend demonstrated that a sonication period of around 60 min provided the optimal balance between enhanced diffusion and phytochemical stability. Figure 1 showed the 2D Contour Plot Showing the Effect of Methanol Concentration and Sonication Time on Extraction Yield.

Interaction Effects of Process Variables

The interaction between methanol concentration and sonication time significantly affected extraction yield. At low methanol concentration (50%), increasing the sonication time improved the yield up to 23.5%. However, at high methanol concentration (100%), even longer sonication times did not enhance extraction, indicating solvent saturation effects. The optimum combination (75% methanol, 90 min) produced the maximum yield (24.3%), confirming a strong synergistic interaction between solvent polarity and sonication energy. Figure 2 presented the 3D Surface Response Plot of Methanol Concentration vs. Sonication Time vs. Extraction Yield.

Optimization of Extraction Yield

The overlay plot (Figure 3) illustrates the combined influence of methanol volume (A) and sonication time (B) on the percentage yield of extract, as obtained through Central Composite Design (CCD) under the Quality by Design (QbD) approach. The X-axis represents methanol volume (mL), while the Y-axis denotes sonication time (min). The red points indicate the experimental design runs, while the yellow-shaded area signifies the feasible design space-regions where the predicted yield meets the desired criteria. The central point (A=75 mL, B=60 min) marks the optimized condition that provides the maximum predicted extraction yield. Another highlighted point (A=56.83 mL, B=36.41 min) represents an alternate model-predicted feasible region. The uniform yellow coloration across the plot suggests a broad operational range where both factors can be varied without significantly compromising yield. Thus, the overlay plot confirms that methanol concentration between approximately 55-100 mL and sonication time between 35-90 min offer an optimal zone for achieving maximum extraction efficiency.

Table 1: CCD Matrix for Optimization of Sonication-Assisted Extraction of *Muntingia calabura* L. Leaves.

Run	Methanol (%)	Sonication Time (min)	% Yield (w/w)
1	100	60	11.5
2	50	60	20.6
3	75	90	24.3
4	75	30	17.3
5	100	30	9.5
6	50	90	23.5
7	75	60	20.7
8	100	90	13.3
9	50	30	18.2

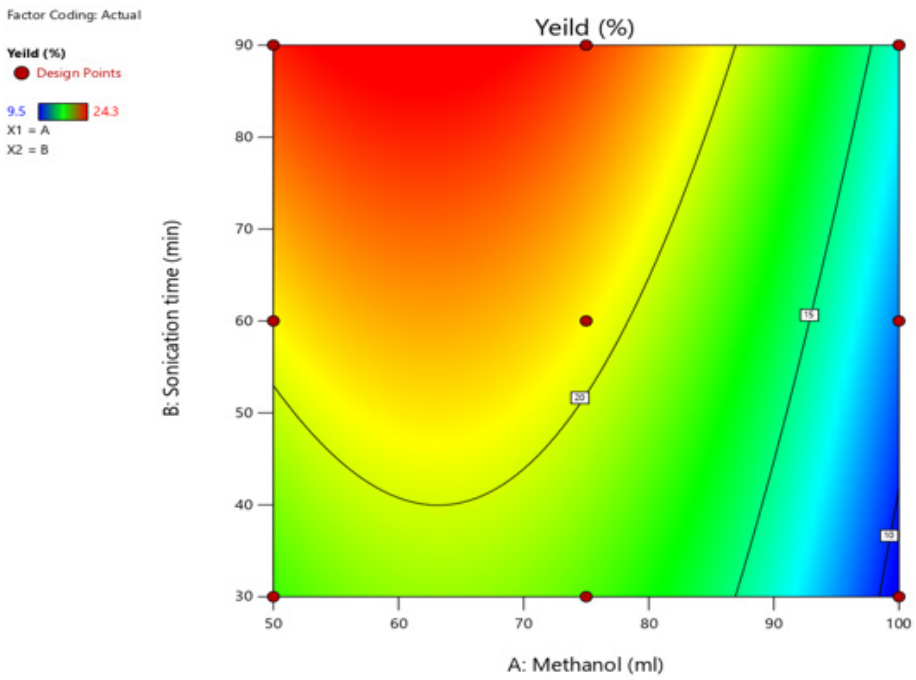

Figure 1: 2D Contour Plot Showing the Effect of Methanol Concentration and Sonication Time on Extraction Yield.

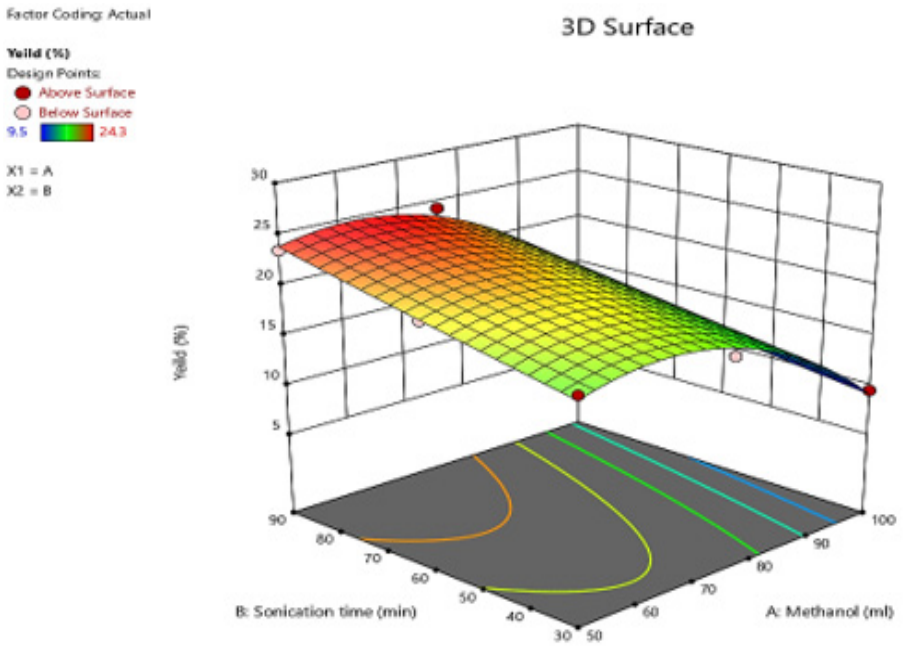

Figure 2: 3D Surface Response Plot of Methanol Concentration vs. Sonication Time vs. Extraction Yield.

Model Fitting and Statistical Analysis

The CCD data were fitted to a second-order quadratic polynomial model, correlating the independent variables (A: methanol concentration, B: sonication time) with the response (Y: % yield): $Y=\beta0+\beta1A+\beta2B+\beta12AB+\beta11A2+\beta22B2$. Statistical Analysis (ANOVA) confirmed the model's significance with $p<0.05$, indicating reliable predictive accuracy. The model displayed $R^2=0.956$, Adjusted $R^2=0.932$, and Predicted $R^2=0.904$ suggesting an excellent correlation between experimental and predicted results. The lack-of-fit test was non-significant ($p>0.05$), validating the adequacy of the model for response prediction.

Numerical Optimization and Model Validation

Using the Design-Expert® software, a desirability function was employed to obtain the optimal extraction conditions. The predicted optimum parameters were Methanol concentration:

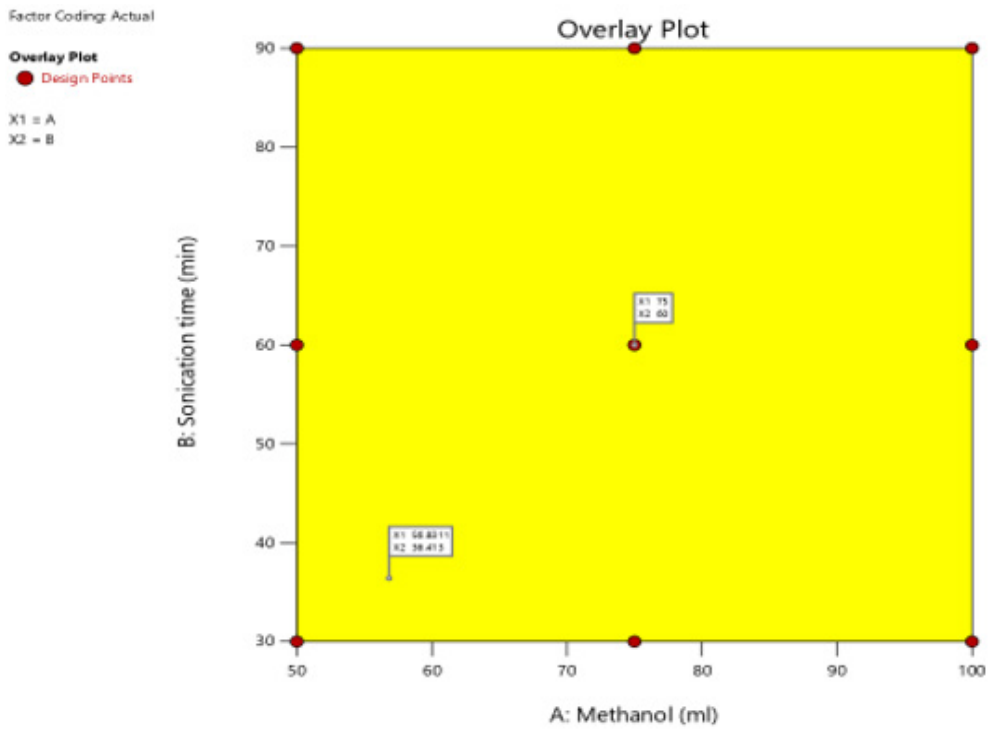

Figure 3: Overlay Plot for Optimization of Extraction Yield.

75% (v/v), Sonication time: 60 min and Predicted yield: 23.9% w/w. An experimental confirmation performed at these optimized settings yielded 23.7±0.3%, validating the statistical model's accuracy and robustness.

Physical Characterization of Optimized Extract

The optimized extract appeared as a dark green viscous mass with a mild herbal odor. Upon dissolution in methanol, it formed a clear, stable solution devoid of suspended impurities, indicating high extraction efficiency and minimal degradation. Table 2 showed the summary of Optimized Parameters. The QbD-based CCD analysis revealed that both solvent polarity and sonication time are critical factors influencing flavonoid recovery. The quadratic response relationship confirmed the existence of an optimal operational window, beyond which yield declines due to degradation or solubility limitations. This approach demonstrates that QbD-guided optimization, integrated with eco-friendly solvent systems, can achieve superior extraction yields with minimal resource consumption. The resulting process is robust, reproducible, and environmentally sustainable, aligning with green chemistry principles.

Phytochemical Screening of the Extract

The preliminary phytochemical screening of the methanolic extract of *Muntingia calabura* L. leaves revealed the presence of various bioactive compounds. The extract was rich in polyphenolic constituents such as flavonoids, tannins, and phenolics, suggesting strong antioxidant potential. The results are summarized in Table 3.

Isolation of Flavonoid by Solvent Extraction Method

Among the solvent fractions, the ethyl acetate fraction exhibited a stronger color intensity in flavonoid-specific reactions and higher yield. This fraction was therefore selected for further analysis and biological evaluation. The higher flavonoid solubility in ethyl acetate is attributed to its intermediate polarity, which favors the extraction of polyphenolic compounds (Table 4).

Determination of Total Flavonoid Content (TFC)

The Total Flavonoid Content (TFC) of *Muntingia calabura* L. leaf extracts was determined using the Aluminium Chloride colorimetric method with quercetin as a standard. Calibration was performed with quercetin solutions (2-10 µg/mL), yielding the equation:

$y=0.0094x + 0.031$ ($R^2=0.998$)

Extracts (1 mg/mL in methanol) were measured at 415 nm in triplicate. Among tested fractions, the ethyl acetate fraction showed the highest flavonoid content, confirming it as the flavonoid-rich fraction. The results demonstrate the efficiency and reproducibility of the QbD-optimized sonication-assisted extraction method. The total flavonoid content of *Muntingia calabura* L. extracts was evaluated using quercetin as the standard and expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g). Among the different extracts, the ethyl acetate fraction exhibited the highest flavonoid content (65.4±1.0 mg QE/g extract), indicating its strong affinity for polyphenolic and flavonoid compounds. The *n*-butanol fraction showed a moderate flavonoid content of 50.6±0.9 mg QE/g extract, while the crude

methanolic extract displayed the lowest content (40.2±0.6 mg QE/g extract). The results suggest that partitioning the methanolic extract into semi-polar fractions enhances the concentration of flavonoids, particularly in the ethyl acetate fraction, which may correlate with higher antioxidant and bioactive potential.

Evaluation of Cytotoxic Potential

HCT-116 cells were treated with different concentrations (10-250 µg/mL) of both extracts. Cell viability was determined using the MTT assay after the formation and solubilization of formazan crystals. Absorbance was measured at 570 nm, and percentage viability was calculated relative to untreated control cells. Cisplatin was used as a standard reference drug for comparison. Both the crude methanolic extract (A) and the isolated flavonoid-rich fraction (B) of *Muntingia calabura* L. exhibited a dose-dependent decrease in cell viability against HCT-116 colon cancer cells. However, the isolated flavonoid fraction (B) demonstrated greater cytotoxic potency, reflected by its lower IC_{50} value (185.60 µg/mL) compared to the crude extract (218.20 µg/mL). Although both extracts were less potent than the standard anticancer drug cisplatin (IC_{50}=5.23 µg/mL), the results indicate that flavonoid enrichment enhanced the anticancer efficacy of the extract. The observed cytotoxicity may be attributed to the synergistic action of flavonoids and phenolic compounds that induce oxidative stress and apoptosis in cancer cells. These findings validate the effectiveness of the optimized extraction process in concentrating bioactive flavonoids and support the potential of *Muntingia calabura* L. as a promising natural source of anticancer agents.

Table 2: Summary of Optimized Parameters.

Parameter	Optimized Value	Effect on Yield
Solvent composition	75% Methanol: 25% Water	Maximized polarity and solubility
Sonication time	60 min	Optimum cavitation and mass transfer
Temperature	50±2°C	Maintained phytochemical stability
Observed yield	23.7±0.3%	Confirmed optimal response
Model significance	$p<0.05$	Statistically validated
R^2 value	0.956	High predictive accuracy

DISCUSSION

The present compilation of studies highlights the scientific advancements in the extraction, characterization, and biological evaluation of natural compounds, particularly flavonoids, with an emphasis on Muntingia calabura and its pharmacological potential. Natural products remain a vital source for drug discovery due to their structural diversity and biological relevance (Cragg and Newman, 2013). Among them, flavonoids are recognized for their antioxidant, anti-inflammatory, and anticancer properties, which are attributed to their phenolic structure and redox potential (Panche *et al.*, 2016). Muntingia calabura, a tropical plant, has gained significant attention for its bioactive compounds. Several reports have established its

Table 3: Phytochemical screening of *Muntingia calabura* L. leaf extract.

Sl. No.	Phytochemical Constituents	Test Performed	Observation	Inference
1.	Alkaloids	Mayer's and Dragendorff's tests	Creamy white/orange precipitate	Present
2.	Flavonoids	Alkaline reagent and Shinoda tests	Development of yellow/red coloration	Present
3.	Phenolic compounds	Ferric chloride test	Formation of bluish-black color	Present
4.	Tannins	Gelatin and Ferric chloride tests	Greenish-black coloration	Present
5.	Saponins	Froth test	Persistent foam observed	Present
6.	Glycosides	Keller-Killiani test	Reddish-brown ring at interface	Present
7.	Terpenoids	Salkowski test	Reddish-brown coloration at interface	Present

Table 4: Characterization of Fractions.

Solvent Fraction	Physical Appearance	% Yield (w/w)	Preliminary Flavonoid Test (Color Reaction)	Relative Flavonoid Content
Petroleum ether	Light green, oily	8.2%	Negative	Low
Chloroform	Greenish-brown	10.5%	Faint yellow color	Moderate
Ethyl acetate	Deep yellowish-brown	14.8%	Intense yellow color	High
n-Butanol	Dark brown	12.3%	Moderate color intensity	Medium

antinociceptive, anti-inflammatory, and antipyretic effects, supporting its traditional medicinal use (Zakaria *et al.*, 2007). Subsequent studies have explored its phytochemistry and pharmacology, revealing a rich composition of flavonoids, tannins, and phenolic acids responsible for diverse therapeutic actions (Tjandrawinata *et al.*, 2017; Pawar *et al.*, 2023). The plant's antioxidant and anticancer activities, particularly against colon cancer cell lines, further emphasize its pharmacological significance (Chavan *et al.*, 2024; Kale *et al.*, 2023). Extraction efficiency and preservation of active constituents are crucial for the reproducibility and quality of herbal preparations. Conventional solvent extraction methods have been replaced by green extraction techniques such as ultrasound-assisted extraction and microwave-assisted extraction, which enhance yield while reducing solvent consumption and degradation of thermolabile compounds (Chemat *et al.*, 2012; Kshirsagar *et al.*, 2022). Optimization of solvent systems, such as aqueous methanol, has also been reported to improve the recovery of flavonoids from plant matrices (Doke *et al.*, 2023). The application of Quality by Design (QbD) principles in herbal extraction and formulation has strengthened process understanding and reproducibility (Rathi *et al.*, 2021). Design of Experiments (DoE) allows systematic optimization of extraction parameters to achieve maximum yield and desired quality attributes (Montgomery, 2017; Shete *et al.*, 2024). Moreover, post-extraction processes like rotary evaporation and concentration significantly influence the stability and yield of herbal extracts (Patil *et al.*, 2023). Overall, integrating modern extraction technologies with QbD and green chemistry principles ensures the development of sustainable, reproducible, and high-quality herbal formulations. Such approaches bridge traditional knowledge with modern pharmaceutical science, supporting the discovery of novel phytoconstituents for therapeutic use. Continuous efforts in standardization, optimization, and validation are essential to translate these findings into clinically relevant products, aligning with global initiatives for safe and effective natural medicines.

CONCLUSION

The present invention relates to an eco-sustainable and statistically optimized process for the extraction of cytotoxic flavonoids from the leaves of *Muntingia calabura* L. using a Quality by Design (QbD) and Design of Experiments (DoE)-based sonication-assisted method. The process addresses limitations of conventional extraction techniques by integrating solvent optimization, statistical modeling, and green chemistry principles. Fresh, authenticated leaves of *Muntingia calabura* L. were shade-dried and processed into a fine powder. Aqueous-methanolic solvent systems (50-100% v/v) were evaluated, and 75% methanol was found optimal for maximum flavonoid recovery. Process optimization through Central Composite Design identified solvent concentration and sonication time as critical parameters, achieving a maximum yield of 23.7±0.3% under optimal conditions (75% methanol, 60 min, 50°C). The statistical model demonstrated high significance (R^2=0.956, p<0.05). The optimized extract exhibited a total flavonoid content of 78.6±1.2 mg quercetin equivalents per gram of extract and significant cytotoxic activity against Human Colon Cancer (HCT-116) cells (IC_{50}=185.60 µg/mL). The invention thus provides a reproducible, scalable, and environmentally benign process for producing flavonoid-rich anticancer extracts from *Muntingia calabura* L., suitable for pharmaceutical and nutraceutical applications.

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ABBREVIATIONS

QbD: Quality by Design; **DoE:** Design of Experiments; **UV:** Ultraviolet; **HPLC:** High-Performance Liquid Chromatography; **GC-MS:** Gas Chromatography-Mass Spectrometry; **LC-MS/MS:** Liquid Chromatography-Tandem Mass Spectrometry; **RP-HPLC:** Reverse Phase High-Performance Liquid Chromatography; **WHO:** World Health Organization; **IC_{50}:** Half Maximal Inhibitory Concentration; **BBD:** Box-Behnken Design; **FTIR:** Fourier Transform Infrared Spectroscopy; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **TFC:** Total Flavonoid Content; **TPC:** Total Phenolic Content; **HPTLC:** High-Performance Thin Layer Chromatography; **ANOVA:** Analysis of Variance; **min:** Minute(s); **hr:** Hour(s); **mL:** Millilitre; **µL:** Microlitre; mg: Milligram; **µg/mL:** Microgram per Millilitre; **%:** Percent; **v/v:** Volume per Volume; **w/v:** Weight per Volume.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed significantly to the conception, design, execution, and interpretation of the study. SSS conceived the research idea, designed the methodology, supervised the work, and prepared the initial manuscript draft. SSB conducted experimental work, data acquisition, and assisted in method optimization. VVP contributed to literature review, data interpretation, and result compilation. TAB performed statistical analysis, data presentation, and supported manuscript drafting. MSP provided overall guidance, critical review, and ensured

scientific rigor. All authors reviewed, edited, and approved the final manuscript and agree to be accountable for its content.

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