

Assessing the Genotoxic and Cytotoxic Potential of Protein Supplements Using *Allium cepa* as a Model Bioindicator

C. A. SriRanjani¹, C. Asha Deepti², K. Bhavya Sri^{3,*}

¹Department of Pharmaceutical Analysis, GITAM School of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, INDIA.

²Department of Pharmaceutical Chemistry, GITAM School of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, INDIA.

³Department of Pharmaceutical Analysis, RBVRR Women's College of Pharmacy, Hyderabad, Telangana, INDIA.

ABSTRACT

Objectives: The increasing consumption of dietary protein supplements raises concerns regarding potential contamination and associated toxicological effects. The present study evaluates the cytotoxic and genotoxic potential of commercially available protein supplements using the *Allium cepa* root tip assay. **Materials and Methods:** Six protein supplements originating from different geographical regions were evaluated. Cytotoxicity was determined by measuring root growth inhibition and calculating the mitotic index, while genotoxicity was assessed through chromosomal aberrations and micronuclei formation in meristematic cells. Heavy metal concentrations were previously determined using ICP-MS. Statistical analysis was performed using Bayesian one-way ANOVA through JASP software. **Results:** A concentration-dependent reduction in root growth and mitotic activity was observed across all samples. At 10% concentration, complete root growth inhibition occurred in all treatments. Vegan protein samples exhibited comparatively higher cytotoxic and genotoxic effects than whey protein samples. Increased chromosomal abnormalities and micronuclei frequency were detected in samples containing elevated heavy metal concentrations. **Conclusion:** The findings indicate that certain protein supplements may exhibit measurable cytotoxic and genotoxic effects, possibly due to contamination with trace metals or processing-related compounds. The *Allium cepa* bioassay proved to be a sensitive and reliable screening tool for evaluating potential toxicological risks in dietary supplements.

Keywords: *Allium cepa* Assay, Bayesian Analysis, Cytotoxicity, Genotoxicity, Heavy Metals, Protein Supplements.

Correspondence:

Dr. K. Bhavya Sri

Professor, Head, Department of
Pharmaceutical Analysis, RBVRR Women's
College of Pharmacy, Hyderabad,
Telangana, INDIA.

Email: bhavya.khagga@gmail.com

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INTRODUCTION

The global consumption of protein supplements has increased substantially due to growing awareness of physical fitness, nutritional requirements, and perceived health benefits. Protein supplements derived from sources such as whey, soy, pea, and rice are widely used to support muscle development, weight management, and overall nutrition. However, the composition and safety of these supplements may vary considerably depending on raw material quality, manufacturing processes, and geographical origin. Such variability raises concerns regarding the presence of organic and inorganic contaminants that may pose potential health risks (Abbate *et al.*, 2015). Cytotoxicity refers to the ability of a substance to inhibit cell growth or

induce cellular damage, whereas genotoxicity involves damage to genetic material that may result in mutations, chromosomal aberrations, or genomic instability. These parameters are critical for evaluating the safety of food and nutritional products. Conventional toxicity assessments often rely on animal models, which are time-consuming, costly, and associated with ethical constraints (Balls, 2002). Consequently, plant-based bioassays have gained acceptance as reliable, cost-effective, and ethically suitable alternatives for preliminary toxicity screening (Grant, 1999).

The *Allium cepa* root tip assay is a well-established and sensitive plant bioassay widely used for assessing cytotoxic and genotoxic effects of environmental pollutants, food additives, and chemical substances (Fiskesjö, 1985). The rapidly dividing meristematic cells of onion roots are particularly sensitive to mitotic disturbances and DNA damage, enabling the detection of chromosomal aberrations, micronuclei formation, and changes in the mitotic index (Rank and Nielsen, 1998). Moreover, the large and few chromosomes of *Allium cepa* facilitate clear cytogenetic observations, making it an effective model for toxicity assessment.



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Protein supplements may pose potential risks due to contamination with heavy metals, pesticides, undeclared anabolic agents, or processing-related byproducts (Leme and Marin-Morales, 2009a; Aina *et al.*, 2024; Nissen and Sharp, 2003). Studies have reported the presence of toxic metals such as lead, cadmium, mercury, and arsenic in dietary supplements, with concentrations varying according to geographical origin and quality control practices (Tamanna and Mahmood, 2015). High-temperature processing may also result in the formation of harmful compounds such as advanced glycation end products, while additives including artificial sweeteners and flavoring agents may contribute to cytotoxic or genotoxic effects (Einhellig, 1996; Mishra, 1993; Rodrigues *et al.*, 2010; Leme and Marin-Morales, 2009b; Sindhuja *et al.*, 2023; Kwasniewska *et al.*, 2012; Gideon *et al.*, 2013; Konuk *et al.*, 2007). Furthermore, plant-based protein sources may contain secondary metabolites that require evaluation for cellular toxicity.

Considering the widespread consumption of protein supplements and the potential variability in their contaminant profiles, systematic evaluation of their cytotoxic and genotoxic effects is essential. Therefore, the present study aims to assess the cytotoxic and genotoxic potential of protein supplements sourced from different geographical regions using the *Allium cepa* root tip assay. By analyzing root growth inhibition, mitotic index, and chromosomal aberrations following exposure to aqueous extracts, this study seeks to provide insight into their cellular safety profile and support consumer protection and regulatory decision-making (Hadwan and Abed, 2016; Habig and Jakoby, 1981; Mesquita *et al.*, 2014; Bradford, 1976; Legendre, 2019). The results of this research will add to the overall picture of the safety implications of protein supplements of varying origin and will inform future quality control and consumer advice.

The present investigation provides a novel toxicological evaluation of commercially available protein supplements using the plant cytogenetic bioassay based on *Allium cepa*. While the *Allium cepa* root tip assay has been extensively employed for assessing environmental pollutants and industrial contaminants, its application to nutraceutical safety assessment remains limited. This study uniquely integrates trace metal profiling of protein supplements with biological cytogenotoxic endpoints, including mitotic index inhibition, chromosomal aberrations, and root growth suppression. Furthermore, the comparative analysis of whey and vegan protein supplements originating from different geographical regions offers insight into potential variations in toxicological profiles associated with raw material sourcing and manufacturing practices. The incorporation of Bayesian statistical analysis using JASP provides a robust analytical framework for interpreting cytogenotoxic responses. Collectively, the findings highlight the potential cellular risks associated with contaminated dietary supplements and emphasize the importance of rigorous quality control and regulatory monitoring to ensure consumer safety.

MATERIALS AND METHODS

The biological materials for this study were consistently sized onion bulbs of a local variety, with an average diameter of 15-22 mm. Six distinct protein powder samples, chosen based on their trace element concentrations as previously quantified by ICP-MS, were utilized for cytotoxicity and genotoxicity assessments using the *Allium cepa* assay. These samples included whey protein concentrate from China (1-WPC), vegan protein concentrate from China (2-VPC), whey protein from Japan (3-WPJ), vegan protein from Japan (4-VPJ), whey protein isolate from India (5-WPI), and vegan protein isolate from India (6-VPI). The selection of these diverse protein sources, varying in origin and type, aimed to provide a comprehensive evaluation of potential cytotoxic and genotoxic effects linked to their trace element profiles.

Analysis for physico-chemical parameters

The physico-chemical characterization of the samples involved the analysis of several standard parameters conducted following the methodologies outlined in APHA/AWWA/WEF (1998). Additionally, the concentrations of selected heavy metals were determined in the samples using standard analytical method and quantified using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Allium cepa Assay Procedure

The *Allium cepa* assay was employed for both macroscopic and microscopic evaluations. To prevent desiccation of the root primordia during the cleaning process, peeled onion bulbs were placed in fresh tap water. Subsequently, test samples were prepared at a concentration of 10%, ensuring the use of high-quality samples with a pH of 7.1. Eighteen onion bulbs were initially set up, for root growth inhibition analysis, six roots exhibiting the most robust growth were selected. For negative control Distilled water is served.

The experimental temperature setup was maintained in darkness at $25 \pm 1^\circ\text{C}$ for a duration of 72 hr, with daily renewal of the test solutions. Root length measurements were taken to assess early seedling growth. For each test sample, the EC_{50} value, representing the concentration at which 50% root growth inhibition was seen of the control (100%), was determined by interpolation from a graph.

For cytological studies, root tips were excised from each bulb after 48 hr of exposure. These tips were hydrolyzed immediately by setting in a solution of Ethan-ol and G-acetic acid (3:1, v/v) and then in 1 N HCl at 65°C for 3 min. Acetocarmine staining technique was adapted on 2 root tips per bulb followed by squashing onto microscope slides for microscopic observation.

$$\text{Mitotic Index} = \frac{\text{Total number of cells observed}}{\text{Number of dividing cells}} \times 100$$

The findings regarding inhibition and aberrations of root growth and chromosome are conferred as the mean $\pm$ standard error, calculated from measurements of 18 onion bulbs for each tested concentration.

To assess the significance of the observed effects, a Bayesian One-Way Analysis of Variance was conducted using JASP (Jeffreys's Amazing Statistics Program).

Where the Bayesian ANOVA indicated a significant effect of concentration, Bayesian post-hoc comparisons, analogous to the frequentist Tukey test, were performed within JASP to determine which specific concentrations exhibited statistically meaningful differences from the control group and from each other, based on the resulting Bayes Factors.

All statistical analyses were performed utilizing the Bayesian statistical methods implemented in JASP.

Macroscopic Observations- Root Length Inhibition

The root lengths of *Allium cepa* exposed to the six different protein samples, across a range of concentrations, exhibited a clear concentration-dependent decrease. Notably, sample concentrations of 0% and 1% did not significantly inhibit root growth. However, at highest concentration of 10% for all protein samples a complete (100%) growth inhibition of root was observed.

The onion bulbs grown in a 0%, 1% and 10% concentration of all the samples and calculated EC₅₀ values (the effective concentration causing 50% root growth inhibition) and length of the roots respectively are shown in Table 1 and Figure 1. Interestingly, the EC₅₀ values were lower for WPC, WPJ, and WPI, indicating a greater inhibitory effect in vegan protein samples concentrations compared to the whey protein samples.

Overall, restricted root growth was observed across all protein sample treatments. The degree of root growth inhibition followed a increasing order: WPC < WPI < WPJ < VPI < VPC < VPJ. This suggests that vegan protein samples generally exhibited a stronger inhibitory effect on root growth compared to the Whey protein samples (Table 2 and Figure 2).

Microscopic Effects- Cell Division and Chromosome Aberrations

The impact of protein powders, varying in geographical origin and heavy metal concentration, on division of cells along with chromosome behavior in *Allium cepa* is summarized in Table 3.

The control group exhibited a normal Mitotic Index (MI) of 12.4, with no observed chromosomal aberrations. In contrast, all tested concentrations of the protein samples induced statistically significant ($p < 0.05$) chromosomal aberrations. Furthermore, a concentration-dependent mitotic index was observed as sample concentration increased, indicating a cytotoxic effect on cell proliferation.

The types of chromosomal aberrations identified in treated roots included, but were not limited to, multiple anaphase, anaphase configurations, mitotic spindle deviations and disturbances, particularly prominent at 10% sample concentrations.

RESULTS

The *Allium cepa* bioassay generated two categories of findings-macroscopic root-growth data and microscopic cytogenetic data which together characterise the cytotoxic and genotoxic behaviour of the six protein supplement samples across the tested concentration range (0%, 1%, and 10%).

Macroscopic observations root length inhibition

The root lengths of *Allium cepa* exposed to the six different protein samples, across a range of concentrations, exhibited a clear concentration-dependent decrease. Notably, sample concentrations of 0% and 1% did not significantly inhibit root growth. However, at the highest concentration of 10% for all protein samples, complete (100%) inhibition of root growth was observed.

The onion bulbs grown in 0%, 1%, and 10% concentrations of all the samples, and the calculated EC₅₀ values (the effective concentration causing 50% root growth inhibition) together with the corresponding root lengths, are shown in Table 1 and Figure 1. Interestingly, the EC₅₀ values were lower for WPC, WPJ, and WPI, indicating a greater inhibitory effect of the vegan protein sample concentrations compared to the whey protein samples.

Table 1: The MRoL for bulbs grown in a 0%, 1% and 10% concentration of all the samples and calculated EC₅₀ values.

Protein Sample	0% MRoL (cm)	1% MRoL (cm)	10% MRoL (cm)	EC ₅₀ Value (%)
WPC	3.4	2.21	0.4	1.5
WPI	3.8	2.02	0.3	1.85
WPJ	2.4	1.86	0.2	2.8
VPI	2.8	1.46	0.1	3.1
VPC	3.6	0.98	0	3.3
VPJ	3.5	0.6	0	3.5

*MRoL-Mean Root Length (cm).

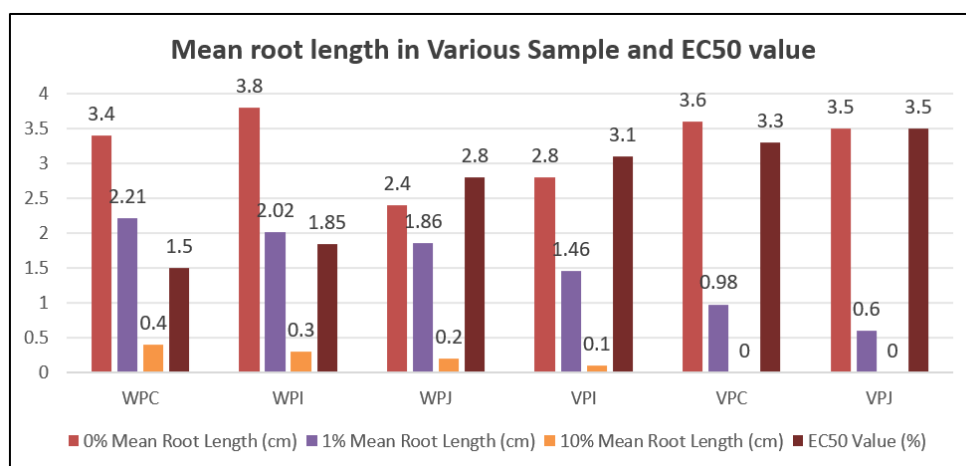


Figure 1: Mean Root Length (MRoL) in Various Sample and EC₅₀ value.

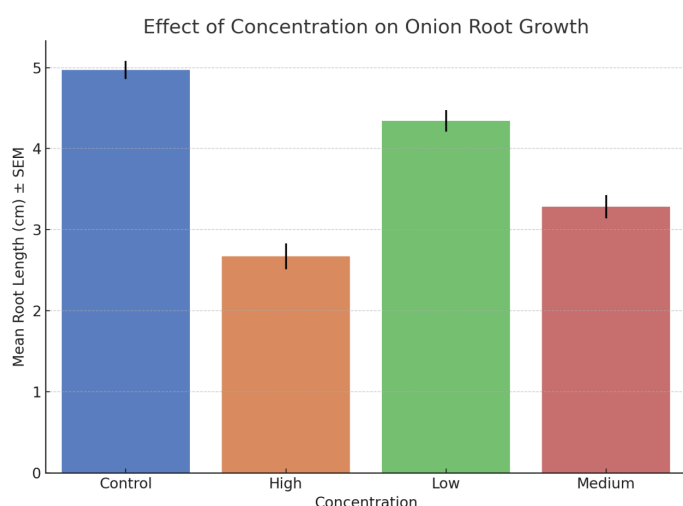


Figure 2: Effect of Concentration on Onion Root Growth.

Overall, restricted root growth was observed across all protein sample treatments. The degree of root growth inhibition followed an increasing order: WPC < WPI < WPJ < VPI < VPC < VPJ. This suggests that vegan protein samples generally exhibited a stronger inhibitory effect on root growth compared to the whey protein samples (Table 2).

The impact of protein powders, varying in geographical origin and heavy metal concentration, on cell division and chromosome behavior in *Allium cepa* is summarized in Table 3.

The control group exhibited a normal Mitotic Index (MI) of 12.4, with no observed chromosomal aberrations. In contrast, all tested concentrations of the protein samples induced statistically significant ($p < 0.05$) chromosomal aberrations. Furthermore, a concentration-dependent decline in mitotic index was observed as sample concentration increased, indicating a cytotoxic effect on cell proliferation.

The types of chromosomal aberrations identified in treated roots included, but were not limited to, multiple anaphase, anaphase

Table 2: Mean Root Length (MRoL) ± SEM.

Concentration	MRoL (cm)	SEM (cm)
Control	4.97	0.11
Low	4.34	0.13
Medium	3.28	0.14
High	2.67	0.16

bridges, mitotic spindle deviations, and disturbances, particularly prominent at 10% sample concentrations (Figure 3).

DISCUSSION

All statistical analyses were performed utilizing the Bayesian statistical methods implemented in JASP (Jeffreys's Amazing Statistics Program), which allowed the effect of protein sample concentration on root growth, mitotic index, and chromosomal aberration frequency to be evaluated with associated Bayes Factors rather than conventional p-values alone. The *Allium cepa* test revealed pronounced cyto-genotoxic effects attributable to the protein powder samples, indicating that exposure to these supplements interfered with both cell viability and genomic integrity in the meristematic root-tip cells. These effects followed a distinct and reproducible pattern across the six samples evaluated: vegan protein samples originating from Japan and China exhibited the highest cytotoxic and genotoxic potential, followed by the vegan protein sample from India. In contrast, whey protein samples, irrespective of their geographical origin, consistently demonstrated the least severe cytotoxic and genotoxic effects, suggesting that the protein source (plant-based versus dairy-based) and its associated manufacturing process may be an important determinant of the observed toxicity.

This study employed the Bayesian statistical methods implemented in JASP for the *Allium cepa* assay specifically to assess the cytotoxic and genotoxic potential of the various protein powders, with particular focus on those samples previously identified as having elevated heavy metal concentrations by ICP-MS. When

Table 3: Cytological effects of onion bulbs grown in a 0%, 1% and 10% concentration of all the samples and calculated Mitotic Inhibition and Aberrant cells.

Protein Sample	CON (%)	No. dividing index	Mitotic Mean Index $\pm$ SE	Mitotic inhibition Mean $\pm$ SE	Aberrant cells (%)
WPC	0	124	12.4 $\pm$ 0.43	-	-
	1	72	7.21 $\pm$ 0.26*	23.8	5.1
	10	35	1.51 $\pm$ 0.01*	41.7	7.4
WPI	0	95	9.45 $\pm$ 0.21*	-	-
	1	72	7.21 $\pm$ 0.26*	65.1	9.3
	10	23	5.64 $\pm$ 0.21*	87.8	11.7
WPJ	0	82	12.4 $\pm$ 0.43	-	-
	1	56	7.21 $\pm$ 0.26*	53.7	8.9
	10	35	1.51 $\pm$ 0.01*	71.9	12.5
VPI	0	115	9.45 $\pm$ 0.21*	-	-
	1	96	7.21 $\pm$ 0.26*	32.8	12.5
	10	32	5.64 $\pm$ 0.21*	61.7	13.6
VPC	0	128	9.45 $\pm$ 0.21*	-	-
	1	81	7.21 $\pm$ 0.26*	33.8	9.5
	10	23	5.64 $\pm$ 0.21*	81.7	13.5
VPJ	0	122	9.45 $\pm$ 0.21*	-	-
	1	64	7.21 $\pm$ 0.26*	42.5	8.6
	10	11	5.64 $\pm$ 0.21*	91.5	12.4

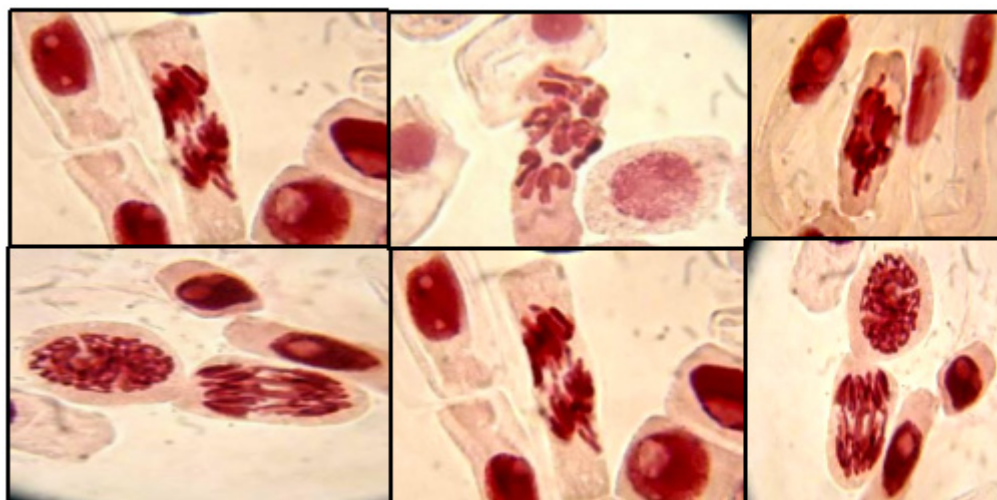


Figure 3: Chromosomal aberrations observed in root tip cells of *Allium cepa*. 1. AnB 2. MuB 3. AnB 4. AnCC 5. TeV 6. PoD. *AnB-Anaphase bridge: *MuB-multiple bridges: *AnB-Fanaphase bridge and fragments. *AnCC-criss-cross at anaphase: *TeV-telophase with vagrant chromosome *PoD-polar deviation. *[Picture taken through Fapiwen Telescope with Smart phone adapter mount].

the heavy metal content of the tested samples was compared with established regulatory allowable limits, concerning levels were indicated for most of the analyzed parameters, most notably the heavy metal content within these protein powders. This correspondence between elevated metal content and the magnitude of the biological response observed in the bioassay supports the interpretation that trace metal contamination is

a plausible contributing factor to the cyto-genotoxic effects recorded.

Both the macroscopic and microscopic analyses derived from the *Allium cepa* test unequivocally demonstrated the cytotoxic nature of these protein powders, an effect primarily evidenced by their significant, concentration-dependent inhibition of root growth relative to the untreated control. Moreover, the microscopic

investigation established the cyto-genotoxic effects of the protein powders at the cellular level, further substantiating their detrimental impact on *Allium cepa* root-tip cell proliferation. The observed abnormalities in cell division, coupled with the induction of various chromosomal aberrations-including Anaphase Bridges (AnB), Multiple Bridges (MuB), Anaphase Chromosome Clumping (AnCC), telophase vagrants (TeV), and Polyploidy (PoD)-were especially prominent at the 10% sample concentration. Taken together, these findings strongly suggest that the observed cytotoxic and genotoxic effects are a direct consequence of the heavy metal contamination and high protein concentrations present in the test samples, rather than an artefact of the assay conditions.

CONCLUSION

The present study demonstrates that several commercially available protein supplements exhibit significant cytotoxic and genotoxic potential when assessed using the *Allium cepa* bioassay. Vegan protein supplements, particularly those sourced from Japan and China, showed the highest toxicity, while whey protein supplements exhibited comparatively lower effects. The observed cellular damage is likely attributable to elevated heavy metal concentrations and processing-related contaminants. These findings emphasize the importance of stringent quality control, regulatory monitoring, and routine safety assessment of dietary protein supplements to ensure consumer safety.

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ABBREVIATIONS

ICP-MS: Inductively Coupled Plasma Mass Spectrometry; **ANOVA:** Analysis of Variance; **JASP:** Jeffreys's Amazing Statistics Program; **MI:** Mitotic Index; **EC₅₀:** Effective concentration causing 50% root growth inhibition; **SEM:** Standard Error of the Mean; **WPC:** Whey Protein Concentrate from China; **VPC:** Vegan

Protein Concentrate from China; **WPJ:** Whey Protein from Japan; **VPJ:** Vegan Protein from Japan; **WPI:** Whey Protein Isolate from India; **VPI:** Vegan Protein Isolate from India; **MROl:** Mean Root Length; **AnB:** Anaphase Bridge; **MuB:** Multiple Bridges; **AnCC:** Anaphase Chromosome Clumping; **TeV:** Telophase with Vagrant Chromosome; **PoD:** Polyploidy or Polar Deviation; **pH:** Potential of Hydrogen; **HCl:** Hydrochloric Acid.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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