Impact of Copper Oxide Nanoparticles on Enhancement of Gymnemic Acid and Phenolic Compounds Using Cell Suspension Culture of *Gymnema sylvestre* (Retz.) R. Br

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Abstract: Gymnema sylvestre is a pharmacological plant which has a rich source of bioactive compounds specifically gymnemic acid (GA) and phenolic compounds (PC) that used for pharmaceutical industries. Sources for naturally occurring bioactive compounds are limited, due to geographical and seasonal variations; on the other hand, it is commercially in demand. Biosynthesis of G. sylvestre phytochemicals through in vitro culture often enhanced by elicitation. The use of cell suspension cultures (CSC) has interested serious attention on the production of essential phytochemicals. The current study is aimed at improving the contents of GA and PC in G. sylvestre CSC using the copper oxide nanoparticles (CuO NPs). Callus was obtained on MS medium with 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L kinetin (KIN), phytoagar (8.0 g/L), and sucrose (30 g/L). The above medium devoid of agar was used for the initiation of CSC. The CSC was treated with three levels of CuO NPs (1, 3 or 5 mg/L) to enhance the production of GA and PC. The greatest amount of GA (89.25 mg/g dry cell mass, DCM), total phenolic (245.10 mg/g), and flavonoid (4.57 mg/g) in CSC were achieved when G. sylvestre cells were treated for 48 h with 3 mg/L CuO NPs. Also, the biomedical potential (antioxidant, antidiabetic, anti-inflammatory, antibacterial, antifungal and anticancer activities) were also high in the CuO NPs (3 mg/L) treated CSC extracts of G. sylvestre. CuO NPs elicitation of CSC significantly increased production of GA (9-fold), and PC than non-elicited CSC in G. sylvestre.

Keywords: Gymnema sylvestre; cell suspension cultures; copper oxide nanoparticles; gymnemic acid; phenolic compounds; pharmacological activity

1. Introduction
Homeopathic plants are the ideal source of lifespan saving medicines for the majority of the peoples in the world. *Gymnema sylvestre* (Retz.) R. Br. (Family: Asclepiadaceae) is a valuable medicinal climber and possesses various bioactive compounds like gymnemic acid (GA), and phenolic compounds (PC) which are used in the therapy of diabetes mellitus [1]. This plant has been used for tea beverages and confection industries and has also been useful in numerous food preparations for the regulation of sugar homeostasis and the control of fatness and blood cholesterol levels [2]. GA has been used for pharmacological potential such as antimicrobial, antihypercholesterolemic, hepatoprotective and anti-saccharine activities [3]. GA has been documented for its role in selectively suppressing sweet flavor feelings in humans. The leaves contain triterpenoid saponins such as oleanane (GA), and dammarane (gymnemasides) classes are primarily considered the active constituents [4]. To avoid abolishing the valuable medicinal plant, the *in vitro* plant tissue or cell suspension cultures (CSCs) represent a good alternative for biotechnological applications. Tissue culture or CSCs perform talented for obtaining secondary metabolites, as they produce high yields of phytochemical production [5]. Elicitation is one of the most effective biotechnological approaches for increasing the production of phytochemicals in tissue culture or CSCs [6]. Elicitors are biological and non-biological molecules to which plant receptors on the cytoplasmic membrane react, and thus, plant cells produce a signal that stimulates the gene’s expression within the pathway, which causes the plant’s secondary metabolites to synthesize. Elicitors can stimulate defense response to defend the cell, tissue, organ, and plant, which is frequently realized by the alteration of various biosynthetic pathways. Consequently, the production of preferred phytochemicals needs external stimuli also referred to as elicitors [7]. Studies on the enhanced accumulation of secondary metabolites by the addition of elicitors to the CSCs is limited [8,9]. Application of elicitors for improvement of GA from *G.*
sylvestre is well accomplished by using callus and CSC methods [10,11,12]. Copper (Cu) is a vital micronutrient for plant development, existence a co-factor in various physiological processes for example cell wall metabolism, photosynthesis, and lignification. However, it is toxic at higher levels [13]. Cu is also present in catalytic enzymes, assists in protein transport, and performances as transcription flag for some genes [14]. Elicitation of CuSO₄ increases the phytochemicals like bacoside, betacyanin, and phenylpropanoids on in vitro tissue cultures of Bacopa monnieri [15], Alternanthera philoxeroides [16] and Ocimum basilicum [14]. Due to its essential role in the growth and a stimulatory effect on phytochemical production, Cu has received noticeable attention.

Nanotechnology is an advanced technology that nearly found implications in every field of science. Modern growths in nanotechnology and extensive uses of engineered nanoparticles (ENPs) have advanced the area of biotechnology. The manufacture of ENPs is expected to upsurge from 0.27 million tons (2012) to 1.663 million tons by 2020 due to its extensive uses in various field science and technology [17]. The nanotechnology produces huge benefits to agriculture field for controlling diseases and crops production [18]. Nanomaterials with a dimension between 1 and 100 nm are often commercial uses. Recently with the development in the area of nanotechnology, researchers are now concentrating on using the nanomaterials as an elicitor to evaluate the stress responses in different economically and medicinally important plants. Currently, NPs extensively used as abiotic elicitors in the field of plant biotechnology to enhance the production of valuable bioactive compounds [19]. Metallic elements the cellular responses are very different when induced by ionic forms compared to the nano-metric forms [20]. In modern times, there has been increasing use of nanoscale nourishments and insecticides in agriculture, and copper-containing nano-pesticides are one of the most famous products on the
market because of their excellent antimicrobial activities [21]. The application of copper oxide nanoparticles (CuO NPs) increases the yield and fruit quality of the tomato [22] and pepper [23]. Copper, silver and gold nanoparticles improved the accumulation of phenolics, flavonoids, and protein in callus cultures of Prunella vulgaris [24,25]. Limited evidence is available on the influence of CuO NPs on plants. However promising results on the use of CuO NPs were reported recently for some plants such as Mentha longifolia [26], Verbena bipinnatifida [27], Ocimum basilicum [28], and Chinese cabbage [29]. However, hitherto there are no reports on the impacts of CuO NPs on the CSC of G. sylvestre. Therefore, this work aimed to study the influence of CuO NPs on the level of phytochemicals (GA and PC) production and also evaluate the antioxidant, antidiabetic, antibacterial, antifungal, anti-inflammatory and anticancer activities.

2. Materials and Methods

2.1. Cell Suspension Cultures

G. sylvestre seeds were disinfected with 2% NaOCl solution for 20 minutes, 0.5% HgCl2 solution for 5 minutes finally, cleaning with germ-free deionized water for five times. Disinfected seeds were grown in MS [30] medium containing 8.0 g/L phytoagar and 30 g/L sucrose. Cultures were placed in a plant growth chamber at a 24 ± 2 °C with16/8-h light/dark for 4-weeks. Leaf segments were incubated on a medium containing 2,4-D (0.5 – 3.0 mg/L) combined with 0.1 mg/L KIN for callus development. The cultures were kept in a plant growth chamber at a 24 ± 2 °C with16/8-h light/dark for 21 days. Friable callus (1 g) was cultured in conical flasks containing MS basal salt solution with 0.1 mg/L KIN and 2.0 mg/L 2,4-D for initiation of CSC. These flasks were aerated in a shaking incubator (110 rpm) in 16/8 h light and
dark at 25 ± 2 °C. The growth of cell suspensions in terms of fresh mass (FM), dry mass, and amounts of phytochemical contents were evaluated after 6, 12, 18 and 24 days of culture.

2.2. Elicitations of CuO NPs in CSC

CuO NPs size at 25–55 nm, (99%) was procured from US Research Nanomaterials, Inc., Houston, TX, USA. Eighteen days old CSC was treated with 0, 1, 3 or 5 mg/L CuO NPs for two days and subcultured into the fresh media lacking CuO NPs. CSC was cultured on a rotatory shaker at 110 rpm in 16/8 h light and dark at 25 ± 2 °C. The cells were collected from the medium for fresh and dry mass determined at 23 days. CSC collected from the medium through filter washed with sterile deionized H₂O and blotted on a germ-free filter paper (Whatman#1) to remove the water drops before FM determinations, and filtered cells were lyophilized as dry mass (DM). *G. sylvestre* cells (50 mg DM) harvested from each treatment were treated with 70% nitric acid for 1 h at 115 °C. Then, the samples were diluted with HPLC water, filtered into Eppendorf tube (1.5 mL) using nylon filters (0.2-µm) and used for ICP-MS (Varian 820-MS, USA) analysis.

2.3. Extraction and Estimation of Gymnemic Acid (GA) in CSC

CSC (500 mg DM) was extracted using an equal volume of EtOH and H₂O, and 10 mL of KOH solution (12%) was added and refluxed. After 1 h, 11 mL of HCl (4 N) was added at room temperature and then refluxed. After 1 h, samples were filtered using nylon filters (0.22 µM) and used for high-performance liquid chromatography (HPLC). HPLC (Waters, Milford, USA) was equipped with a variable dual Waters 2487 PDA (Photo Diode Array) detector and wavelength at 210 nm. Separations were done with C18 (5 µm, 2.1 mm × 100 mm) column with ACN: H₂O
(80:20) at a flow rate of 1 mL/min at 27 ºC [4]. The GA standard was acquired from Chromadex, USA. The conversion of gymnemagenin to GA was calculated using the formula, Molecular mass conversion of gymnemagenin to gymnemic acid (809.0/506.7) = conversion [4].

2.4. Total Phenolic and Flavonoids in CSC

A spectrophotometric method using Folin–Ciocalteu (FC) reagent was followed to determine the total phenolic content (TPC). CuO NPs treated and non-treated CSC samples (100 μL, 100 mg/mL) were added with FC reagent followed by 15% sodium carbonate. The absorbance was read at 755 nm. Total flavonoid content (TFC) of CuO NPs treated and non-treated CSC samples (100 μL, 100 mg/mL) were measured using the calorimetric method [31].

2.5. Pharmaceutical Activities in CSC

2.5.1. Antioxidant Activity

CuO NPs treated and non-treated CSC extracts were done the antioxidant activity. The radical scavenging activity was assessed by DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay [25,32]. The reducing potential of *G. sylvestre* cell extracts was determined based on the previously published protocols [25,33]. The total antioxidant potential of extracts was assessed using the phosphomolybdenum method, as formerly reported [25].

2.5.2. Antidiabetic Activity

CuO NPs treated and non-treated CSC extracts were carried out antidiabetic activity. Alpha-amylase inhibition assay was completed by the dinitroalicylic acid method [25,34]. Nonenzymatic glycosylation of hemoglobin activity was estimated as described earlier [34].
2.5.3. Anti-inflammatory Activity

CuO NPs treated and non-treated CSC extracts were achieved anti-inflammatory activity. The lipoygenase activity was estimated according to Shah et al. 2013 [35]. Inhibition of albumin denaturation was determined according to the method [36].

2.5.4. Antibacterial and Antifungal Activities

CuO NPs treated and non-treated CSC extracts were examined for their antimicrobial activity using a described procedure [25,29]. The microorganisms of Staphylococcus aureus, Bacillus subtilis (Gram-positive) and Pseudomonas aeruginosa, Escherichia coli (Gram-negative bacteria) and fungus (Aspergillus niger, Fusarium oxysporum, and Candida albicans) using disc diffusion method [25,29].

2.5.5. Anticancer Activity

The cancer cell lines (HT-29 and the MCF-7) were treated with 12.5-200 μg/L G. sylvestre cell extracts, and cytotoxicity of CuO NPs treated and non-treated CSC extracts were evaluated by MTT assay [25].

2.6. Statistical Analysis

All the tests were performed in triplicate (n = 3) as mean ± SD and followed by DMRT and significance was determined at P≤0.05 level.

3. Results and Discussion
3.1. Gymnemic Acid (GA) Content in Cell Suspension Cultures (CSC)

Callus formation was noticed in all studied media. The callus induction was considerably improved with increasing the levels of 2,4-D up to 2.0 mg/L. However, it was dropped when the 2,4-D greater than 2.0 mg/L. The best callus initiation frequency (94.66%) was attained on MS medium with 2.0 mg/L 2,4-D and 0.1 mg/L KIN (Fig. 1a). Similarly, the combination of 2,4-D and KIN enhanced the callus production in G. sylvestre [37]. The maximum accumulation of biomass was recorded at 18 days (12.26 g of dry cell mass (DCM) and GA production (10.20 mg/g DCM) (Fig. 1b). Succeeding these time passage, it was perfect that the biomass growth closely correlated with GA accumulation. Parallel results were attained during the CSC of Polygonum multiflorum for the production of anthraquinones [38].

3.2. Biomass, Copper and GA content in CuO NPs treated and non-treated CSC

The Cu level of CuO NPs treated and non-treated CSC was analyzed by ICP-MS. The upsurge in Cu accumulation was noted when the cells were exposed with CuO NPs. The Cu level was higher (1.1 mg/L) in 5 mg/L CuO NPs treated CSC (Fig. 2a). Similarly, copper accumulation was enhanced at greater amount of CuO NPs in B. rapa [29]. However, the greatest 14.51g DCM was obtained on medium with 1 mg/L CuO NPs. Increasing the level of CuO NPs above 1 mg/L decreased the DCM (Fig. 2b). Similar pattern observation was also noted in the CSC of bitter gourd after AgNPs elicitation. By our results, a higher dose of CuO NPs decreased both FM and DM of hairy root culture in Chinese cabbage [25]. Cu has been studied as a stimulatory element for in vitro culture in many plants [39]. Previous studies reported that CuO NPs functions as a stimulator of phytochemical productions of in vitro culture in Stevia rebaudiana [40]. We investigated the impact of CuO NPs on the GA content in CSC
was identified by HPLC. The content of GA in elicited CSC was significantly higher (4 to 9-fold) than non-elicited CSC (Fig. 2b). An increase of nine fold increments of GA (89.25 mg/g DCM) was evident with 3 mg/L CuO NPs (Fig. 2b). However, a high dose of CuO NPs inhibited the synthesis of GA (Fig. 2b). The abiotic elicitation of G. sylvestre with cadmium chloride was produced the maximum amount of 59.97 mg/g DCW of GA, i.e., a 6.8-fold increase in comparison to the non-treated CSC [12]. GA was improved in the CSC of G. sylvestre by using signaling molecules such as methyl jasmonate and salicylic acid [41]. An upsurge of 7.78 fold increment of GA was apparent with linolenic acid treatment compared to that of the non-elicited roots of G. sylvestre [4]. It can be concluded that CuO NPs are effective in improving the production of GA when compared to metal salts and signaling molecules in the CSC of G. sylvestre.

3.3 Total Phenolic Compounds and Flavonoids in CSC

Several studies reported the inclusion of an abiotic or biotic elicitor to the growth medium notably upsurge the synthesis of bioactive compounds by activating a defense system. We investigated the impact of CuO NPs on the accumulation of total phenolics and flavonoids (TPC and TFC) in CSC of G. sylvestre. In the current investigation, it was found that CuO NPs (3.0 mg/L) yielded 245.10 mg/g DCM TPC and 4.57 mg/g DCM TFC (Fig. 3a, b). However, CSC elicited with 5 mg/L of CuO NPs decrease the amount of TPC and TFC. In Brassica rapa, TPC and TFC in hairy roots were improved by the elicitation CuO NPs [29] and AgNPs in CSC of bitter gourd [25]. CuO NPs have a useful role, and it is mainly due to the accumulation of Cu in cells and play a key role in plant biochemistry [42]. Elicitation with SiO₂ NPs elevates the PC accumulation in hairy roots of Dracocephalum kotschyi [43]. The mechanism of elicitor’s impact
on cell, organ, and plant is not well-documented; however, biotic or abiotic elicitors easily attack
the cell wall receptors and therefore, triggered the defense signals like electrolyte leakage,
oxidative burst, active oxygen specious, phosphorylation and dephosphorylation of proteins in
response to the stimulator. Thus, phytoalexins like PC by secondary messenger molecules
include H$_2$O$_2$ were increased. The over-expression of PAL (defense-gene) leads to the enhanced
production of PC [44]. Elicitation of magnetite nanoparticles and static magnetic field elicitation
has increased the content of PC, polyphenol oxidase, and PAL activities in the CSC of
Dracocephalum polychaetum [9]. Our results propose that CuO NPs treatment improved the
amount of TPC and TFC in CSC of G. sylvestre.

3.4. Antioxidant Activity

The main anti-oxidant compounds are bioactive compounds, whose production upsurges
when a particular factor in the media is changed, this factor cause’s stress, which leads to
improve the accumulation of bioactive compounds such as GA, phenols, and flavonoids.
Previously reported that the scavenging activity (57.10%), reducing potential absorbance at 0.15
and antioxidant capacity was found to be 17.54 mg/g expressed in G. sylvestre leaf extracts [46].
The phenolic and flavonoids were significantly greater in CSC treated with CuO NPs, that
correlated with their antioxidant activity (Fig 4). The free radical scavenging activity of the CSC
extracts was confirmed through DPPH assay. Of the different concentrations of CuO NPs treated
CSC, 3 mg/L CuO NPs treated CSC extract was displayed the highest antioxidant activity of
86.15% as compared to the control (72.62%) (Fig. 4a). The CuO NPs elicited CSC exhibited the
highest reducing potential compared with non-elicited CSC (Fig. 4b). The reducing potential is
commonly connected with the number of reductones in the test samples [4]. The chelating
capacity is essential to determine the antioxidant power of crude extract or single compound because it decreases the number of metal ions by catalyzing MDA [45]. Figure 4c depicts the antioxidant activity of the CuO NPs (3 mg/L) elicited CSC was higher (80.55 mg/g) than non-elicited CSC (69.50 mg/g). Consistent with our study, elicitation of polyunsaturated fatty acids triggered the antioxidant defense system and also a substantial quantity of GA and PC in hairy root cultures of *G. sylvestre* [4]. Correspondingly, CuO NPs elicitation in hairy roots of Chinese cabbage [29] and regenerated shoots of *Stevia rebaudiana* [40] improved their antioxidant potential.

### 3.5. Antidiabetic Activity

Oxidative stress in the body is one of the most serious providers to the occurrence of diabetes. The free radical scavengers are used to manage the oxidative damage and to constrain the enzymes like α-amylase and α-glucosidase which are responsible for the cause of diabetes [47]. The inhibition of carbohydrate-hydrolyzing enzymes such as α-amylase can be an important approach to lower postprandial blood glucose levels. In this study, the activity of the α-amylase enzyme was expressively inhibited in CuO NPs-elicited CSC extracts (Fig. 5a). The results displayed that α-amylase was dramatically suppressed in a dose-dependent manner after incubation with different amount of extracts. CuO NPs-elicited CSC extracts (100 μg/mL) 86.75% and non-elicited CSC 70.50% of alpha-amylase enzyme inhibition (Fig. 5a). In the meantime, acarbose exhibited 92.00% of inhibition (Fig. 5a). GA fraction showed a significant reduction in amylase activity (14.25%) of *G. sylvestre* leaves [48]. The increase in the non-enzymatic glycosylation of hemoglobin in CuO NPs treated and non-treated CSC extracts. The inhibition of glycosylation was concentration-dependent increases in seen with CuO NPs treated and non-
treated CSC extracts, and tocopherol, which was used as a standard. The inhibition of
glycosylation in CuO NPs treated (3 mg/L) and non-treated CSC extracts were 90.50% and
75.25%, respectively. An effective treatment for diabetes can be given by using the bioactive
elements present in the extracts of the plants. GA obtained from G. sylvestre not only inhibits
glucose absorption in the small intestine but also suppresses hyperglycemia and
hyperinsulinemia in an oral glucose tolerance test. The efficiency of GA in inhibiting glucose
absorption in the small intestine was found to be increased by a combined effect with acarbose
and voglibose [49]. GA and PC have potent inhibitory effects on α-amylase and α-glucosidase
[49,50].

3.6. Anti-inflammatory Activity

Inflammation is a biological reaction triggered by the disturbance of the tissue homeostasis,
occurring in response to the presence of a biological, chemical, or physical agent in the body. PC
that can interfere with these mechanisms by preventing a prolonged inflammation could be
useful for human health [45]. The PC frequently reduced the inflammatory process by altering
cyclooxygenase and lipoxygenase activities. Lipoxygenase inhibitors are involved in various
inflammatory diseases like cancer, asthma, leukemia, lymphoma, autoimmune disorders and
increase the immune response to viral and bacterial infections [35]. The anti-inflammatory
capacity was evaluated by lipoxygenase activity (Fig. 5c). CuO NPs-elicited CSC extracts
showed maximum activity which is about 75.55% higher activity than that of non-elicited CSC
extracts 63.00% inhibition. The inhibitory effects PC on 15-lipoxygenase and lipoxygenase have
been well documented [51]. Denaturation of protein molecules is well recognized in the literature,
and it is due to an inflammation process in conditions like arthritis. Inhibition of protein
disruption might be responsible for the anti-rheumatic activity of nonsteroidal anti-inflammatory drugs [52]. CuO NPs-elicited CSC extracts can inhibit the membrane stabilization 86.25%, and it is near to standard aspirin 90.75% (Fig. 5d). CuO NPs-elicited CSC extracts strongly inhibited the denaturation of protein in membrane stabilization test. Flavonoids could constrain enzymes, along with reactive C protein or adhesion molecules [51].

3.7. Antibacterial and Antifungal Activities

Antimicrobial activity of *G. sylvestre* was evaluated against various pathogens, specifically, *B. subtilis, S. aureus, E. coli* and while no activity was noted against gram-negative bacteria [53]. The antimicrobial properties of GA and PC against pathogenic bacteria and fungus have been reported [25,54]. Figure 6 shows that the CuO NPs treated CSC extracts of *G. sylvestre* showed strong antibacterial and antifungal activities compared to non-treated CSC extracts. CuO NPs treated CSC extracts showed more distinct activities against Gram-positive than Gram-negative bacteria. These results are expected to due to the absence of lipopolysaccharide membrane surrounding the cell wall of Gram-positive bacteria allowing increased permeability of *Hypericum* antimicrobial metabolites into cells [55]. Earlier, AgNPs-treated plants and CuO NPs-elicited hairy root extracts had greater antimicrobial activities in *Achillea millefolium* [56] and Chinese cabbage [29]. The leaf extracts of *G. sylvestre* exhibited good antimicrobial activity [54]. GA and PC have potent antimicrobial activities [25,54]. Our data confirm that the CuO NPs treated CSC extracts have shown potent antimicrobial activity against clinically relevant microorganisms.

3.8. Anticancer Activity
GA, saponins and PC could be used for anticancer activities [25,57]. An earlier study has demonstrated that *G. sylvestre* extracts against the human lung adenocarcinoma and breast carcinoma cell lines [58]. Screening cytotoxic activity of the CuO NPs treated and non-treated CSC extracts against cancer cells (MCF-7, and HT-29) were investigated. The cancer cells were exposed to various levels of CuO NPs treated and non-treated CSC extracts. The cancer inhibition percentage increased with increasing concentrations of CSC extracts (Fig. 7). The greater inhibition was noted at 200 μg/mL of CSC extracts (Fig. 7a, b), at which the CuO NPs treated CSC extracts exhibited high cancer inhibition whereas the non-treated CSC extracts displayed less inhibition. Similarly, *Gymnema sylvestre* and *Eclipta prostrata* extracts obtained from the AgNPs treated plants were also displayed higher cytotoxicity against HeLa cells [57]. This high cytotoxic activity in CuO NPs-elicited CSC may be due to the high amount of GA and PC. In agreement with various earlier studies which confirmed that the CuO NPs-elicited hairy roots displayed higher cytotoxic activities than non-elicited hairy roots [29].

4. Conclusions

CuO NPs used as abiotic elicitors for improved biomass and bioactive compounds (GA and PC) production in CSC of *G. sylvestre*. In addition, antioxidant, antidiabetic, anti-inflammatory, antibacterial, antifungal and anticancer activities were also increased in CuO NPs-elicited CSC extracts. This investigation will deliver a reference for forthcoming studies on the potential relation of these bioactive compounds to plant abiotic stress response in *G. sylvestre*. Therefore, our protocol could be useful for the industrial production of GA and PC and their uses for pharmaceutical activities concerned with significant health benefits using cell suspension cultures of *G. sylvestre*. 
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Conflicts of Interest: The authors declare no conflict of interest.

References


Fig. 1 a. Effect of different concentrations of 2,4-D in combination with 0.1 mg/L KIN for callus induction in *G. sylvestre*, b. Biomass accumulation and gymnemic acid production on MS liquid medium with 2,4-D (2.0 mg/L) and KIN (0.1 mg/L) and sucrose (30 g/L) at different growth period. Different letters indicate a significant difference at $P \leq 0.05$. 
Fig. 2 Effect of CuO NPs on copper content, biomass accumulation and gymnemic acid (GA) production in *G. sylvestre.* a. Copper content, b. Biomass accumulation and GA production. Different letters indicate a significant difference at $P \leq 0.05$. 
Fig. 3 Effect of CuO NPs on total phenolic and flavonoid contents (TPC and TFC) in cell suspension cultures of *G. sylvestre*. a. TPC, b. TFC. Different letters indicate a significant difference at $P \leq 0.05$. 
Fig. 4. Effect of CuO NPs on antioxidant activities in cell suspension culture of *G. sylvestre*. a. Free radical-scavenging activity by DPPH method, b Total Fe$^{3+}$– Fe$^{2+}$ reductive potential reference antioxidants (butylated hydroxytoluene), c Total antioxidant capacity (TAC) by phosphomolybdenum method [TAC was expressed as equivalents of α-tocopherol (μg/g of extract)]. Different letters indicate a significant difference at $P \leq 0.05$. 
Fig. 5 Effect of CuO NPs on antidiabetic and anti-inflammatory activities cell suspension cultures of *G. sylvestre*. **a.** In vitro α-amylase activity, **b.** Non-enzymatic glycosylation of hemoglobin activity, **c.** Lipoxygenase inhibition activity, **d.** Albumin denaturation inhibition assay. Different letters indicate a significant difference at $P \leq 0.05$. 

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Fig. 6 Effect of CuO NPs on antimicrobial activity in cell suspension cultures of *G. sylvestre* using disc diffusion method. Different letters indicate a significant difference at *P* ≤ 0.05.
**Fig. 7** Effect of CuO NPs on cell viability of MCF-7 and HT-29 cell lines in cell suspension cultures of *G. sylvestre*. **a.** MCF-7, **b.** HT-29. Different letters indicate a significant difference at $P \leq 0.05$. 

![Graph showing inhibition (%) vs. Concentration (µg/L) for different treatments.](image-url)