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Phytotoxicity of Copper & Silver Nanoparticles on Seed Germination and Biochemistry in *Cassia Fistula* and *Clitoria Ternatea*

• **Deepshikha Pandey** • **Sweety** • **Worthyson Sakshi**

• **Hena Naz**

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Corresponding Author : Hena Naz

Abstract : *The present paper elaborate the consequences of oxidative stress caused by synthesized copper and silver nanoparticles in two different plants viz; Cassia fistula and Clitoria ternatea. The Seeds of these plants were treated with Cu-CuO based nanoparticles and Ag nanoparticles at concentration of 1000 mg/l under normal conditions for 6 hours. The effect of these two Nanoparticles (NPs) on seeds germination and biochemistry has been observed. The results obtained showed significant reduction in growth at 1000 µg/ml NPs. Copper showed more stress to the germinated seedlings (50-60%) than silver (20-30%).The chlorophyll content in the fresh leaves of germinated seedlingsshowed reduction in both, chlorophyll a and chlorophyll b in NPs treated than control.Exposure to 1000 µg/ml of Cu and Ag nanoparticles reported a significant retardation on total content of primary metabolites and enhancement on total content of secondary metabolites. The obtained results and reviews put forward the prospects of research direction of the environmental behavior and the biological toxicity of NPs, hoping to bring new ideas to the further research on NP phytotoxicity.*

Keywords: *phytotoxicity, nanoparticles, seed germination, plant growth.*

Deepshikha Pandey
B.Sc. III year, Botany (Hons.),
Session : 2015-2018, Patna Women's College,
Patna University, Patna, Bihar, India

Sweety
B.Sc. III year, Botany (Hons.),
Session : 2015-2018, Patna Women's College,
Patna University, Patna, Bihar, India

Worthyson Sakshi
B.Sc. III year, Botany (Hons.),
Session : 2015-2018, Patna Women's College,
Patna University, Patna, Bihar, India

Hena Naz
Assistant Professor, Deptt. of Botany,
Patna Women's College,
Bailey Road, Patna–800 001, Bihar, India
E-mail : henanaz64@gmail.com

Introduction :

In the 21st century, nanotechnology has received much importance due to its capabilities of modulating metals in their nanoparticles. It is a multidisciplinary field, as it combines the knowledge from different disciplines: chemistry, physics, and biology amongst others (Schmid et al, 2006; Schmid, 2010), and it has excellent prospects for exploitation across the medical, pharmaceutical, biotechnology, engineering, manufacturing, telecommunications and information technology markets.

Nanoparticles (NPs) are atomic or molecular aggregates with at least one dimension between 1 and 100nm (Ball 2002), that can drastically modify their physico-chemical properties compared to the bulk material (Nel et al. 2006). The novel properties of NPs, characterized by their small size and unique physical and chemical properties, have led to a drastic increase in the incorporation of NPs into commercial products. This rapid expansion has increased concern over the impact of Nano scale particles (NSP) on the environment and biota. Some metal and metal oxide (MeO) NPs, such as those made from gold, silver, copper ,copper oxide (CuO), zinc oxide (ZnO), and titanium dioxide (TiO2), are nanomaterials commonly employed in consumer products including sunscreen, food preservatives, clothing, electronics, transistors, polymers, medicines, and pesticides. The increased use of NPs will predictably lead to accidental introduction into the environment via consumer use or manufacturing and its unique properties allow them to interact with biological system and can act as a promoter or can cause phytotoxicity.

In order to develop a comprehensive toxicity profile for manufactured nanoparticles, their phytotoxicity has to be investigated. The most common monitored parameters include the germination rate and root/stem growth rate. Recently, the number of leaves and chlorophyll content of exposed plants were included as new monitored parameters for phytotoxicity tests. Rico et al.(2011) and Peralta-Videa et al.(2011) are the pioneers of studies dealing with the nonotoxicity of nanoparticles on vascular plants.

In this study, the main goal is to find out if any phytotoxicitydevelops by copper and silver nanoparticles in *Cassia fistula* and *Clitoria ternatea,*which are easily available in our locality.These plants have great medicinal properties as they contain important micro and macro nutrients, antioxidant and various essential properties required to treat recent serious diseases. We selected Ag and Cu-CuO based NPs because the commercial use of these NPs has significantly increased (Sun et al., 2014) and have found their way into the environment. Therefore, the potential of nanoparticles to affect human health and the environment is of significant concern.

Materials and Methods :

Seeds of *Cassia fistula*(Amaltas)and*Clitoria ternatea* (Aprajita) were collected from Patna Women's College and Patna Science College. After authentication seeds were collected in bulk and washed under running tap water to remove any adhering dirt. Then sterilized (with 0.5 % $HgCl_2$), dried in sunlight and store in dry and clean air tight container.

Copper and silver nanoparticles were selected for this whole experiment as it is widely used in industries and industrial products as well as it can be easily prepared in labs.

Copper Nanoparticles were prepared by chemical reduction method (Audil Rashid et al. 2016).The precipitates formed were separated from the solution by filtration and washed, dried at room temperature and were stored in glass vial for further analysis.

Silver Nanoparticles were prepared by chemical reduction method (AstaSileikaite et al. 2006). AgNPs weresynthesized from silver nitrate solution using sodium citrate as a reductant. Then it was cooled at room temperature and stored in brown bottle.

The chemically synthesized nanoparticles were characterized using UV-Vis spectrophotometer at the range of 200-800 nm.

The direct contact between nanoparticles and seed/plant roots is an essential requirement for a phytotoxicity study in plant model experiments (Gorka and Liu 2016). Taking that into account, a pre-sowing of seed treatment with colloidal solutions of nanoparticles was performed (Figure1).

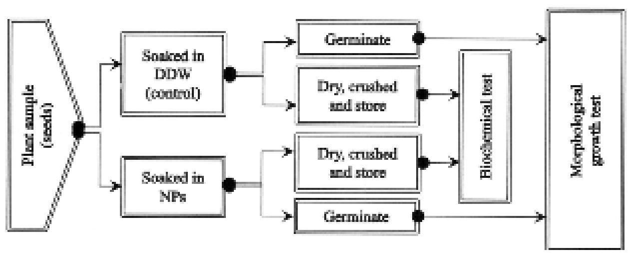


Fig. 1. Flow chart of experimentalsetup

Some seeds of *Cassis* and *Clitoria* are soaked with double distilled water or DDW (control) and some in nanoparticles (both NPs at 1000 mg/ml) for 6 hours. Few seeds are allowed to germinate and other seeds are dried, crushed and stored for biochemical test.

Seeds of both plants(treated and non-treated with control) were allowed to grow in petriplates with cotton and distilled water medium under natural light for 14 days (Figure 2).

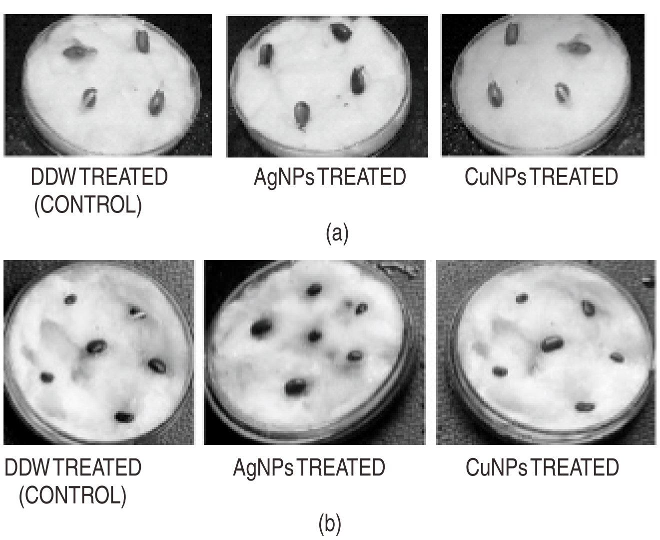


Fig. 2. Germination setup of Amaltas (a) &Aprajita (b) seeds in AgNPs & CuNPs with control.

The parameters selected for measurement of phytotoxicity during seed germination were:

Percentage (%) of Germination was calculated using the following formula (Razzaq et al., 2016):-

$$\text{Percentage (\%) of Germination} = \frac{\text{No. seeds germinated}}{\text{Total seeds}} \times 100$$

The seedling vigour index was calculated by using the method suggested by Abdul-Baki and Anderson (1973) and expressed as index numbers.

Seedling vigour index = [root length (cm) + shoot length (cm)] × germination (%)

Effect of seedlings on plant height (root and shoot length), leaf colour, biomass was measured after 14 days of germination (Ahmad et al., 2011).

Chlorophyll content was estimated by Arnon (1949) method. The absorbance of the solution was estimated at 645nm and 663nm wavelength against the solvent (acetone) blank.

The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the following equation:

- Total Chlorophyll = Chlorophyll a + Chlorophyll b
- Chlorophyll a = 12.7(A663) - 2.69(A645)
- Chlorophyll b = 22.9(A645) - 4.68(A663)

Phytochemical screening - The powdered seed samples were screened for the presence of some primary (Amino acid, Protein and Sugar) and secondary metabolites (Alkaloids, Flavonoids and Tannin). Total Amino acid (TAA) was measured by method of Sircelj et.al. (2005) and the colour intensity was measured at 570nm of UV-Vis spectrophotometer. Total Soluble Protein (TSP) content was estimated by Lowry et al. (1951). The absorbance was calculated at 510 nm.Total Soluble Sugar (TSS) content was estimated by the method of Dey and Harborne (1990). Absorbance was recorded at 490 nm.Total Alkaloid contents in the plant sample were estimated by Harbone (1973) method.The result was expressed in gram.Total Flavonoid content (TFC) in the plant sample was estimated by Dewanto et al. (2002) method. Absorbance was measured at 510 nm.Total Tannin content (TTC) was estimated by Folin-Denis reagent method (Anonymous, 1980). Absorbance was measured at 760 nm.

Results and Discussion :

Characterization of NPs used in bioassays is an essential step because the ability of penetration into plant tissues strongly depends on the physicochemical properties of NPs. Silver and Copper nanoparticles were synthesized by the chemical reduction process (Figure 3). In case of silver nanoparticles, colour of the solution changes from colourless to grey. It was characterized with UV-vis spectrometer at a wavelength range from 200 nm –800 nm. Size of the nanoparticle ranges from 42 to 48.75nm, and was confirmed from the result (i.e., 44 to 56.55nm) obtained by Udapudi et al. (2012). In case of copper nanoparticles, colour of the solution changes from yellow to ochre. It was characterized by UV-vis Spectrophotometer at wavelength range of 300nm to 700nm. Size of the nanoparticle ranges from 14nm to 25nm.

(A) Synthesized AgNPs (B) Synthesized CuNPs

Fig. 3. Silver and copper nanoparticles synthesized by chemical reduction method

Seed germination is extensively used in phytotoxicity test because it is simple, low cost and most suitable for unstable samples or chemicals (Munzuroglu and Geckil 2002; Wang et al. 2001).

Results obtained signify that the copper and silver nanoparticles exhibited significant effects on seed germination (Table 1). We have observed that germination of the Clitoria and Cassia was stressed by copper 60% and 50% respectively while Ag NPs stressed only 30% and 20 % respectively with the control percentage (100% in both). Results are about in line with Mustafa et al (2017) whose study shows 49% of stress induced by Cu NPs and 31% stress induced by Ag NPs. The corresponding growth of Cassia fistula and Clitoria ternatea in 14 days experiment demonstrate that stress caused by Cu is maximum than Ag and control.

Table 1. Morphological growth result of both the plants at 14th day of experiment

S.No.	Physical parameter	CASSIA FISTULA NONTREATED (CONTROL)	CASSIA FISTULA TREATED Ag NPS	CASSIA FISTULA TREATED Cu NPS	CLITORIA TERNATEA NONTREATED (CONTROL)	CLITORIA TERNATEA TREATED Ag NPS	CLITORIA TERNATEA TREATED Cu NPS
1.	% of germination	100%	80%	50%	100%	70%	40%
2	vigour index	2460	1720	635	1530	917	336
3.	Day of emergence of first leaf	3rd Day	4th day	7th day	3rd Day	4th day	6th day
4.	No. of leaves	6	6	4	8	6	4
5.	Colour of leaf	Dark Green	Light green	Light green	Dark Green	Light green	Light green
6.	Plant height(cm)	24.6	21.5	12.7	15.3	13.1	8.4
7	Shoot length (cm)	9.3	7.7	5.1	7.8	6.9	5.1
8.	Root length(cm)	15.3	13.8	7.6	7.5	6.2	3.3
9.	Plant fresh weight(mg)	0.65	0.58	0.49	0.36	0.31	0.25
10.	Plant dry weight(mg)	0.37	0.23	0.19	0.21	0.11	0.09

The visible changes in colour of growing leaves i.e., dark green coloured leaves in control and comparatively light colour leaves in Ag and Cu-NPs treated are observed. Yellowish green leaf colour observed in NPs treated plants indicates its influence on chlorophyll and other pigment contents in the plants and from this we could suspected that photosynthesis may be getting affected in NPs treated plants.

Figure 4 represents reduction in fresh and dry biomassin ***Clitoria*** and ***Cassia*** by Cu NP and Ag NP treatment. The copper nanoparticles have reduced the root length, shoot length and plant biomass and exhibited more stress as compared to AgNPs in ***Cassia*** and ***Clitoria***. Lee et al (2008) also reported similar results.

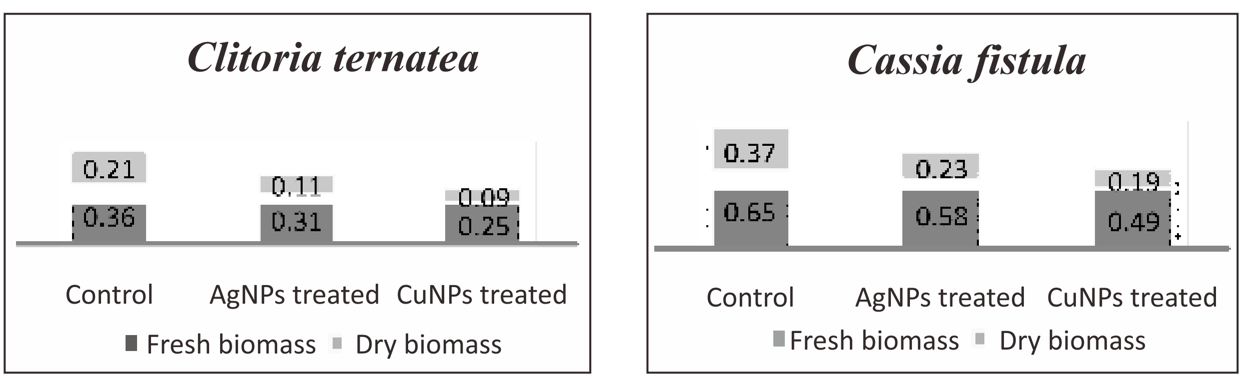


Fig. 4. Biomass of different plant samples

Response of samples to CuNPs and AgNPs (at 1000mg/L concentrations) shows distinct effects on chlorophyll content. The total chlorophyll content, chl. a and chl. b content are being decreased comparatively more due to the effect of Cu NPs than Ag NPs followed by control (Figure 5 and 6).

The results are in agreement with Abdolsamad et al (2015) which showsdecreasing effects on the chlorophyll content of AgNPs at the concentration of 400 µg/ ml -1000 µg/ ml (d” 0.4-10 mg/ml) but increasing effects in the 100µg/ml(0.1 mg/ml)concentration .

According to report of Racuciu et al (2007), chlorophyll content of maize plants was found to be increased by low concentration (10-50 µl/l) while it was found to be inhibited by higher concentrations of nanoparticle.

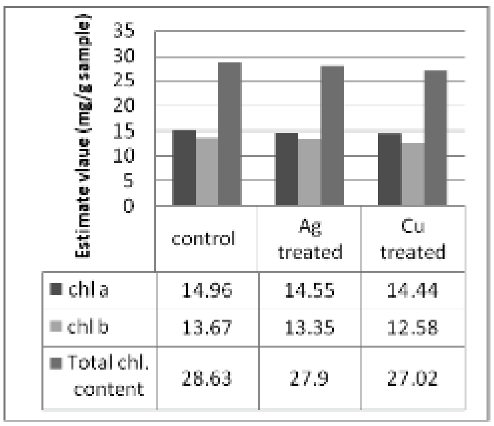


Fig. 5. Chlorophyll content in different samples of *Clitoria ternatea*

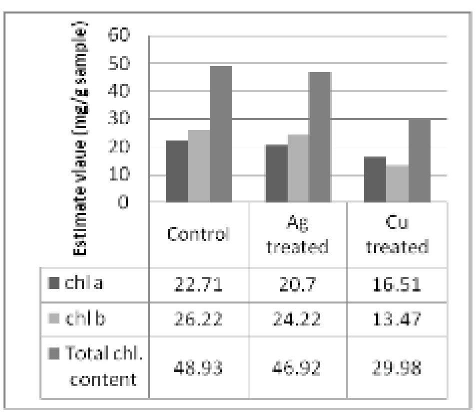


Fig. 6. Chlorophyll content in different samples of *Cassia fistula*

Figures 5 and 6 also signifies that copper stress caused a reduction in Chl. a (6.20mg/g in ***Cassia*** and 0.52mg/g in ***Clitoria***) and Chl. b (2.75mg/g in ***Cassia*** and 1.11 mg/g in ***Clitoria***), but this reduction was more in Chl. b than Chl. a.

Many studies found the reduction of chlorophyll by the treatment of copper (Ahmed et al. 2010; Zengin and Kirbag 2007; Khatun et al. 2008).The reduction of chlorophyll in broad bean under treatment of copper can be due to the inhibition of the enzymes acting on the synthesis of the chlorophyll or the degradation of chlorophyll (Mourato et al. 2009).

Both Ag and Cu NPs show significant effects on primary and secondary metabolites of the seed extracts of Amaltas and Aprajita.

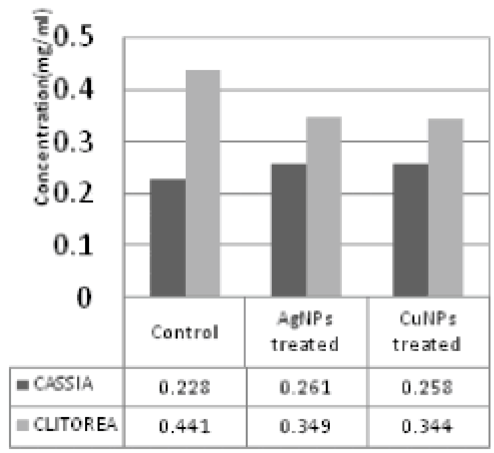


Fig. 7. Total amino acid estimation in different samples of *Cassia fistula and Clitoria ternatea*

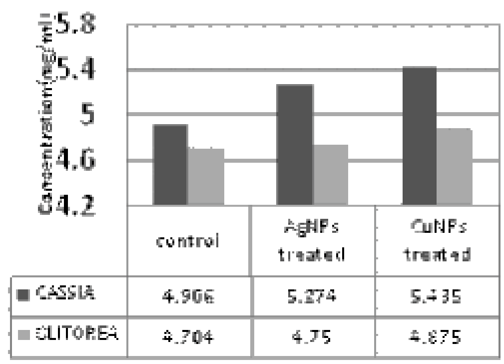
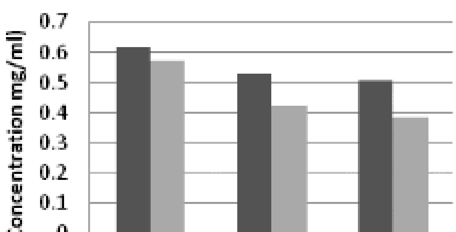


Fig. 8. Sugar estimation in different samples of Cassia fistula and Clitoria ternatea

Figure 7, shows decline of amino acid content from control to AgNPs to CuNPS treated samples. The CuNPs treated sample have minimum amount of amino acid which shows its stress to the plant samples.

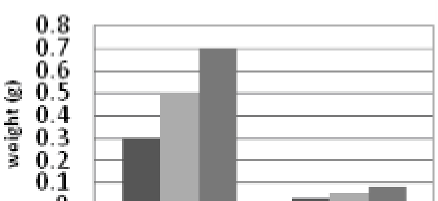
The obtained result is in agreement with the results of previous experiments of Olkhovych et al (2016).

Total soluble sugar (TSS) results (Figure 8) show that the copper at high concentration (1000mg/L) cause reduction (by 0.531 mg/ml in *Cassia* and 0.171 mg/ml in *Clitoria*) whichindicate the stress obtained by it. AgNPs shows comparatively less stress than CuNPs. The results are not in agreement with Benouis and Reguieg (2016) who state the application of copper to deferential dose causes an increase in levels of soluble sugars.



	Control	AgNPs treated	CuNPs treated
CASSIA	0.618	0.532	0.51
CLITOREA	0.571	0.42	0.385

Fig. 9. Protein estimation in different samplesof *Cassia fistula and Clitoria ternatea*

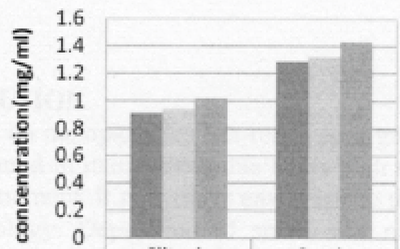


	Cassia fistula	Clitoria ternatea
Control	0.3	0.03
AgNPs treated	0.5	0.05
CuNPs treated	0.7	0.08

Fig. 10. Total alkaloid content in different samples of *Cassia fistula and Clitoria ternatea*

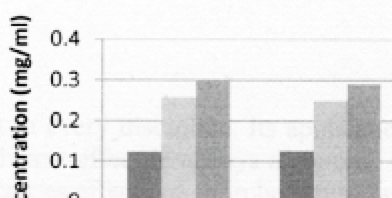
Total soluble protein (TSP) observation from the Figure 9 revealed synthesis of stressed in the plant which is treated with copper nanoparticles and silver nanoparticles. The stress induced by copper (0.510 mg/ml) is minimum than silver nanoparticles (0.532 mg/ml) followed by control (0.618 mg/ml). Stress could be the main cause behind this decrement of total protein content (Zaka et al. 2016).

The alkaloidis one of the important secondary metabolite. From the observation (Figure 10), it is visual that there is increase in alkaloid content when treated with CuNPs and AgNPs followed by control. The observation is in agreement with Zaka et al (2016), who reported that CuNPs produce considerably higher alkaloid content.



	Clitoria ternatea	Cassia fistula
Control	0.911	1.287
AgNPs treated	0.945	1.321
CuNPs treated	1.02	1.43

Fig. 11. Total flavonoid content in different samples of *Cassia fistula and Clitoria ternatea*



	Cassia fistula	Clitoria ternatea
Control	0.123	0.126
AgNPs treated	0.258	0.25
CuNPs treated2	0.3005	0.291

Fig. 12. Total tannin content in different samples of *Cassia fistula and Clitoria ternatea*

As shown in Figure 11, Total flavonoid content (TFC) is comparatively higher in samples treated with CuNPs as compared to Ag and control nanoparticles treated samples which conclude that CuNPs are responsible for inducing more stress as compared to AgNPs and control. The observations are in line with Zaka et al (2016) who reported similar findings in an attempt to check the effect of NPs for TFC in *Eruca sativa.*

The experimental result (Figure 12) shows that there is increment in total tannin content (TTC) when treated with CuNPs and AgNPs. CuNPs treated samples show high tannin content than AgNPs and control. The result is in agreement with the paper of Zaka et al (2016).

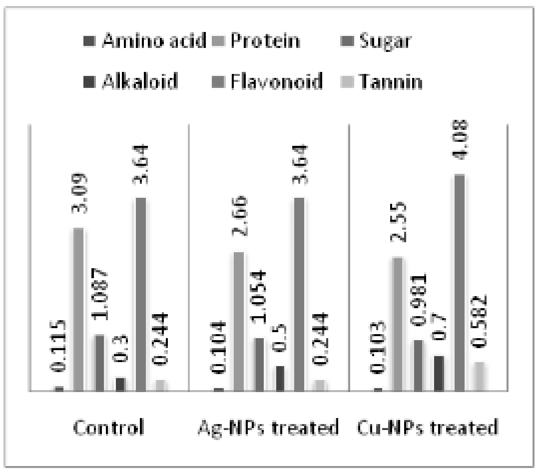


Fig. 13. Quantitative estimation of phytoconstituents of *Cassia fistula*

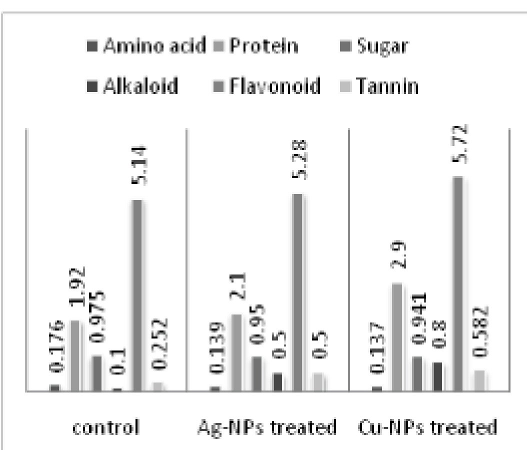


Fig. 14. Quantitative estimation of phytoconstituents of *Clitoria ternatea*

The quantitative estimation of phytochemicals (Figures 13 and 14) signifies the copper NPs stress to both plants compared to silver and control. The concentration of primary metabolites contents decreases and secondary metabolites contents increaseddue to stress induced by copper and silver nanoparticles at 1000 mg/L concentration.

Conclusion :

In this study, the toxicity of two different metal oxide NPs (Cu and Ag) with controls were tested on *Cassia fistula and Clitoria ternatea* and the highest toxicity was exhibited by Cu, followed by Ag and control. Overall results and observations indicate that the nanosize metal ion/nps if released into cultivation media and accumulated in seedlings contribute significantly to the phytotoxicity. More investigations are needed to determine the negative impact of nanoparticles on various plants and its consequences in other living organisms.

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