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QUALITATIVE AND QUANTITATIVE PROFILING OF PHYTOCHEMICALS IN PHALSA LEAVES INFLUENCED BY CHEMICAL MUTAGENS

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Phalsa (*Grewia asiatica* L.) is an underutilized fruit belonging to the Tiliaceae family, characterized by a chromosome count of 2n = 18. It stands as one of the oldest fruits, renowned for its exceptional nutritional profile and various health benefits (Jamil *et al.*, 2022). The species name "Grewia" was bestowed by Nehemiah Grew, a pioneer in the field of plant physiology (Sinha *et al.*, 2015). Phalsa is recognized for its high nutrient content and is often utilized in dietary therapy (Hiwale, 2015). It boasts a plethora of medicinal properties, including anti-diabetic, anti-hyperglycemic, radio-protective, antibacterial, antipyretic, anti-fungal, analgesic, and antiviral attributes (Sinha *et al.*, 2015). Rich in terpenoids, anthocyanins, phenols, tannins, flavonoids, and various other compounds, Phalsa holds significant pharmacological potential (Wani *et al.*, 2015; Khan *et al.*, 2018). A study conducted by Kaur *et al.* (2018) unveiled the presence of alkaloids, flavonoids, glycosides, carbohydrates, saponins, and tannins in the leaves of *G. asiatica.* Phalsa seeds serve as essential propagating material, highlighting the need for improvements in germination to produce healthy germplasm.

Mutations are instrumental in enhancing plant growth traits, and chemical mutagens have been pivotal in horticultural crop improvement efforts (Adeoti *et al.*, 2021; Singh and Singh, 2001). Chemical agents like colchicine, functioning as anti-microtubule agents, induce polyploidy by blocking microtubule polymerization, thereby bringing about significant morphological, cytological, and histological changes in various plants (Meyers and Levin, 2006; Otto, 2007). Ethyl methane sulphonate (EMS) is highly effective in inducing variations through point mutations by altering nucleotides (McCallum *et al.*, 2000). Similarly, sodium azide remains a potent mutagen (Siddiqui *et al*., 2007). Despite the wealth of literature on mutagenic effects, there is a dearth of research on their impact on phytochemicals. Thus, this investigation aims to shed light on the chemical mutagenic impact on the qualitative and quantitative phytochemicals of phalsa.

The research experiment was conducted at the Horticultural experimental block of Khalsa College, Amritsar, Punjab, during the period of 2022-23. The experimental site is situated in the central plains of Punjab, with an average elevation of 228.86 meters above Mean Sea Level (MSL) and receives an annual rainfall of 735 mm. Seeds of 6-year-old phalsa plants (bush type) were subjected to pre-treatment with colchicine, sodium azide, and ethyl methane sulphonate at concentrations of 0.1% and 0.5% each, for a duration of twelve hours. Untreated seeds were dipped in distilled water. A total of seven treatments were applied, with each treatment consisting of 25 plants, distributed across three replications. The seeds were sown in a mixture of soil and sand (1:1 ratio) during the first week of July. After 120 days from seed sowing, five leaves were randomly selected from plants in each replication, totaling fifteen leaves per treatment, and collected for analysis. For data analysis, a Randomized Block Design with three replications was employed as the statistical design. This design allows for rigorous evaluation and comparison of the effects of different treatments on the phalsa plants while accounting for potential variability within the experimental blocks.

A) Leaf attributes

The number of leaves was determined by calculating the average total number of fully grown leaves per seedling of selected plants. Five mature leaves from each selected plant in different treatments were collected and weighed individually on an electronic weighing balance. Subsequently, the leaves were dried in paper bags for approximately 10 days. After drying, the dry weight of each leaf was measured using a weighing balance. Finally, the leaf area was calculated using a leaf area meter. These steps were undertaken to accurately assess the effects of the treatments on leaf morphology and growth parameters.

B) Qualitative phytochemical leaf analysis

For the qualitative phytochemical analysis of phalsa leaves, plant extracts were prepared by shade-drying the leaves for approximately 10 days. The dried leaves were then crushed and ground into a fine powder using an electric grinder. Subsequently, 50 g of the dry leaf powdered sample was soaked in 500 ml of distilled water overnight in a tightly sealed jar for maceration, specifically for alkaloids, flavonoids, and terpenoids

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analysis. After filtration, the extract was boiled for five minutes and then cooled (Khanal, 2021). To test for alkaloids, two drops of Mayer's reagent were added to 2 ml of the plant extract. The presence of white-colored precipitates confirmed the presence of alkaloids (Pundlik and Patil, 2019). For flavonoids analysis, 2 ml of NaOH (2%) was added to 2 ml of plant extract in a test tube. A few drops of HCl were then added to the precipitates, and the appearance of colorless precipitates indicated the presence of flavonoids (Khanal, 2021). For saponins analysis, 4 g of dry leaf powder was added to 50 ml of distilled water and boiled for half an hour before filtration. The solution was then cooled and vigorously shaken for 1-2 minutes. The occurrence of constant froth confirmed the presence of saponins (Pundlik and Patil, 2019). The determination of iridoids was performed by taking 1 g of leaf powder and adding 5 ml of 1% HCl (aqueous). After 3 to 6 hours, 0.1 ml of 0.20 % $CuSO_4.5H_2O$ in water and 0.5 ml of concentrated HCl were added, followed by heating for a short period. The presence of a blue color indicated the presence of iridoids (Pundlik and Patil, 2019). Lastly, terpenoid analysis was conducted by mixing 2 ml of chloroform with 0.5 ml of plant extract, followed by the addition of 2 ml of H_2SO_4 with gentle shaking. The presence of a reddish-brown color indicated the presence of steroids, terpenoids, and glycosides (Khanal, 2021). These methods allowed for the comprehensive qualitative analysis of phytochemicals present in phalsa leaves.

C) Quantitative phytochemical leaf analysis

Quantitative phytochemical leaf analysis was conducted following the methods outlined by Harborne (1973). To determine alkaloid content (%), 5 grams of dried leaves were powdered and immersed in 100 milliliters of 10% acetic acid for four hours, then filtered. The filtrate was evaporated until 1/4th of the original volume remained, and sodium hydroxide was added drop by drop to precipitate the alkaloids. After filtration, the precipitates were cleaned with NaOH and dried at 60 ℃ for half an hour. Alkaloid content was calculated using the formula: Alkaloid (%) = $(w_2 - w_1) / w_1 \times 100$, where w_1 represents the empty filter paper weight and w_2 represents the paper weight plus alkaloid extract. For flavonoid content (%), dry leaf powdered material (5 grams) was boiled in 100 milliliters of water with 2 milliliters of HCl solution for 30 minutes. After cooling, the mixture was filtered, and the aqueous layer was removed. The filter paper remnants were then dried at 60 ℃ for 30 minutes, and the weight of flavonoids was calculated using the formula: Flavonoid (%) = $(w_2 - w_1)$ / weight of sample × 100, where w_1 represents the empty filter paper weight and w_2 represents the weight of paper plus flavonoid extract. Saponin content (%) was examined by boiling macerated plant extract (25 milliliters) in a flask with 100 milliliters of 50% alcohol for half an hour. After filtration, 2 grams of charcoal were added to the cooled filtrate, followed by an equal volume of acetone to precipitate. The precipitated residue was collected and calculated as: Saponins (%) = $(w_2 - w_1) / w_1 \times 100$, where w_1 represents the filter paper weight and w_2 represents the residue weight. To determine terpenoid content (%), 10 grams of dried leaf powder (w_1) was soaked in 90 milliliters of ethanol, filtered, and then 10 milliliters of petroleum ether were added. After filtration, the extract was dried, and the terpenoid content was calculated using the formula: Terpenoid (%) = $(w_1 - w_2) / w1 \times 100$, where w_1 represents the dried plant extract and w_2 represents the extract after drying. These methods facilitated the quantitative analysis of alkaloids, flavonoids, saponins, and terpenoids in phalsa leaves, providing valuable insights into their phytochemical composition.

Using the method outlined by Singleton and Rossi (1965), the total phenolic content was measured as follows: one gram of fresh leaf tissue was homogenized with 5 ml of 80% methanol in a mortar and pestle, repeated three times. The pooled extract volume was adjusted to 50 ml with distilled water, from which 0.5 ml was taken and added to a test tube containing 0.2 ml of 1 N Folin-Ciocalteu's phenol reagent and 3.3 ml of distilled water. With constant stirring, 1 ml of $NaNO_3$ was added, followed by incubation for 30 minutes at room temperature. After the reaction, a blue-colored complex was formed and observed at 700 nm. The total phenolic content was then calculated as milligrams of gallic acid (GA) equivalents per 100 grams of leaf sample.

D) Statistical analysis

The data were statistically analyzed using IBM-SPSS Statistics (Version 29.0). To determine significant differences between means, Duncan's Multiple Range Test was employed.

Number of leaves

Significant variations ($p \leq 0.05$) in leaf production were observed in response to the concentration of the chemical mutagens applied (Table 1). An increase in mutagenic dose corresponded to a reduction in leaf formation. Untreated seeds yielded the maximum number of leaves (18.11), comparable to colchicine 0.1%, which exhibited an average of 17.70 leaves per plant. Among the treated seeds, colchicine resulted in higher leaf production compared to EMS and sodium azide. A reduction of 34.18% was noted in colchicine concentrations ranging from 0.1% to 0.5%. The lowest leaf production was observed in the sodium azide treatments, with an average of 8.50 leaves per plant. The variation in leaf production across doses can be attributed to abnormalities in meiotic divisions induced

by mutagens, which serve as a reliable indicator for mutagen assessment (Kumar and Rai, 2007). Higher dosages of mutagens can induce dwarfism and inhibit tissue growth. Similar results have been reported in other crops such as *Nigella sativa* (Amin *et al.*, 2019), fenugreek (Naaz *et al.*, 2022), and sorghum (Murali *et al.*, 2013). Studies on jute by Nura *et al.* (2011) have also shown increased leaf production with colchicine, consistent with the findings of this study. Additionally, Kaur and Kaur (2023) reported higher leaf production with colchicine and lower with sodium azide in jamun.

Leaf fresh & dry weight basis (g)

A significant reduction was observed (Table 1) at the maximum dose of colchicine, EMS, and sodium azide mutagens. The maximum fresh leaf weight (2.47 g) was noted in the colchicine 0.1% treatment, with a reduction of 45.74% at colchicine 0.5%. Similarly, in EMS treatments, the highest fresh leaf weight (2.26 g) was observed in EMS 0.1% treated seeds, followed by a reduction of 36.84% in sodium azide treated seeds with 0.1%, and a maximum reduction of 74.08% in sodium azide 0.5% treated seeds. Regarding leaf dry weight, the maximum (1.31 g) was recorded in plants raised after the application of colchicine 0.1%, followed by untreated seeds with 1.24 g, which were statistically comparable. However, a significant reduction in dry weight was observed with increased mutagen dosage. EMS treatment followed a similar trend to colchicine, with a reduction of 52.84% at EMS 0.5% compared to 0.1% EMS. Sodium azide exhibited the lowest dry weight (0.41 g) at a concentration of 0.5%.

The higher incidence of abnormalities in leaf weight observed at higher doses of mutagens may be attributed to the improper functioning of various phytohormones or disturbances in the chlorophyll apparatus (Swaminathan, 1965). Similar morphological variations with increased mutagen concentration have been reported by Mallick *et al.* (2016) in *Kinnow* mandarin and Al Qurainy (2009) in *Eruca sativa*. Reductions in fresh weight with increased mutagen dosage have also been documented by Kaur and Kaur (2023) in jamun.

Leaf area (cm^2)

Significant effects ($p \leq 0.05$) on leaf area were observed across various concentrations of chemical mutagens studied. Compared to non-mutated plants, a notable reduction was observed with increasing doses of EMS, colchicine, and sodium azide mutagens. The maximum reduction (75.11%) was noted in seedling leaves from seeds treated with sodium azide 0.5%. On the other hand, the maximum leaf area (49.32 cm^2) was achieved in the colchicine 0.1% treatment, followed by 48.19 cm^2 in untreated seeds. The morphological alterations induced by chemical mutagen treatments have been documented by several researchers (Sharma *et al.*, 2013; Wi *et al.*, 2005). The present study aligns with these findings, demonstrating a reduction in leaf area with higher mutagen doses, likely due to damage to the cellular framework and degradation of endogenous auxins (Moore, 2012). Similar reductions in leaf area observed in kinnow studies have been reported by Mallick *et al.* (2016).

Qualitative phytochemical analysis of phalsa leaves

The studied phytochemicals i.e. secondary metabolites found in plants are required in smaller quantities for various biological functions (Tiwari *et al.*, 2011). Preliminary screening of the chemical mutagenic effect involved qualitative analysis. Alkaloids, flavonoids, saponins, and terpenoids were detected in seedlings treated with various concentrations of mutagens, while iridoids were absent across all treatments. Notably, the

Table 1. Effect of chemical mutagens on leaf attributes of phalsa

Treatments	Number of leaves	Leaf fresh weight (g)	Leaf dry weight (g)	Leaf area (cm^2)
T_1 - EMS 0.1%	16.53 ± 0.29^{b}	2.26 ± 0.02^{c}	1.23 ± 0.10^{b}	45.22 ± 0.33^{c}
T_2 - EMS 0.5%	9.71 ± 0.19^{e}	0.91 ± 0.02^{f}	0.58 ± 0.05^{e}	18.51 ± 0.84^{f}
T_3– Colchicine 0.1%	17.70 ± 0.19^{a}	2.47 ± 0.03^{a}	1.31 ± 0.10^{a}	49.32 ± 0.49^{a}
T_4 - Colchicine 0.5%	11.65 ± 0.33^{d}	1.34 ± 0.03^{e}	0.83 ± 0.04^{d}	27.26 ± 0.46^{e}
T_5 - Sodium azide 0.1%	12.27 ± 0.10^{c}	1.56 ± 0.02^{d}	0.90 ± 0.08^{c}	34.21 ± 0.65^{d}
T_6 - Sodium azide 0.5%	8.50 ±0.22^{f}	0.64 ± 0.02^{g}	0.41 ± 0.03^{f}	12.25 ± 0.57^{g}
T_7 – Control	18.11 ±0.36^{a}	2.40 ± 0.06^{b}	1.24 ± 0.14^{ab}	48.19 ± 0.82^{b}
Mean ± S.D.	13.49 ± 3.72	1.65 ± 0.70	0.93 ± 0.34	33.56 ± 14.07
C.D. ($p \leq 0.05$)	0.48	0.02	0.05	0.34

Values in the same column not followed by the same letter are significantly different at $p \leq 0.05$

Table 2. Effect of chemical mutagens on qualitative phytochemical analysis of phalsa leaves

Mutagen	Doses	Color	Taste	Odour	Alkaloids	Flavonoids	Saponins	Irodoids	Terpenoids
EMS	0.1%	Green	Bitter	Disagreeable	++	+++	++	-	++
	0.5%	Green	Bitter	Disagreeable	++	++	+	-	+
Colchicine	0.1%	Green	Bitter	Disagreeable	++	++++	++	-	++
	0.5%	Green	Bitter	Disagreeable	+	+	+	-	+
Sodium Azide	0.1%	Green	Bitter	Disagreeable	+	++	+	-	+
	0.5%	Green	Bitter	Disagreeable	+	+	+	-	+
Control	0.0%	Green	Bitter	Disagreeable	+	++	++	-	++

(++++) – very high; (+++) – high; (++) – high; (+) – moderate; (-) – absent

color, taste, and odor remained unaffected by mutagen concentrations. Alkaloid concentrations were influenced by mutagen dosage, with moderate levels observed at lower doses of colchicine and EMS, but reduced at higher doses (0.5%). Sodium azide-treated seedlings consistently exhibited lower alkaloid levels. Flavonoid content was confirmed in all treatments, with higher availability observed at lower concentrations (0.1%) of EMS and colchicine, and moderate levels at 0.5%. Saponin presence was moderate across mutagen treatments, with lower concentrations observed at higher (0.5%) mutagen concentrations. Iridoids were consistently absent in all treatments. Terpenoids were present in moderate amounts across mutagen treatments, with lower concentrations observed at lower mutagen concentrations. This aligns with the findings of Pundlik (2021), who also confirmed the absence of iridoids in phalsa leaves.

Quantitative phytochemical analysis of phalsa leaves

The highest flavonoid content (60.48%) was observed in plant leaves treated with colchicine 0.1%, followed by 54.68% in EMS 0.1% treatment (Table 3). On the other hand, the lowest flavonoid content (44.44%) was measured in leaves treated with sodium azide 0.5%. This trend was consistent across alkaloids, saponins, and terpenoids (Table 3), with maximum levels (16.56%, 3.85%, and 19.48% respectively) recorded in colchicine 0.1% treatment. Conversely, the lowest content (11.56%, 1.11%, and 13.73%) of these bioactive compounds was extracted from leaves treated with sodium azide 0.5%. Additionally, the highest total phenolic content (39.60 mg/g FW) was observed in leaves treated with colchicine 0.1% (Table 3). However, the phenolic content decreased to 35.16 mg/g FW at the highest dose of colchicine (0.5%). In comparison, sodium azide-treated seedlings exhibited a 9.64% reduction in phenolic content compared to untreated seeds.

The secondary metabolites possess antimutagenic properties, which can reduce the mutagenic frequency through various mechanisms, including the scavenging of free radicals. Therefore, the increased phytochemical composition observed as a result of mutagenic application might be attributed to the plants' response to oxidative stress induced by abiotic factors (Mezzouge *et al.*, 2006). This finding is supported by the research

Table 3. Effect of chemicals on quantitative phytochemical analysis of phalsa leaves

Treatments	Flavonoids (%)	Alkaloids (%)	Saponins (%)	Terpenoids (%)	Phenols (mg GAE/100 g FW)
T_1– EMS 0.1%	54.68 ± 0.10^{b}	15.69 ± 0.18^{b}	2.89 ± 0.15^{b}	18.47 ±0.20^{b}	36.63 ± 0.15^{c}
T_2– EMS 0.5%	51.32 ± 0.08^{c}	12.51 ± 0.20^{f}	1.56 ± 0.15^{e}	14.46 ±0.22^{f}	38.48 ± 0.16^{b}
T_3– Colchicine 0.1%	60.48 ± 0.28^{a}	16.56 ± 0.33^{a}	3.85 ± 0.09^{a}	19.48 ± 0.20^{a}	39.60 ± 0.05^{a}
T_4– Colchicine 0.5%	43.30 ± 0.10^{f}	13.80 ± 0.19^{d}	2.12 ± 0.12^{c}	15.59 ± 0.31^{e}	35.16 ± 0.08^{d}
T_5– Sodium azide 0.1%	46.22 ± 0.19^{d}	14.32 ±0.25^{c}	1.81 ± 0.05^{d}	16.44 ± 0.26^{d}	33.15 ± 0.11^{e}
T_6– Sodium azide 0.5%	42.18 ± 0.19^{g}	11.56 ± 0.19^{g}	1.11 ± 0.13^{f}	13.73 ± 0.13^{g}	27.45 ± 0.25^{g}
T_7 – Control	44.44 ± 0.28^{e}	13.23 ± 0.11^{e}	2.20 ± 0.17^{c}	17.38 ± 0.10^{c}	30.38 ± 0.33^{f}
Mean ± S.D.	48.94 ± 6.44	13.95 ±1.66	2.22 ± 0.86	16.50 ± 1.99	34.41 ± 4.16
C.D. (p≤0.05)	0.36	0.33	0.20	0.42	0.36

Values in the same column not followed by the same letter are significantly different at p≤0.05

Table 4. Correlation analysis for leaf attributes and phytochemical analysis of phalsa leaves

	No. of leaves	LFW*	LDW^	LA#	Flavonoids	Alkaloids	Saponins	Terpenoids	Phenols
No. of leaves	1								
LFW*	0.993	1							
LDW^	0.981	0.995	1						
LA#	0.983	0.996	0.995	1					
Flavonoids	0.523	0.552	0.563	0.537	1				
Alkaloids	0.733	0.794	0.829	0.802	0.785	1			
Saponins	0.819	0.845	0.862	0.830	0.819	0.934	1		
Terpenoids	0.932	0.960	0.967	0.958	0.714	0.924	0.939	1	
Phenols	0.285	0.333	0.385	0.334	0.821	0.664	0.649	0.485	1

*LFW – Leaf fresh weight, ^LDW – Leaf dry weight, #LA – Leaf area

conducted by Mostafa (2015) on *Khaya senegalensis* (Mahogany) trees, which aligns with the present results. While the presence of phytochemicals in phalsa has been confirmed in previous research studies (Pundlik, 2021), literature specifically addressing the effects of chemical mutagens on phytochemicals is currently lacking.

The research evidence highlights the significance of phenols as crucial secondary metabolites, directly contributing to the antioxidant activity of plants (Naaz *et al.*, 2022). Phenols serve as antimicrobial agents, providing defense against harmful radiation, and are utilized by the epidermal layer of plants as part of their protective screening mechanism (Carletti *et al.*, 2003). The summarized data indicates that an increase in phenolic content is vital for enhancing antioxidant properties, demonstrating a positive correlation with higher concentrations of EMS treatments (Lee *et al.*, 2009). Research outcomes by Khater *et al.* (2018) and Hanafy and Akladious (2018) have also reported increased total phenolic content in fenugreek, further supporting these findings.

The correlation analysis of phytochemical traits in phalsa revealed notable patterns. Flavonoids exhibited a high positive correlation with alkaloids, saponins, and phenols, and a moderate positive association with terpenoids (Table 4). Alkaloids displayed a very high positive correlation with saponins and terpenoids, yet a moderate correlation with phenols. Similarly, saponins showed a very high positive correlation with terpenoids, but a moderate positive correlation with phenols. Phenols and terpenoids exhibited a relatively low positive correlation with each other.

The research findings demonstrated that the application of colchicine at 0.1% concentration notably enhanced the phytochemical composition of phalsa seeds. However, higher doses of mutagens adversely affected growth, leading to the degeneration of cell organelles and disruption of cellular mechanisms due to the accumulation of free radicals in plant tissues, ultimately resulting in reduced phytochemical content. Interestingly, certain phytochemicals, such as phenols, exhibited an increase in response to higher doses of EMS, possibly as a stress response to the presence of free radicals. In conclusion, the study suggests that chemical mutagenic treatments could serve as a viable approach for generating superior mutants in future horticultural crop improvement strategies.

Authors' contribution

Conceptualization and designing of research work (AK); Conduct of field/lab experiment and data collection (LK); Data analysis and interpretation (LK, AK); Manuscript preparation (AK, LK).

Declaration

Authors declare that they do not have any conflict of interest.

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