

PHYTOCHEMICAL FINGERPRINTING AND EVALUATION OF *In Vitro* CYTOTOXIC ACTIVITY OF *Piper betle* LEAVES

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Abstract. The phytochemical profile and multi-biological activities of three *Piper betle* varieties (Black, White and Hybrid) were carried out using comprehensive biochemical and cytotoxic assays. Methanolic leaf extracts were analysed through qualitative phytochemical screening, quantitative estimation of total phenolics and flavonoids, antioxidant assays, lipid peroxidation inhibition, GC-MS profiling and MTT cytotoxicity assessment against MCF-7 breast cancer cells. All varieties contained major secondary metabolites, including flavonoids, phenols, alkaloids and terpenoids. Among them, the Hybrid variety recorded the highest phenolic content and demonstrated superior antioxidant capacity, while the Black variety showed elevated flavonoid levels and strong anti-peroxidative activity. GC-MS analysis confirmed the presence of several pharmacologically active constituents, such as benzoic acid derivatives and triazine compounds. Cytotoxic evaluation revealed a dose-dependent reduction in MCF-7 cell viability, with an IC₅₀ of 41.22 µg/mL. Collectively, the findings identified that the hybrid *Piper betle* variety is the most potent and underscoring the therapeutic potential of *P. betle* as a natural source of antioxidant and anticancer agents suitable for future drug development.

Keywords: Black *Piper betle*, white *Piper betle*, hybrid *Piper betle*, Phytochemical profiling, antioxidant activity, lipid peroxidation, MCF-7 cells.

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1. Introduction

Medicinal plants serve as important sources of therapeutic agents due to their rich diversity of bioactive phytochemicals and broad pharmacological activities.

Approximately 80% of the global population relies on traditional plant-based remedies, reflecting their long-standing efficacy and safety (Adams, 2017). The rising incidence of chronic diseases, including cancer and metabolic and neurodegenerative disorders, has intensified research interest in plant-derived compounds capable of mitigating oxidative stress and disease progression (Aggarwal *et al.*, 2009). Phytochemicals such as phenolics, flavonoids, alkaloids and terpenoids are recognized for their potent antioxidant, anti-inflammatory and cytoprotective properties, making them valuable candidates for safer therapeutic applications (Assidqi *et al.*, 2022). Oxidative stress, caused by excessive reactive oxygen species (ROS), leads to lipid peroxidation, DNA damage and carcinogenesis (Ayala *et al.*, 2014). Lipid peroxidation generates toxic aldehydes that contribute to inflammation and tumor growth (Azwanida *et al.*, 2015). Concerns over the toxicity of synthetic antioxidants have encouraged the exploration of natural antioxidants from medicinal plants (Bajpai *et al.*, 2020) and several studies demonstrate a positive correlation between antioxidant activity and anticancer potential in plant extracts. Breast cancer remains a leading cause of mortality among women worldwide, with more than 1.3 million new cases reported in 2008 (Bali, 2025). Standard treatments like chemotherapy, radiotherapy and hormonal therapy often produce severe side effects and face growing challenges of drug resistance (Biswas *et al.*, 2022; Boontha *et al.*, 2019; Brand-Williams *et al.*, 1995). These limitations highlight the urgent need for effective and less toxic plant-derived anticancer agents (Chang *et al.*, 2002).

Piper betle L. (*Piperaceae*), widely used in Asian traditional medicine, contains numerous bioactive compounds such as eugenol, hydroxychavicol and chavibetol which contribute to its antimicrobial, antioxidant and anti-inflammatory actions (Cragg *et al.*, 2005; Gottesman *et al.*, 2002). The plant exhibits multiple biological activities, including immunomodulatory, antidiabetic, anti-allergic and antiproliferative effects (Halliwell, 2012). Extracts of *Piper betle* have shown cytotoxicity against various cancer cell lines, including Raji, KB, HeLa and MCF-7 cells, with hydroxychavicol demonstrating strong pro-apoptotic activity (Halliwell *et al.*, 2015; Aktepe *et al.*, 2025; Kumar *et al.*, 2013).

However, limited studies have compared the phytochemical diversity and biological potency of different *Piper betle* varieties such as Black, White and Hybrid leaves. Extraction methods and solvent polarity are also known to influence the chemical composition and bioactivity of plant extracts (Longley *et al.*, 2005), necessitating a systematic comparative approach. Gas Chromatography-Mass Spectrometry (GC-MS) provides detailed phytochemical profiling by identifying key bioactive constituents (Madhumita *et al.*, 2020). Alongside this, the DPPH scavenging test, reducing-power assay, lipid peroxidation inhibition assay and MTT cytotoxicity assay collectively offer comprehensive insight into antioxidant and anticancer potential. The MTT assay remains widely used due to its sensitivity and suitability for high-throughput analysis (Mosmann, 1983).

The present study performs a comparative evaluation of three *P. betle* varieties black, white and hybrid through phytochemical fingerprinting and multiple biological assays. By examining phytochemical composition, phenolic and flavonoid content, antioxidant activity, anti-lipid peroxidative effects, GC-MS metabolite profiling and cytotoxicity against MCF-7 cells, this research identifies the most biologically potent variety and supports the therapeutic relevance of *Piper betle* as a promising natural antioxidant and anticancer source.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used were of analytical grade. Methanol, Folin-Ciocalteu reagent, gallic acid, aluminum chloride, quercetin, DPPH, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ferrous sulfate and other analytical reagents were purchased from standard suppliers. Cell culture media, fetal bovine serum (FBS), antibiotics, trypsin and MTT reagent were obtained from HiMedia Laboratories (Mumbai, India).

2.2. Collection and authentication of plant material

Fresh leaves of *Piper betle* (Black, White and Hybrid varieties) were collected from Ambur, Vellore District, Tamil Nadu, India. Leaves were washed, shade-dried at room temperature, powdered and stored in airtight containers. Authentication was carried out by a qualified botanist using morphological and taxonomic criteria.

2.3. Preparation of methanolic extracts

About 25 g of dried leaf powder from each variety was extracted with methanol using maceration for 48-72 hours with frequent shaking. The extract was filtered and concentrated using a rotary evaporator at 40-45°C, then stored at 4°C for further analysis. The percentage yield of black, white and hybrid *Piper betle* leaf was 7.23, 6.82 and 7.46, respectively (Nalina *et al.*, 2007).

2.4. Qualitative phytochemical screening

Phytochemical tests were performed using standard qualitative procedures to determine the presence of alkaloids, tannins, saponins, flavonoids, phenols, glycosides, terpenoids and other secondary metabolites based on characteristic colour reactions and precipitate formation (Ohkawa *et al.*, 1979).

2.5. Determination of Total Phenolic Content (TPC)

Total phenolic content was quantified using the Folin-Ciocalteu colorimetric method. Briefly, 0.5 mL of extract was mixed with Folin-Ciocalteu reagent followed by sodium carbonate solution. After incubation at room temperature, absorbance was recorded at 765 nm using a UV-visible spectrophotometer. Gallic acid was used as the calibration standard and results were expressed as mg gallic acid equivalents (GAE) per gram of extract (Pramod *et al.*, 2021).

2.6. Determination of Total Flavonoid Content (TFC)

TFC was determined using the aluminium chloride colorimetric assay. Extracts were treated with aluminium chloride and potassium acetate and absorbance was measured at 415 nm. Quercetin served as the standard and results were expressed as mg quercetin equivalents (QE)/g extract (Pramod *et al.*, 2021).

2.7. DPPH radical scavenging assay

Antioxidant potential was assessed by DPPH scavenging activity. Extracts (20-100 µg/mL) were mixed with DPPH solution and incubated for 30 minutes in the dark.

Absorbance was measured at 517 nm. Ascorbic acid served as the positive control. Percentage inhibition was calculated as described by Brand-Williams et al. (1995).

2.8. Reducing power assay

The reducing ability of the extracts was assessed by mixing them with phosphate buffer and potassium ferricyanide, followed by incubation at 50°C. After adding TCA, the mixture was centrifuged and the supernatant was treated with ferric chloride. Absorbance was recorded at 700 nm; higher absorbance indicated stronger reducing ability.

2.9. Lipid peroxidation inhibition assay

Anti-peroxidative activity was examined using goat liver homogenate. Extract concentrations (50-250 µg/mL) were incubated with the homogenate and ferrous sulfate to induce lipid peroxidation. TBA reagent was added to form a pink-colored MDA-TBA complex and absorbance was measured at 532 nm. Tocopherol served as the standard. Percentage inhibition was calculated relative to control (Rice -Evans *et al.*, 1997).

2.10. GC-MS analysis

GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer system and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-5MS (5% Diphenyl/95% Dimethyl poly siloxane), 30x0.25mmX0.25mm df and mass detector. The carrier gas at a constant flow of 1 ml/min and an injection volume of 2 µl was employed (split ratio of 10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 minutes isothermal at 280°C. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time is 36 min (Singh *et al.*, 2019).

2.11. Cytotoxicity evaluation (MTT Assay)

Cytotoxic activity against MCF-7 human breast cancer cells was evaluated using the MTT assay. The monolayer cell culture was trypsinized and the cell count was adjusted to 5.0×10^4 cells/ml using respective media containing 10% FBS. To each well of the 96-well microtiter plate, 100µl of the diluted cell suspension (5,000cells/well) was added. Cells were seeded in a 96-well plate and treated with extract concentrations ranging from 3.12 to 100 µg/mL for 24 hours. MTT solution was added, incubated and formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm. IC₅₀ values were calculated according to Mosmann's method (1983).

2.12. Statistical analysis

Data were expressed as mean ± standard deviation (SD) of triplicate measurements. Data were analyzed by one-way analysis of variance (ANOVA) and follow-up comparison between the groups was made using Tukey's multiple-comparison test at $p < 0.05$.

3. Results

3.1. Qualitative phytochemical screening

The qualitative phytochemical screening of methanolic extracts of *Piper betle* leaves (Black, White and Hybrid varieties) revealed the presence of major secondary metabolites, including alkaloids, flavonoids, carbohydrates, glycosides, terpenoids, proteins and phenols in all three varieties, while saponins, tannins and anthraquinones were absent (Table 1). The uniform occurrence of key bioactive compounds such as flavonoids and phenols indicates that all three varieties possess similar qualitative phytochemical profiles, suggesting comparable antioxidant and therapeutic potential at the qualitative level.

Table 1. Phytochemical constituents in methanolic extracts of *Piper betle* leaves

No	Phytochemical	Piper betle leaf (Black)	Piper betle leaf (White)	Piper betle leaf (Hybrid)
1	Alkaloids	+	+	+
2	Flavonoids	+	+	+
3	Carbohydrates	+	+	+
4	Glycoside	+	+	+
5	Saponins	-	-	-
6	Tannins	-	-	-
7	Terpenoids	+	+	+
8	Protein	+	+	+
9	Anthraquinone	-	-	-
10	Phenol	+	+	+

Note: Presence (+), Absence (-) of phytochemicals

3.2. Total phenolic and flavonoid content

The total phenolic and flavonoid contents of the methanolic extracts varied among the three *Piper betle* varieties. As shown in Figure 1, the Hybrid variety exhibited the highest phenolic content (5.98 ± 0.99 mg GAE/g), followed by the Black (5.07 ± 0.21 mg GAE/g) and White varieties (5.03 ± 0.21 mg GAE/g). In contrast, flavonoid levels (Figure 2) were highest in the Black variety (7.22 ± 0.34 mg QE/g), while the White (4.14 ± 0.23 mg QE/g) and Hybrid (4.32 ± 0.22 mg QE/g) samples showed comparatively lower values. These results indicate that the Hybrid variety is richer in phenolics, whereas the Black variety contains the highest flavonoid concentration.

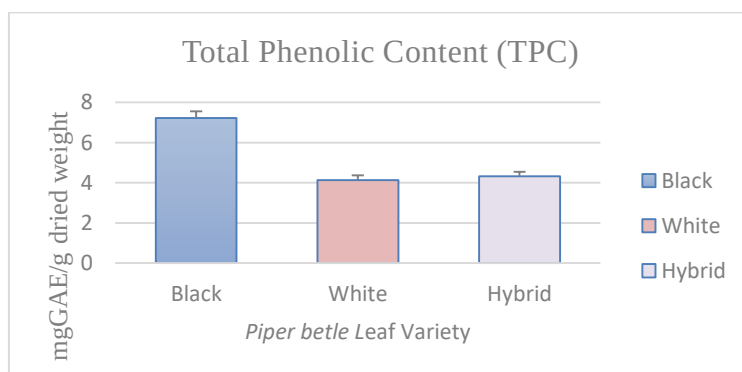


Figure 1. Total phenolic content of methanolic extracts of *Piper betle* leaves

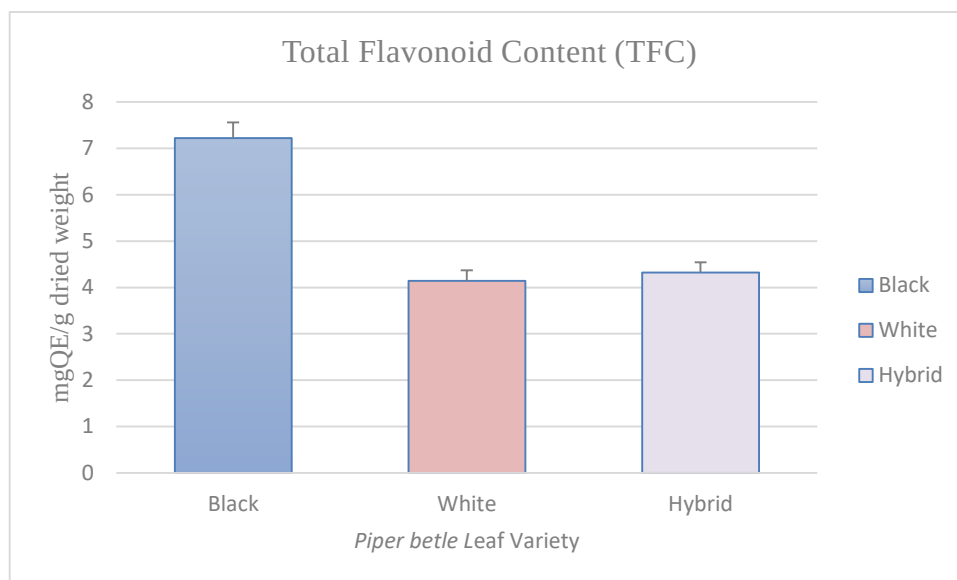


Figure 2. Total flavonoid content of methanolic extracts of *Piper betle* leaves

3.3. DPPH radical scavenging activity

The antioxidant potential of the methanolic extracts of *Piper betle* leaves was evaluated using the DPPH radical scavenging assay. As shown in Figure 3, all extracts exhibited a dose-dependent increase in free radical scavenging activity across the tested concentrations (20-100 $\mu\text{g/mL}$). Among the three varieties, the Hybrid extract demonstrated the highest activity, reaching $69.91 \pm 1.44\%$ at 100 $\mu\text{g/mL}$, followed by the White variety ($64.59 \pm 0.95\%$) and the Black variety ($47.91 \pm 1.80\%$). Although all extracts showed lower activity compared to the standard vitamin C ($89.10 \pm 1.12\%$ at 100 $\mu\text{g/mL}$), the Hybrid and White varieties displayed notably stronger radical scavenging potential than the Black variety. These findings suggest that phenolic-rich varieties, particularly the Hybrid leaf, possess superior antioxidant activity.

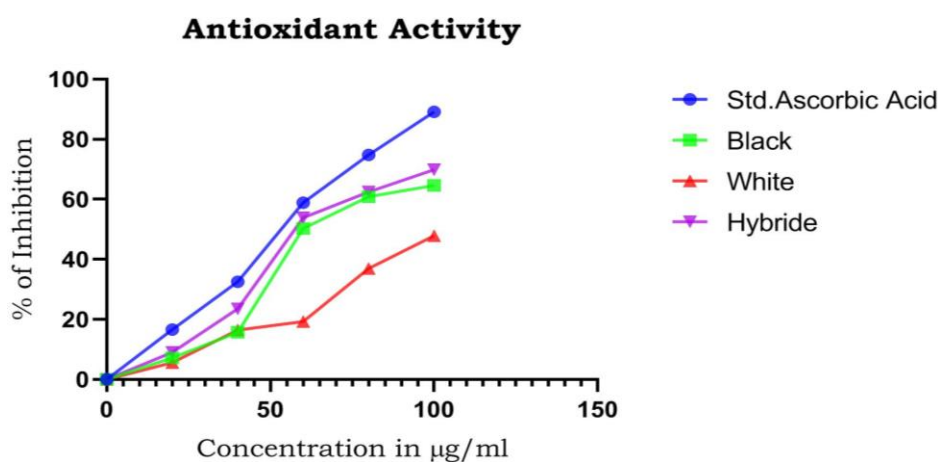


Figure 3. DPPH radical scavenging activity of methanolic extracts of *piper betle* leaves

3.4. Reducing power assay

The reducing power of the methanolic extracts of *Piper betle* leaves increased progressively with concentration, as shown in Figure 4. Among the three varieties, the

Hybrid extract displayed the highest reducing ability, reaching 0.31 ± 0.01 absorbance at $100 \mu\text{g/mL}$, followed by the Black variety (0.28 ± 0.02) and the White variety (0.23 ± 0.02). Although all extracts showed lower reducing power compared to the ascorbic acid standard, the Hybrid variety consistently demonstrated superior electron-donating capacity across the tested concentrations.

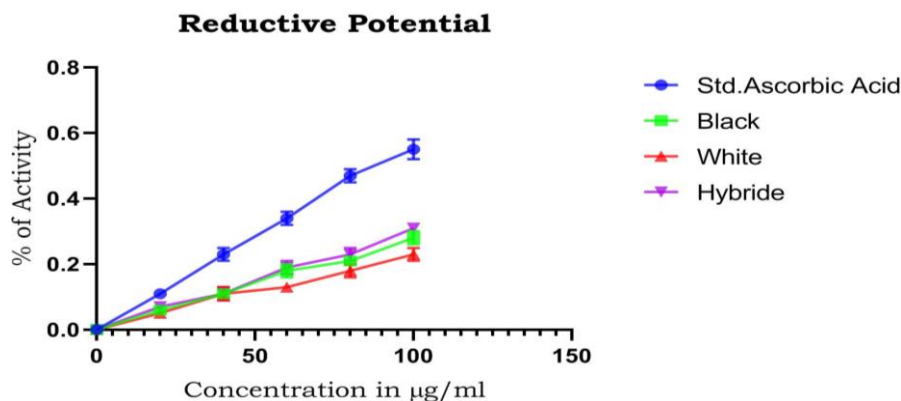


Figure 4. Reducing power assay of methanolic extracts of *Piper betle* leaves

3.5. Lipid peroxidation inhibition assay

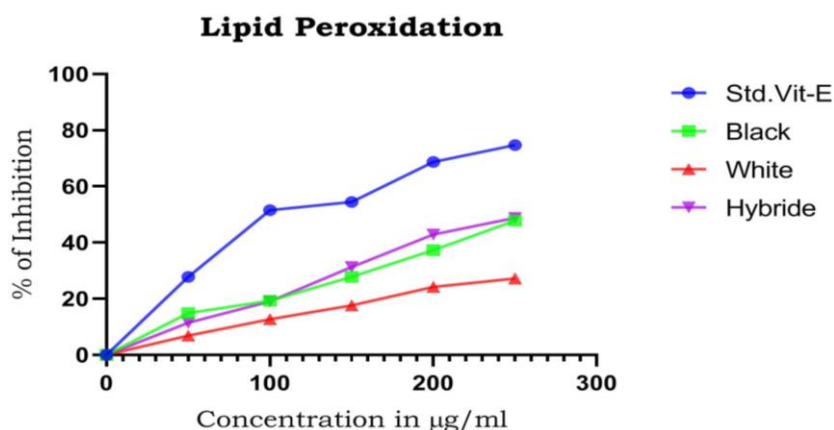


Figure 5. Lipid peroxidation inhibition activity of methanolic extracts of *Piper betle* leaves

As shown in Figure 5, all methanolic extracts of *Piper betle* leaves exhibited a concentration-dependent inhibition of lipid peroxidation. The Hybrid variety showed the strongest inhibitory activity across most concentrations, reaching $48.81 \pm 1.05\%$ at $250 \mu\text{g/mL}$, closely followed by the Black variety ($47.82 \pm 1.21\%$). The White extract displayed comparatively lower inhibition ($27.22 \pm 1.04\%$ at $250 \mu\text{g/mL}$). Although all extracts demonstrated lower activity than the standard tocopherol, the Hybrid and Black varieties consistently exhibited superior anti-peroxidative potential, indicating stronger membrane-protective antioxidant activity.

3.6. GC-MS analysis of *Piper betel* leaf (hybrid)

The GC-MS analysis of the methanolic extract of *Piper betle* identified several bioactive compounds eluting between 5 and 14 minutes (Figure 6). The major constituents were Benzoic acid, 3-methyl-2-trimethyl and 2-Chloro-4,6-di(1-

piperidinyl)-1,3,5-triazine (each 9.56% peak area), both known for antimicrobial and anticancer activities. Other notable compounds included 1,3,4-Thiadiazol-2-amine, 5-methyl (4.51%) and Anthranilic acid, N-methyl (3.28%), associated with anti-inflammatory and therapeutic effects. Minor components such as Acetamide derivatives, Acetyl cyanide and N-Ethylformamide also contributed to the extract's pharmacological potential. Overall, the chromatographic profile indicates the presence of multiple bioactive molecules supporting the medicinal value of *Piper betle* leaves (Soltanbeigi & Özlman, 2026).

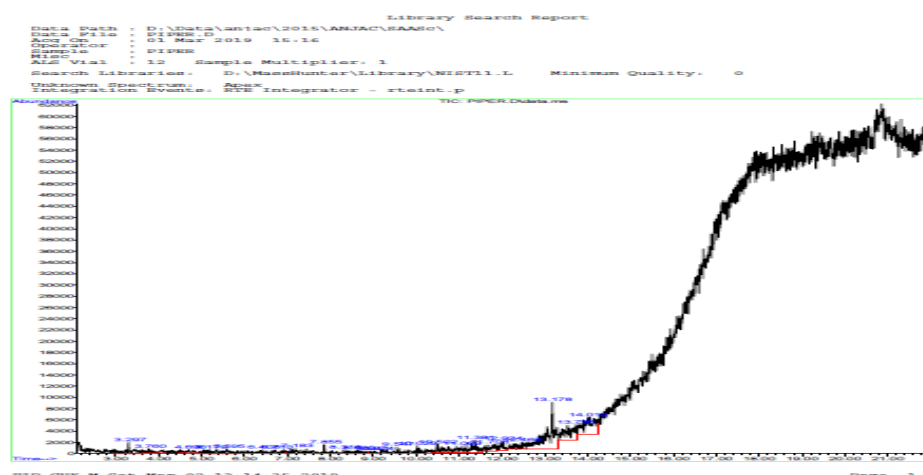


Figure 6. GC-MS chromatogram of methanolic extract of *Piper betle*

3.7. Cytotoxic effect of Piper betle leaf (hybrid)

The methanolic extract of *Piper betle* leaves exhibited dose-dependent cytotoxicity against MCF-7 cells (Figure 7). Cell viability decreased from 80.45% at 3.12 µg/ml to 36.88% at 100 µg/ml, with an IC₅₀ value of 41.22 µg/ml, indicating moderate cytotoxic activity. These results suggest the extract's potential as a source of anticancer agents.

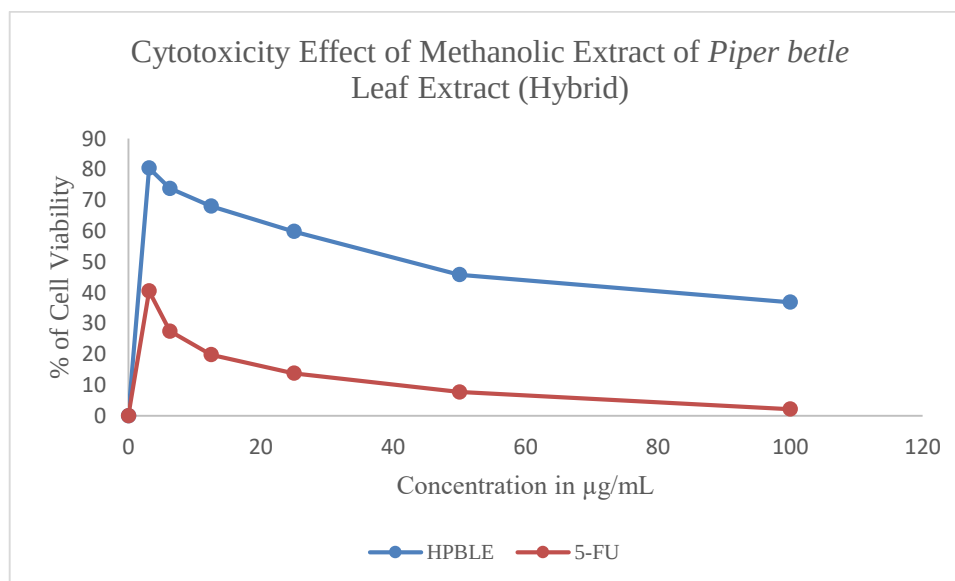


Figure 7. Cytotoxic effect on MCF-7 cells

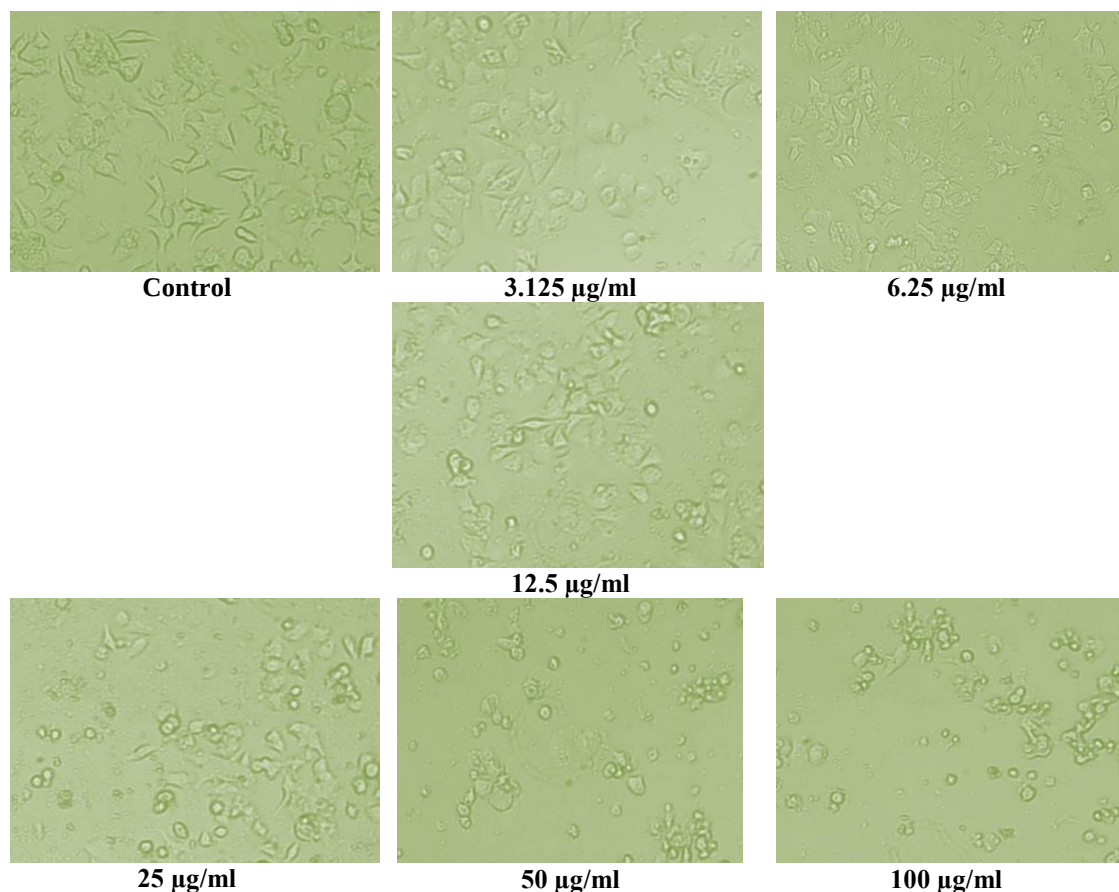


Figure 8. Cytotoxic effect of *Piper betle* leaf methanolic extract on MCF-7 cells

4. Discussion

The present study provides a comparative evaluation of three *Piper betle* varieties (Black, White and Hybrid) in terms of phytochemical composition and multi-biological potency, including antioxidant, anti-peroxidative and cytotoxic activities. Qualitative phytochemical screening revealed the presence of key secondary metabolites, such as flavonoids, phenols, alkaloids and terpenoids, in all three varieties, consistent with previous reports highlighting the rich phytochemical profile of *Piper betle* leaves (Halliwell *et al.*, 2015; Singleton *et al.*, 1965). The uniform presence of these compounds suggests that all varieties possess inherent antioxidant and therapeutic potential.

Quantitative analysis showed that the Hybrid variety contained the highest phenolic content, whereas the Black variety had the highest flavonoid concentration (Nohutçu & Tunçtürk, 2026). These findings correlate with the antioxidant assays, where the Hybrid extract exhibited superior DPPH radical scavenging and reducing power, reflecting the contribution of phenolic compounds to electron-donating and free radical-neutralizing activity (Sofowora, 2008; Thi *et al.*, 2023; Özer, 2026). The lipid peroxidation inhibition assay further confirmed that the Hybrid and Black varieties effectively suppressed oxidative damage in goat liver homogenates, suggesting their capacity to protect cellular membranes from ROS-mediated lipid peroxidation (Ayala *et al.*, 2014; Torre *et al.*, 2016).

GC-MS profiling identified several bioactive constituents with known pharmacological effects, including Benzoic acid derivatives, 2-Chloro-4,6-di(1-

piperidinyl)-1,3,5-triazine and 1,3,4-Thiadiazol-2-amine, which have been previously associated with antimicrobial, anti-inflammatory and anticancer activities (Valko *et al.*, 2007). The presence of multiple active compounds likely underpins the observed antioxidant and cytotoxic effects. Cytotoxic evaluation against MCF-7 human breast cancer cells demonstrated a dose-dependent inhibition of cell viability, with an IC₅₀ value of 41.22 µg/ml for the methanolic extract. This moderate cytotoxic activity aligns with reports that *Piper betle* extracts exert antiproliferative effects through induction of apoptosis and modulation of oxidative stress in cancer cells (Veettil *et al.*, 2022; Karafakioğlu *et al.*, 2026). The higher flavonoid content in the Black variety may contribute to its cytotoxic efficacy, supporting the notion that flavonoids play a significant role in anticancer mechanisms by inducing cell cycle arrest and apoptosis.

Overall, these results suggest that *Piper betle* leaves, particularly the Hybrid and Black varieties, exhibit significant antioxidant, anti-peroxidative and cytotoxic activities. The combination of phenolics, flavonoids and other bioactive compounds may act synergistically to mitigate oxidative stress and inhibit cancer cell proliferation. This study underscores the therapeutic potential of *Piper betle* as a natural source of bioactive compounds for the development of antioxidant and anticancer agents. Future investigations should focus on mechanistic studies, *in vivo* evaluations and isolation of specific bioactive molecules to further elucidate their pharmacological relevance.

5. Conclusion

This study provides a systematic comparative assessment of the phytochemical composition and multi-biological potency of Black, White and Hybrid *Piper betle* varieties. All three extracts contained major secondary metabolites; however, the Hybrid variety exhibited the highest phenolic content and demonstrated superior antioxidant and reducing capacities. The Black variety, rich in flavonoids, showed strong anti-peroxidative potential, indicating the functional contribution of flavonoid compounds to oxidative protection. GC-MS profiling confirmed the presence of several pharmacologically relevant biomolecules, further validating the therapeutic significance of *P. betel*. The methanolic extracts also displayed moderate cytotoxicity against MCF-7 breast cancer cells, with dose-dependent reductions in cell viability. Collectively, the findings highlight the Hybrid *Piper betle* variety as the most biologically potent, supporting its potential application as a natural antioxidant and anticancer agent. Future studies should isolate the active constituents and evaluate molecular mechanisms to enhance drug development prospects.

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