Comparative Assessment of Phytochemical Profiles in Tissue Culture-raised, Field-raised, and Commercial Extract of *Bacopa monnieri* (L.) Wettst.

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Abstract. The medicinal efficacy of *Bacopa monnieri* (L.) Wettst. is closely linked to its diverse secondary metabolites, including phenolics, flavonoids, and alkaloids, though commercial use is limited by phytochemical variability in wild and cultivated plants. This study comparatively evaluated phytochemical profiles of tissue culture-raised plants, field-grown counterparts, and standardized commercial extracts (used as a reference sample for comparative assessment of metabolite concentrations relative to raw plant sources) using systematic qualitative screening and quantitative estimation. Total phenolics, flavonoids, and alkaloids were quantified using Folin-Ciocalteu, aluminium chloride, and BCG-chloroform assays, respectively. The study demonstrates that extracts obtained from in-vitro raised plants exhibited approximately 2.40-fold higher total phenolic content, and 1.48-fold higher alkaloid content compared to the field-raised samples, along with a slight increase (~1.04-fold and ~1.62 fold, respectively) over commercial product. Although the commercial sample showed highest flavonoid content, tissue culture-raised extract still demonstrated a ~3.22-fold higher flavonoid concentration compared to field-raised plant extract. Therefore, this result aims to elucidate the inconsistencies in metabolite accumulation across different production systems and evaluate whether biotechnological interventions through plant tissue culture provide more consistent and enriched source of secondary metabolites compared to traditional field cultivated and processed commercial products.

1. Introduction

Bacopa monnieri, also known as Brahmi has been in use for over 5000 years in countries native to its occurrence like India, China and Thailand. In India, the plant is widely used in ayurvedic practices to treat various ailments such as epilepsy and insomnia [1,2]. It is known for its nootropic effects involved in enhancing memory, boosting brain functions and mitigating the effects of certain neurodegenerative diseases such as Alzheimer's and Parkinson's [2,3]. The therapeutic properties of *B. monnieri* are attributed to its broad metabolite profiles consisting of phenolics, flavonoids, alkaloids and terpenes. Owing to these properties, the market demand for *B. monnieri* has significantly increased. The demand for raw material was estimated to be approximately 1000 tonnes per year [4]. Moreover, in terms of priority, the plant ranks second in the list of major Indian medicinal plants used primarily for its therapeutic and commercial values [1]. Also commercially, the processed counterparts such as oil, powder and extracts are even valued at higher prices as compared to whole plants. However, a major challenge in the pharmaceutical application of the plant is phytochemical inconsistency in field cultivated plants [5]. The metabolite profiles in field-raised plants are highly influenced by fluctuating environmental conditions and seasonal cycles [6].

Alternatively, plant tissue culture techniques such as micropropagation provide a robust strategy to address these limitations as it enables rapid, year-round production of biomass with a uniform phytochemical profile [7]. Moreover, in-vitro systems also potentially maximize the yield of active metabolites which are otherwise subject to unpredictability of natural habitats [8].

Even though, a comprehensive research gap exists despite the technological advancement regarding the comparative metabolite benchmarking of in vitro-raised plants against field-raised and finished commercial extracts. Contemporary studies focus primarily on the characteristic bioactive compounds within the plant, undermining the broad quantitative profiles of total metabolites such as phenolics, terpenoids, flavonoids and alkaloids, which defines the plant's holistic quality [9].

Therefore, the goal of the current study was to conduct a comparative assessment of phytochemical profiles of tissue culture-raised, field-raised, and commercial extracts of *B. monnieri*. Following qualitative and quantitative analysis of total secondary metabolites in in-vitro and ex-vitro grown plants, this study seeks to identify the potential source for pharmaceutical applications. Moreover, the study also aims to highlight upon the variability of metabolites across processed commercial product and naturally sourced plant extracts.

2. Materials and methods

2.1 Plant material

Tissue culture-raised plant

In-vitro raised *Bacopa monnieri* plants were obtained through micropropagation of whole leaf and nodal segments. The cultures were established on Murashige and Skoog (MS) medium supplemented with Indole-3-butyric acid (IBA) (3mg/ml) and Kinetin (Kn) (1mg/ml) under controlled environmental conditions such as 2000 lux intensity of light, 25±1° C temperature and 20-30% humidity.

Field-raised plant

Field-grown plants were collected from Glasshouse, Department of Biotechnology and Bioinformatics, JUIT, Waknaghat (Figure 1).

Figure 1. *Bacopa monnieri* (L.) Wettst.

2.2 Commercial extract sample (H1)

A commercially available *Bacopa monnieri* extract product was procured from a local pharmacy. The product label information (250 mg of whole plant extract per tablet) was recorded. The commercial product was subjected to the same phytochemical analyses as plant samples to enable direct comparison.

2.3 Sample preparation

Fresh and whole plant material (2.5g for each sample type) were washed, air dried and grounded using liquid nitrogen. Additionally, 2.5g of commercial extract tablet was also grounded. Subsequently, all three samples were subjected to solvent extraction (80% methanol) via maceration for 24 hours at room temperature. The prepared extract was then filtered, centrifuged (10,000 rpm; 5 mins) and concentrated using Rota Evaporator (45° C; 70 rpm). The concentration of the working extract was standardized to 1mg/ml and 4mg/ml depending upon the assay.

2.4 Phytochemical analysis

2.4.1 Qualitative phytochemical screening

Standard phytochemical tests were performed to detect the presence of secondary metabolites in the prepared samples [10]. Mayer's test, alkaline reagent test, ferric chloride test, froth test and Salkowski's test were performed for the detection of alkaloids, flavonoids, phenolics, saponins and terpenoids respectively.

2.4.2 Quantitative phytochemical estimation

Standard phytochemical assays were performed for quantitative estimation of secondary metabolites in the three samples.

● **Phenolics estimation**

TPC (Total Phenolic Content) in the plant sample was determined following Folin-Ciocalteau assay. Gallic acid standards of varying concentrations (0.02 mg/ml, 0.04 mg/ml, 0.06 mg/ml, 0.08 mg/ml and 0.1 mg/ml) were prepared. Subsequently, standard solutions (GA) and methanolic stock solution (1 mg/ml working conc.) of the three test samples were mixed with FC reagent and 7.5% Sodium Carbonate. The resulting reaction mixture was incubated for 30 minutes in dark condition and the absorbance (OD) was recorded at 750 nm via UV-Vis Spectrophotometer. Finally, the corresponding values were expressed as mg GAE (Gallic Acid Equivalent)/g of extract.

● **Alkaloids estimation**

TAC (Total Alkaloid Content) of the samples was estimated using Bromocresol Green-Chloroform (BCG) assay. Atropine standards of varying concentrations (0.02 mg/ml, 0.04 mg/ml, 0.06 mg/ml, 0.08 mg/ml and 0.1 mg/ml) were prepared followed by the addition of phosphate buffer (0.05 M, pH 4.7), BCG solution (0.04% w/v) and chloroform to the test samples (4 mg/ml working conc.) and standard solutions. The lower organic layer of the mixtures was then collected and the absorbance was recorded at 470 nm. The final results were expressed as mg AE (Atropine Equivalent)/g of extract.

● **Flavonoids estimation**

TFC (Total Flavonoid Content) was determined using Potassium acetate-Aluminium Chloride assay. $AlCl_3$ (10%) and Potassium acetate (1M) solutions were added to the quercetin standard solutions (0.02 mg/ml, 0.04 mg/ml, 0.06 mg/ml, 0.08 mg/ml and 0.1 mg/ml) and test samples (4 mg/ml working conc.) followed by 40 minutes of incubation in dark condition. The absorbance (OD) of the reaction mixtures was recorded at 415 nm using UV-Vis spectrophotometer and final values were expressed as mg QE (Quercetin Equivalent)/g of extract.

2.4.3 Calibration Curve Construction

Calibration curves were constructed by measuring the absorbance values of standard solutions at specific wavelengths: Gallic acid at 750 nm for phenolics, Atropine at 470 nm for alkaloids, and Quercetin at 415 nm for flavonoids. Absorbance versus concentration plots were generated and linear regression equations ($y = mx + c$) were calculated to determine correlation coefficients (R^2). The obtained calibration curves were used to calculate phytochemical concentrations in plant extracts and commercial sample.

2.4.4 Methodology flowchart

The overall methodology is represented as a flowchart in Figure 2.

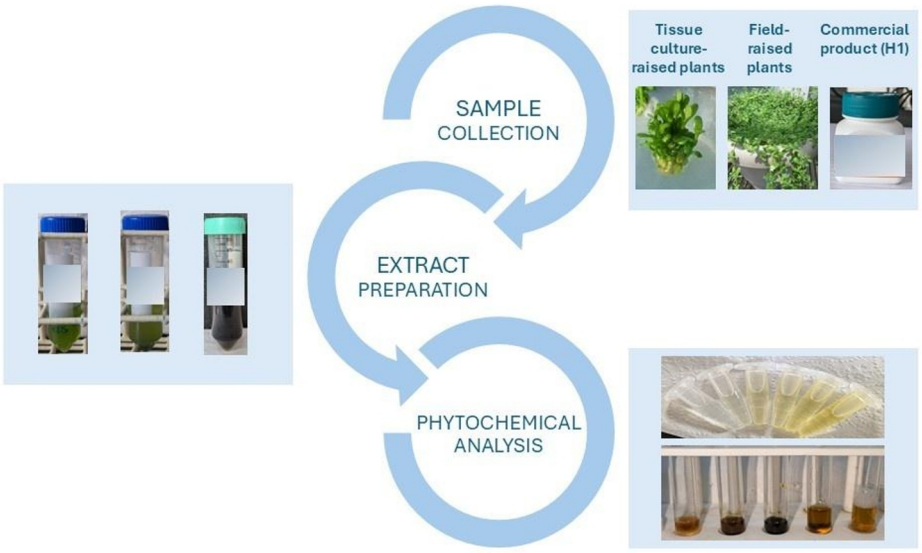

Figure 2. Methodology flowchart

3. Results

3.1 Qualitative Phytochemical Screening

The defined tests detected the presence of all the targeted metabolites in the three samples (Table 1). However, the commercial extract (H1) reflected strongest response visibly.

Table 1: Comparative qualitative phytochemical profiles of three *Bacopa monnieri* samples

TEST	METABOLITE	TISSUE CULTURE-RAISED	FIELD-RAISED	COMMERCIAL PRODUCT (H1)
Mayer's	Alkaloids	+	+	+
Ferric chloride	Phenolics	+	+	+
Alkaline reagent	Flavonoids	+	+	+
Froth	Saponins	+	+	+
Salkowski's	Terpenoids	+	+	+

3.2 Calibration Curves

The representative calibration curves showed strong linear relationships between concentration and absorbance. Standard curves for gallic acid (Figure 3), atropine (Figure 4), and quercetin (Figure 5) exhibited R^2 values of 0.99, 0.98 and 0.99 respectively, indicating high analytical reliability.

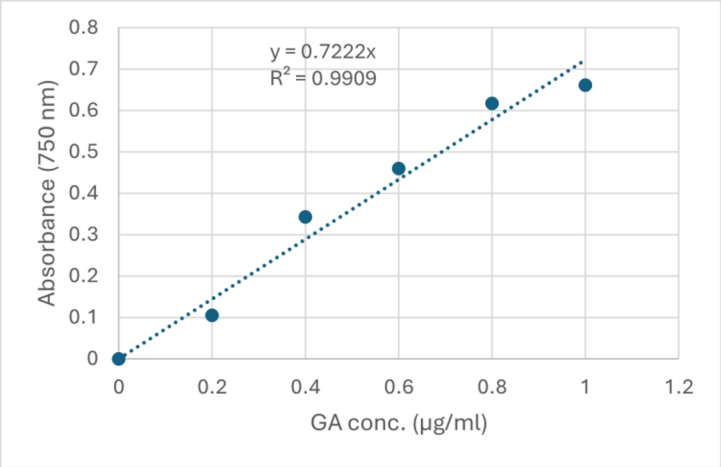

Figure 3. Calibration curve for gallic acid (total phenolics)

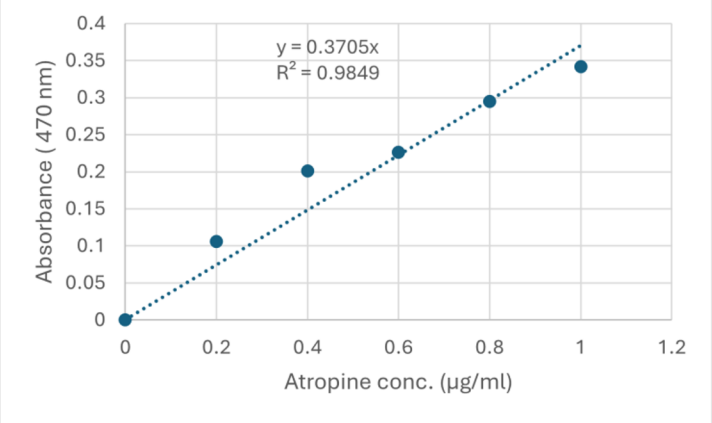

Figure 4. Calibration curve for atropine (total alkaloids)

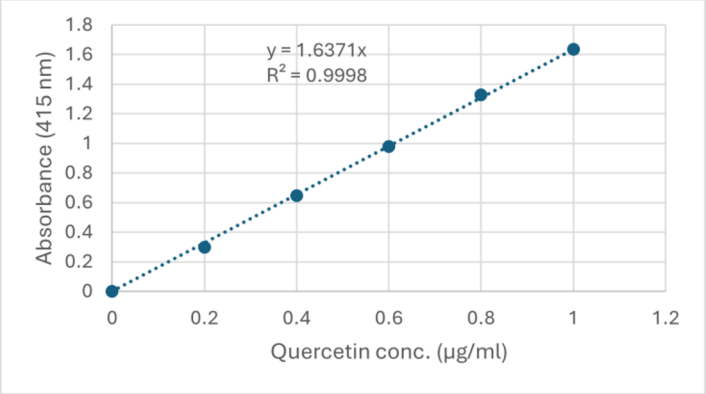

Figure 5. Calibration curve for quercetin (total flavonoids)

3.3 Quantitative Phytochemical Estimation

Following the validation of analytical method through calibration curve analysis of standard compounds, assay specific linear regression equations (y = 0.7222x (Gallic acid); y = 0.3705x (Atropine); y = 1.6371x (Quercetin)) was utilized to convert absorbance values of the test samples into metabolite concentration (x). Further, calculations were performed to express total metabolite content (TPC, TAC and TFC) relative to the three test samples. This was achieved by incorporating the calculated metabolite concentrations (C), extract volume (V), dilution factor (DF), and sample mass (M), allowing the results to be expressed as standard equivalent per gram of extract (Equation 1).

$$TMC = (C \times V \times DF) / M \quad ...(1)$$

Accordingly, tissue culture-derived plant extract showed highest values for total phenolic and alkaloid contents (954 mg GAE/g extract and 47.75 mg AE/g extract respectively), whereas, the commercial extract demonstrated the highest flavonoid content (35.5 mg QE/g extract). Moreover, the amount of all the targeted secondary metabolites was higher in tissue-culture raised plant extract as compared to field-raised plant extract. Detailed quantitative values are presented in Table 2.

Table 2: Comparative phytochemical content in three *Bacopa monnieri* samples

	SAMPLE SOURCE	TPC (mg GAE/g extract)	TAC (mg AE/g extract)	TFC (mg QE/g extract)
1	Tissue culture-raised plants	954	47.75	29
2	Field-raised plants	398	32.25	9
3	Commercial product (H1)	918	29.5	35.5

4. Discussion

The greater amount of phytochemical presence in in-vitro grown plants in comparison to field-raised plants can be attributed to the differences in controlled environmental conditions of laboratory plant culture room and glass house such as nutrient availability, temperature, humidity and light.

Additionally, the inclusion of a commercial product enabled benchmarking of in-vitro grown plant material against market formulations. The findings highlighted the potential role of tissue culture propagation in producing standardized extract in commercial product. Comparative analysis also provided valuable insights into authenticity and consistency of herbal products. However, as detailed information relating to the commercial product such as type of plant material used (in-vitro raised or field raised), names of additional fillers and binders, extraction solvent and techniques was not mentioned in the label, therefore, the presence of targeted metabolites cannot be confirmed as the chemical properties of additives can also influence and alter the phytochemical profile of the commercial extract.

Subsequently, the probability of false positive and negative results is highly plausible. This possibility can be specifically highlighted by comparing the qualitative and quantitative phytochemical profiles of the commercial sample. While qualitative screening referred commercial sample as the strongest extract in terms of metabolite concentration, however, quantitative estimation revealed that the concentration of detected secondary metabolites like phenolics and alkaloids were higher in tissue culture-raised plant extract.

5. Conclusion

Equivalent biomass-based evaluation of phytochemical content revealed the presence of metabolites in greater amount in tissue culture-raised plants when compared to field-raised plants. The study demonstrates that extracts obtained from in-vitro raised plants exhibited approximately 2.40-fold higher total phenolic content, and 1.48-fold higher alkaloid content compared to the field-raised samples, along with a slight increase (~1.04-fold and ~1.62 fold, respectively) over commercial product. Although the commercial sample showed the highest flavonoid content, the tissue culture-raised extract still demonstrated a ~3.22-fold higher flavonoid concentration compared to field-raised plant extract. This finding reflects tissue culture as a sustainable and efficient strategy for enhancing metabolite yield and supporting pharmaceutical applications of *Bacopa monnieri*. Although, comparative analysis also provided valuable insights into authenticity and consistency of the selected commercial product (H1), however, preliminary qualitative and quantitative analyses alone do not provide strong evidence regarding the authenticity of the product. Therefore, the application of advanced analytical technique like High Performance Liquid Chromatography (HPLC) is strongly recommended for quantifying the characteristic chemical marker of the targeted plant species.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Author contribution statement

Hemant Sood: conceptualization and methodology design; Yajuna Sharma: experimental work, data analysis, and manuscript preparation; Nancy Singla: reviewing and editing the manuscript.

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