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

4

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

35

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

38

39 1. Introduction

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40 Homeopathic plants are the ideal source of lifespan saving medicines for the majority of the
41 peoples in the world. *Gymnema sylvestre* (Retz.) R. Br. (Family: Asclepiadaceae) is a valuable
42 medicinal climber and possesses various bioactive compounds like gymnemic acid (GA), and
43 phenolic compounds (PC) which are used in the therapy of diabetes mellitus [1]. This plant has
44 been used for tea beverages and confection industries and has also been useful in numerous food
45 preparations for the regulation of sugar homeostasis and the control of fatness and blood
46 cholesterol levels [2]. GA has been used for pharmacological potential such as antimicrobial,
47 antihypercholesterolemic, hepatoprotective and anti-saccharine activities [3]. GA has been
48 documented for its role in selectively suppressing sweet flavor feelings in humans. The leaves
49 contain triterpenoid saponins such as oleanane (GA), and dammarane (gymnemasides) classes
50 are primarily considered the active constituents [4]. To avoid abolishing the valuable medicinal
51 plant, the *in vitro* plant tissue or cell suspension cultures (CSCs) represent a good alternative for
52 biotechnological applications. Tissue culture or CSCs perform talented for obtaining secondary
53 metabolites, as they produce high yields of phytochemical production [5]. Elicitation is one of
54 the most effective biotechnological approaches for increasing the production of phytochemicals
55 in tissue culture or CSCs [6]. Elicitors are biological and non-biological molecules to which
56 plant receptors on the cytoplasmic membrane react, and thus, plant cells produce a signal that
57 stimulates the gene's expression within the pathway, which causes the plant's secondary
58 metabolites to synthesize. Elicitors can stimulate defense response to defend the cell, tissue,
59 organ, and plant, which is frequently realized by the alteration of various biosynthetic pathways.
60 Consequently, the production of preferred phytochemicals needs external stimuli also referred to
61 as elicitors [7]. Studies on the enhanced accumulation of secondary metabolites by the addition
62 of elicitors to the CSCs is limited [8,9]. Application of elicitors for improvement of GA from *G.*

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63 *sylvestre* is well accomplished by using callus and CSC methods [10,11,12]. Copper (Cu) is a
64 vital micronutrient for plant development, existence a co-factor in various physiological
65 processes for example cell wall metabolism, photosynthesis, and lignification. However, it is
66 toxic at higher levels [13]. Cu is also present in catalytic enzymes, assists in protein transport,
67 and performances as transcription flag for some genes [14]. Elicitation of $CuSO_4$ increases the
68 phytochemicals like bacoside, betacyanin, and phenylpropanoids on *in vitro* tissue cultures of
69 *Bacopa monnieri* [15], *Alternanthera philoxeroides* [16] and *Ocimum basilicum* [14]. Due to its
70 essential role in the growth and a stimulatory effect on phytochemical production, Cu has
71 received noticeable attention.

72 Nanotechnology is an advanced technology that nearly found implications in every field of
73 science. Modern growths in nanotechnology and extensive uses of engineered nanoparticles
74 (ENPs) have advanced the area of biotechnology. The manufacture of ENPs is expected to
75 upsurge from 0.27 million tons (2012) to 1.663 million tons by 2020 due to its extensive uses in
76 various field science and technology [17]. The nanotechnology produces huge benefits to
77 agriculture field for controlling diseases and crops production [18]. Nanomaterials with a
78 dimension between 1 and 100 nm are often commercial uses. Recently with the development in
79 the area of nanotechnology, researchers are now concentrating on using the nanomaterials as an
80 elicitor to evaluate the stress responses in different economically and medicinally important
81 plants. Currently, NPs extensively used as abiotic elicitors in the field of plant biotechnology to
82 enhance the production of valuable bioactive compounds [19]. Metallic elements the cellular
83 responses are very different when induced by ionic forms compared to the nano-metric forms
84 [20]. In modern times, there has been increasing use of nanoscale nourishments and insecticides
85 in agriculture, and copper-containing nano-pesticides are one of the most famous products on the

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86 market because of their excellent antimicrobial activities [21]. The application of copper oxide
87 nanoparticles (CuO NPs) increases the yield and fruit quality of the tomato [22] and pepper [23].
88 Copper, silver and gold nanoparticles improved the accumulation of phenolics, flavonoids, and
89 protein in callus cultures of *Prunella vulgaris* [24,25]. Limited evidence is available on the
90 influence of CuO NPs on plants. However promising results on the use of CuO NPs were
91 reported recently for some plants such as *Mentha longifolia* [26], *Verbena bipinnatifida* [27],
92 *Ocimum basilicum* [28], and Chinese cabbage [29]. However, hitherto there are no reports on the
93 impacts of CuO NPs on the CSC of *G. sylvestre*. Therefore, this work aimed to study the
94 influence of CuO NPs on the level of phytochemicals (GA and PC) production and also evaluate
95 the antioxidant, antidiabetic, antibacterial, antifungal, anti-inflammatory and anticancer activities.
96

97 **2. Materials and Methods**

98 *2.1. Cell Suspension Cultures*

99 *G. sylvestre* seeds were disinfected with 2% NaOCl solution for 20 minutes, 0.5% $HgCl_2$
100 solution for 5 minutes finally, cleaning with germ-free deionized water for five times.
101 Disinfected seeds were grown in MS [30] medium containing 8.0 g/L phytoagar and 30 g/L
102 sucrose. Cultures were placed in a plant growth chamber at a 24 ± 2 ℃ with16/8-h light/dark for
103 4-weeks. Leaf segments were incubated on a medium containing 2,4-D (0.5 – 3.0 mg/L)
104 combined with 0.1 mg/L KIN for callus development. The cultures were kept in a plant growth
105 chamber at a 24 ± 2 ℃ with16/8-h light/dark for 21 days. Friable callus (1 g) was cultured in
106 conical flasks containing MS basal salt solution with 0.1 mg/L KIN and 2.0 mg/L 2,4-D for
107 initiation of CSC. These flasks were aerated in a shaking incubator (110 rpm) in 16/8 h light and

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108 dark at 25 ± 2 °C. The growth of cell suspensions in terms of fresh mass (FM), dry mass, and
109 amounts of phytochemical contents were evaluated after 6, 12, 18 and 24 days of culture.
110

111 *2.2. Elicitations of CuO NPs in CSC*

112 CuO NPs size at 25–55 nm, (99%) was procured from US Research Nanomaterials, Inc.,
113 Houston, TX, USA. Eighteen days old CSC was treated with 0, 1, 3 or 5 mg/L CuO NPs for two
114 days and subcultured into the fresh media lacking CuO NPs. CSC was cultured on a rotatory
115 shaker at 110 rpm in 16/8 h light and dark at 25 ± 2 °C. The cells were collected from the
116 medium for fresh and dry mass determined at 23 days. CSC collected from the medium through
117 filter washed with sterile deionized H_2O and blotted on a germ-free filter paper (Whatman#1) to
118 remove the water drops before FM determinations, and filtered cells were lyophilized as dry
119 mass (DM). *G. sylvestre* cells (50 mg DM) harvested from each treatment were treated with 70%
120 nitric acid for 1 h at 115 °C. Then, the samples were diluted with HPLC water, filtered into
121 Eppendorf tube (1.5 mL) using nylon filters (0.2-µm) and used for ICP-MS (Varian 820-MS,
122 USA) analysis.
123

124 *2.3. Extraction and Estimation of Gymnemic Acid (GA) in CSC*

125 CSC (500 mg DM) was extracted using an equal volume of EtOH and H_2O, and 10 mL of
126 KOH solution (12%) was added and refluxed. After 1 h, 11 mL of HCl (4 N) was added at room
127 temperature and then refluxed. After 1 h, samples were filtered using nylon filters (0.22 µM) and
128 used for high-performance liquid chromatography (HPLC). HPLC (Waters, Milford, USA) was
129 equipped with a variable dual Waters 2487 PDA (Photo Diode Array) detector and wavelength at
130 210 nm. Separations were done with C18 (5 µm, 2.1 mm × 100 mm) column with ACN: H2O

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131 (80:20) at a flow rate of 1 mL/min at 27 ℃ [4]. The GA standard was acquired from Chromadex,
132 USA. The conversion of gymnemagenin to GA was calculated using the formula, Molecular
133 mass conversion of gymnemagenin to gymnemic acid (809.0/506.7) = conversion [4].
134
135 *2.4. Total Phenolic and Flavonoids in CSC*
136 A spectrophotometric method using Folin−Ciocalteu (FC) reagent was followed to determine
137 the total phenolic content (TPC). CuO NPs treated and non-treated CSC samples (100 µL, 100
138 mg/mL) were added with FC reagent followed by 15% sodium carbonate. The absorbance was
139 read at 755 nm. Total flavonoid content (TFC) of CuO NPs treated and non-treated CSC samples
140 (100 µL, 100 mg/mL) were measured using the calorimetric method [31].
141
142 *2.5. Pharmaceutical Activities in CSC*
143 *2.5.1. Antioxidant Activity*
144 CuO NPs treated and non-treated CSC extracts were done the antioxidant activity. The radical
145 scavenging activity was assessed by DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay [25,32]. The
146 reducing potential of *G. sylvestre* cell extracts was determined based on the previously published
147 protocols [25,33]. The total antioxidant potential of extracts was assessed using the
148 phosphomolybdenum method, as formerly reported [25].
149
150 *2.5.2. Antidiabetic Activity*
151 CuO NPs treated and non-treated CSC extracts were carried out antidiabetic activity. Alpha-
152 amylase inhibition assay was completed by the dinitrosalicylic acid method [25,34]. Non-
153 enzymatic glycosylation of hemoglobin activity was estimated as described earlier [34].

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154

155 *2.5.3. Anti-inflammatory Activity*

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

159

160 *2.5.4. Antibacterial and Antifungal Activities*

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

166

167 *2.5.5. Anticancer Activity*

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

171

172 *2.6. Statistical Analysis*

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

175

176 **3. Results and Discussion**

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177 *3.1. Gymnemic Acid (GA) Content in Cell Suspension Cultures (CSC)*

178 Callus formation was noticed in all studied media. The callus induction was considerably
179 improved with increasing the levels of 2,4-D up to 2.0 mg/L. However, it was dropped when the
180 2,4-D greater than 2.0 mg/L. The best callus initiation frequency (94.66%) was attained on MS
181 medium with 2.0 mg/L 2,4-D and 0.1 mg/L KIN (Fig. 1a). Similarly, the combination of 2,4-D
182 and KIN enhanced the callus production in *G. sylvestre* [37]. The maximum accumulation of
183 biomass was recorded at 18 days (12.26 g of dry cell mass (DCM) and GA production (10.20
184 mg/g DCM) (Fig. 1b). Succeeding these time passage, it was perfect that the biomass growth
185 closely correlated with GA accumulation. Parallel results were attained during the CSC of
186 *Polygonum multiflorum* for the production of anthraquinones [38].

187

188 *3.2. Biomass, Copper and GA content in CuO NPs treated and non-treated CSC*

189 The Cu level of CuO NPs treated and non-treated CSC was analyzed by ICP-MS. The
190 upsurge in Cu accumulation was noted when the cells were exposed with CuO NPs. The Cu level
191 was higher (1.1 mg/L) in 5 mg/L CuO NPs treated CSC (Fig. 2a). Similarly, copper
192 accumulation was enhanced at greater amount of CuO NPs in *B. rapa* [29]. However, the
193 greatest 14.51g DCM was obtained on medium with 1 mg/L CuO NPs. Increasing the level of
194 CuO NPs above 1 mg/L decreased the DCM (Fig. 2b). Similar pattern observation was also
195 noted in the CSC of bitter gourd after AgNPs elicitation. By our results, a higher dose of CuO
196 NPs decreased both FM and DM of hairy root culture in Chinese cabbage [25]. Cu has been
197 studied as a stimulatory element for *in vitro* culture in many plants [39]. Previous studies
198 reported that CuO NPs functions as a stimulator of phytochemical productions of *in vitro* culture
199 in *Stevia rebaudiana* [40]. We investigated the impact of CuO NPs on the GA content in CSC

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200 was identified by HPLC. The content of GA in elicited CSC was significantly higher (4 to 9-fold)
201 than non-elicited CSC (Fig. 2b). An increase of nine fold increments of GA (89.25 mg/g DCM)
202 was evident with 3 mg/L CuO NPs (Fig. 2b). However, a high dose of CuO NPs inhibited the
203 synthesis of GA (Fig. 2b). The abiotic elicitation of *G. sylvestre* with cadmium chloride was
204 produced the maximum amount of 59.97 mg/g DCW of GA, i.e., a 6.8-fold increase in
205 comparison to the non-treated CSC [12]. GA was improved in the CSC of *G. sylvestre* by using
206 signaling molecules such as methyl jasmonate and salicylic acid [41]. An upsurge of 7.78 fold
207 increment of GA was apparent with linolenic acid treatment compared to that of the non-elicited
208 roots of *G. sylvestre* [4]. It can be concluded that CuO NPs are effective in improving the
209 production of GA when compared to metal salts and signaling molecules in the CSC of *G.*
210 *sylvestre*.
211
212 *3.3 Total Phenolic Compounds and Flavonoids in CSC*
213 Several studies reported the inclusion of an abiotic or biotic elicitor to the growth medium
214 notably upsurge the synthesis of bioactive compounds by activating a defense system. We
215 investigated the impact of CuO NPs on the accumulation of total phenolics and flavonoids (TPC
216 and TFC) in CSC of *G. sylvestre*. In the current investigation, it was found that CuO NPs (3.0
217 mg/L) yielded 245.10 mg/g DCM TPC and 4.57 mg/g DCM TFC (Fig. 3a, b). However, CSC
218 elicited with 5 mg/L of CuO NPs decrease the amount of TPC and TFC. In *Brassica rapa*, TPC
219 and TFC in hairy roots were improved by the elicitation CuO NPs [29] and AgNPs in CSC of
220 bitter gourd [25]. CuO NPs have a useful role, and it is mainly due to the accumulation of Cu in
221 cells and play a key role in plant biochemistry [42]. Elicitation with SiO_2 NPs elevates the PC
222 accumulation in hairy roots of *Dracocephalum kotschyi* [43]. The mechanism of elicitor's impact

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223 on cell, organ, and plant is not well-documented; however, biotic or abiotic elicitors easily attack
224 the cell wall receptors and therefore, triggered the defense signals like electrolyte leakage,
225 oxidative burst, active oxygen specious, phosphorylation and dephosphorylation of proteins in
226 response to the stimulator. Thus, phytoalexins like PC by secondary messenger molecules
227 include H_2O_2 were increased. The over-expression of *PAL* (defense-gene) leads to the enhanced
228 production of PC [44]. Elicitation of magnetite nanoparticles and static magnetic field elicitation
229 has increased the content of PC, polyphenol oxidase, and PAL activities in the CSC of
230 *Dracocephalum polychaetum* [9]. Our results propose that CuO NPs treatment improved the
231 amount of TPC and TFC in CSC of *G. sylvestre*.
232
233 *3.4. Antioxidant Activity*
234 The main anti-oxidant compounds are bioactive compounds, whose production upsurges
235 when a particular factor in the media is changed, this factor cause's stress, which leads to
236 improve the accumulation of bioactive compounds such as GA, phenols, and flavonoids.
237 Previously reported that the scavenging activity (57.10%), reducing potential absorbance at 0.15
238 and antioxidant capacity was found to be 17.54 mg/g expressed in *G. sylvestre* leaf extracts [46].
239 The phenolic and flavonoids were significantly greater in CSC treated with CuO NPs, that
240 correlated with their antioxidant activity (Fig 4). The free radical scavenging activity of the CSC
241 extracts was confirmed through DPPH assay. Of the different concentrations of CuO NPs treated
242 CSC, 3 mg/L CuO NPs treated CSC extract was displayed the highest antioxidant activity of
243 86.15% as compared to the control (72.62%) (Fig. 4a). The CuO NPs elicited CSC exhibited the
244 highest reducing potential compared with non-elicited CSC (Fig. 4b). The reducing potential is
245 commonly connected with the number of reductones in the test samples [4]. The chelating

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246 capacity is essential to determine the antioxidant power of crude extract or single compound
247 because it decreases the number of metal ions by catalyzing MDA [45]. Figure 4c depicts the
248 antioxidant activity of the CuO NPs (3 mg/L) elicited CSC was higher (80.55 mg/g) than non-
249 elicited CSC (69.50 mg/g). Consistent with our study, elicitation of polyunsaturated fatty acids
250 triggered the antioxidant defense system and also a substantial quantity of GA and PC in hairy
251 root cultures of *G. sylvestre* [4]. Correspondingly, CuO NPs elicitation in hairy roots of Chinese
252 cabbage [29] and regenerated shoots of *Stevia rebaudiana* [40] improved their antioxidant
253 potential.
254

255 *3.5. Antidiabetic Activity*

256 Oxidative stress in the body is one of the most serious providers to the occurrence of diabetes.
257 The free radical scavengers are used to manage the oxidative damage and to constrain the
258 enzymes like α-amylase and α-glucosidase which are responsible for the cause of diabetes [47].
259 The inhibition of carbohydrate-hydrolyzing enzymes such as α-amylase can be an important
260 approach to lower postprandial blood glucose levels. In this study, the activity of the α-amylase
261 enzyme was expressively inhibited in CuO NPs-elicited CSC extracts (Fig. 5a). The results
262 displayed that α-amylase was dramatically suppressed in a dose-dependent manner after
263 incubation with different amount of extracts. CuO NPs-elicited CSC extracts (100 µg/mL) 86.75%
264 and non-elicited CSC 70.50% of alpha-amylase enzyme inhibition (Fig. 5a). In the meantime,
265 acarbose exhibited 92.00% of inhibition (Fig. 5a). GA fraction showed a significant reduction in
266 amylase activity (14.25%) of *G. sylvestre* leaves [48]. The increase in the non-enzymatic
267 glycosylation of hemoglobin in CuO NPs treated and non-treated CSC extracts. The inhibition of
268 glycosylation was concentration-dependent increases in seen with CuO NPs treated and non-

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269 treated CSC extracts, and tocopherol, which was used as a standard. The inhibition of
270 glycosylation in CuO NPs treated (3 mg/L) and non-treated CSC extracts were 90.50% and
271 75.25%, respectively. An effective treatment for diabetes can be given by using the bioactive
272 elements present in the extracts of the plants. GA obtained from *G. sylvestre* not only inhibits
273 glucose absorption in the small intestine but also suppresses hyperglycemia and
274 hyperinsulinemia in an oral glucose tolerance test. The efficiency of GA in inhibiting glucose
275 absorption in the small intestine was found to be increased by a combined effect with acarbose
276 and voglibose [49]. GA and PC have potent inhibitory effects on α-amylase and α-glucosidase
277 [49,50].
278
279 *3.6. Anti-inflammatory Activity*
280 Inflammation is a biological reaction triggered by the disturbance of the tissue homeostasis,
281 occurring in response to the presence of a biological, chemical, or physical agent in the body. PC
282 that can interfere with these mechanisms by preventing a prolonged inflammation could be
283 useful for human health [45]. The PC frequently reduced the inflammatory process by altering
284 cyclooxygenase and lipoxygenase activities. Lipoxygenase inhibitors are involved in various
285 inflammatory diseases like cancer, asthma, leukemia, lymphoma, autoimmune disorders and
286 increase the immune response to viral and bacterial infections [35]. The anti-inflammatory
287 capacity was evaluated by lipoxygenase activity (Fig. 5c). CuO NPs-elicited CSC extracts
288 showed maximum activity which is about 75.55% higher activity than that of non-elicited CSC
289 extracts 63.00% inhibition. The inhibitory effects PC on 15-lipoxygenase and lipoxygenase have
290 been well documented [51]. Denaturation of protein molecules is well recognized in the literature,
291 and it is due to an inflammation process in conditions like arthritis. Inhibition of protein

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292 disruption might be responsible for the anti-rheumatic activity of nonsteroidal anti-inflammatory
293 drugs [52]. CuO NPs-elicited CSC extracts can inhibit the membrane stabilization 86.25%, and it
294 is near to standard aspirin 90.75% (Fig. 5d). CuO NPs-elicited CSC extracts strongly inhibited
295 the denaturation of protein in membrane stabilization test. Flavonoids could constrain enzymes,
296 along with reactive C protein or adhesion molecules [51].
297
298 *3.7. Antibacterial and Antifungal Activities*
299 Antimicrobial activity of *G. sylvestre* was evaluated against various pathogens, specifically, *B.*
300 *subtilis, S. aureus*, *E. coli* and while no activity was noted against gram-negative bacteria [53].
301 The antimicrobial properties of GA and PC against pathogenic bacteria and fungus have been
302 reported [25,54]. Figure 6 shows that the CuO NPs treated CSC extracts of *G. sylvestre* showed
303 strong antibacterial and antifungal activities compared to non-treated CSC extracts. CuO NPs
304 treated CSC extracts showed more distinct activities against Gram-positive than Gram-negative
305 bacteria. These results are expected to due to the absence of lipopolysaccharide membrane
306 surrounding the cell wall of Gram-positive bacteria allowing increased permeability of
307 *Hypericum* antimicrobial metabolites into cells [55]. Earlier, AgNPs-treated plants and CuO
308 NPs-elicited hairy root extracts had greater antimicrobial activities in *Achillea millefolium* [56]
309 and Chinese cabbage [29]. The leaf extracts of *G. sylvestre* exhibited good antimicrobial activity
310 [54]. GA and PC have potent antimicrobial activities [25,54]. Our data confirm that the CuO NPs
311 treated CSC extracts have shown potent antimicrobial activity against clinically relevant
312 microorganisms.
313
314 *3.8. Anticancer Activity*

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315 GA, saponins and PC could be used for anticancer activities [25,57]. An earlier study has
316 demonstrated that *G. sylvestre* extracts against the human lung adenocarcinoma and breast
317 carcinoma cell lines [58]. Screening cytotoxic activity of the CuO NPs treated and non-treated
318 CSC extracts against cancer cells (MCF-7, and HT-29) were investigated. The cancer cells were
319 exposed to various levels of CuO NPs treated and non-treated CSC extracts. The cancer
320 inhibition percentage increased with increasing concentrations of CSC extracts (Fig. 7). The
321 greater inhibition was noted at 200 µg/mL of CSC extracts (Fig. 7a, b), at which the CuO NPs
322 treated CSC extracts exhibited high cancer inhibition whereas the non-treated CSC extracts
323 displayed less inhibition. Similarly, *Gymnema sylvestre* and *Eclipta prostrata* extracts obtained
324 from the AgNPs treated plants were also displayed higher cytotoxicity against HeLa cells [57].
325 This high cytotoxic activity in CuO NPs-elicited CSC may be due to the high amount of GA and
326 PC. In agreement with various earlier studies which confirmed that the CuO NPs-elicited hairy
327 roots displayed higher cytotoxic activities than non-elicited hairy roots [29].
328

329 **4. Conclusions**

330 CuO NPs used as abiotic elicitors for improved biomass and bioactive compounds (GA and PC)
331 production in CSC of *G. sylvestre*. In addition, antioxidant, antidiabetic, anti-inflammatory,
332 antibacterial, antifungal and anticancer activities were also increased in CuO NPs-elicited CSC
333 extracts. This investigation will deliver a reference for forthcoming studies on the potential
334 relation of these bioactive compounds to plant abiotic stress response in *G. sylvestre*. Therefore,
335 our protocol could be useful for the industrial production of GA and PC and their uses for
336 pharmaceutical activities concerned with significant health benefits using cell suspension
337 cultures of *G. sylvestre*.

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338

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341

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343

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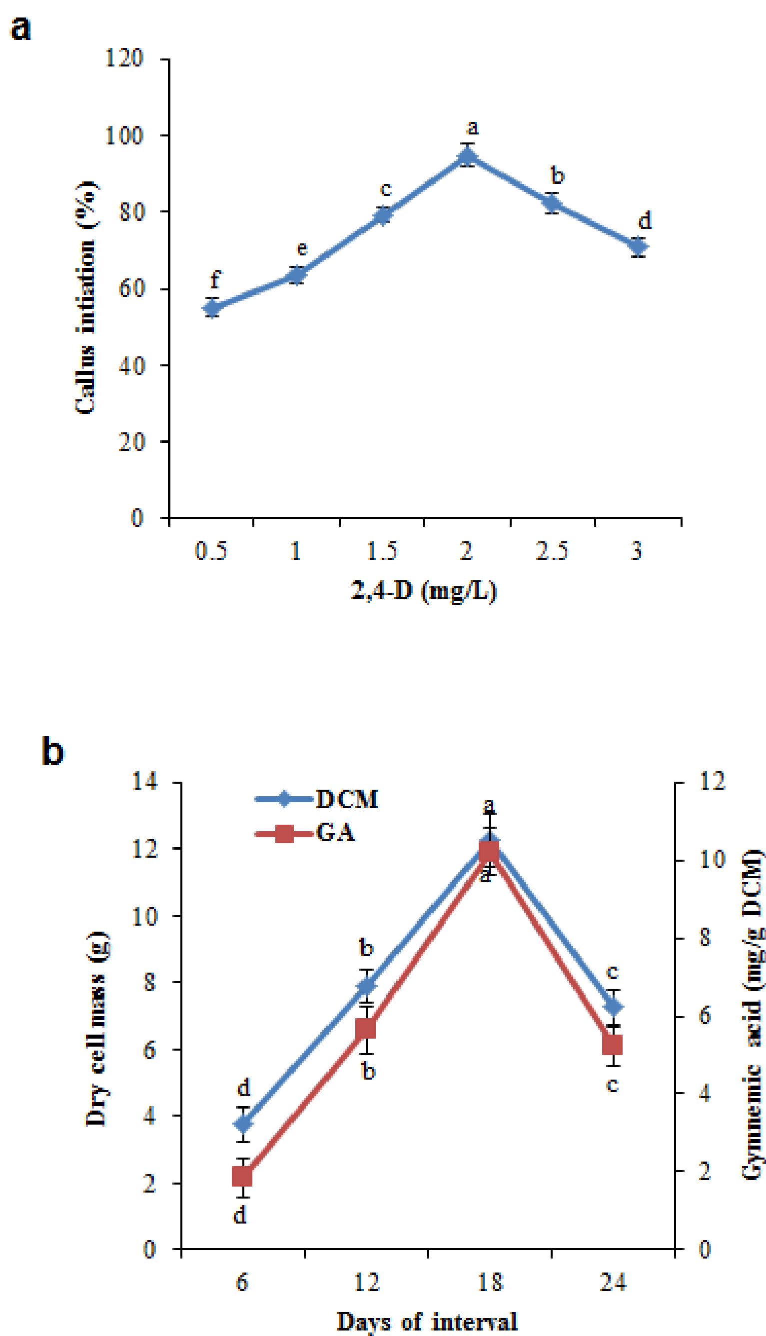

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521 **Fig. 1 a.** Effect of different concentrations of 2,4-D in combination with 0.1 mg/L KIN for callus
522 induction in *G. sylvestre*, **b**. Biomass accumulation and gymnemic acid production on MS liquid
523 medium with 2,4-D (2.0 mg/L) and KIN (0.1 mg/L) and sucrose (30 g/L) at different growth
524 period. Different letters indicate a significant difference at $P \leq 0.05$.

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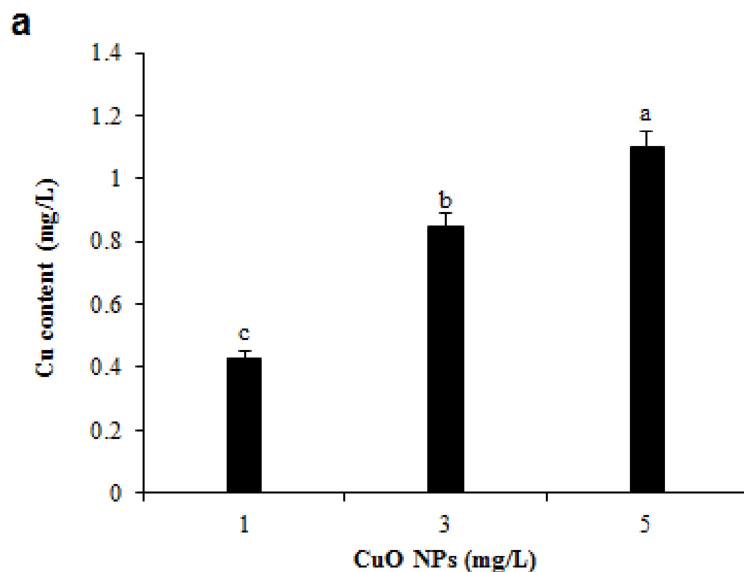

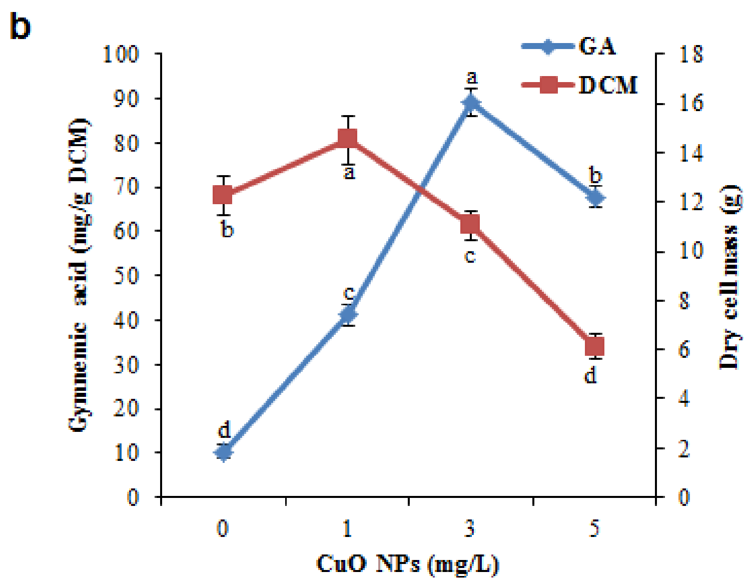

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529 **Fig. 2** Effect of CuO NPs on copper content, biomass accumulation and gymnemic acid (GA)
530 production in *G. sylvestre*. **a**. Copper content, **b**. Biomass accumulation and GA production.
531 Different letters indicate a significant difference at $P \leq 0.05$.

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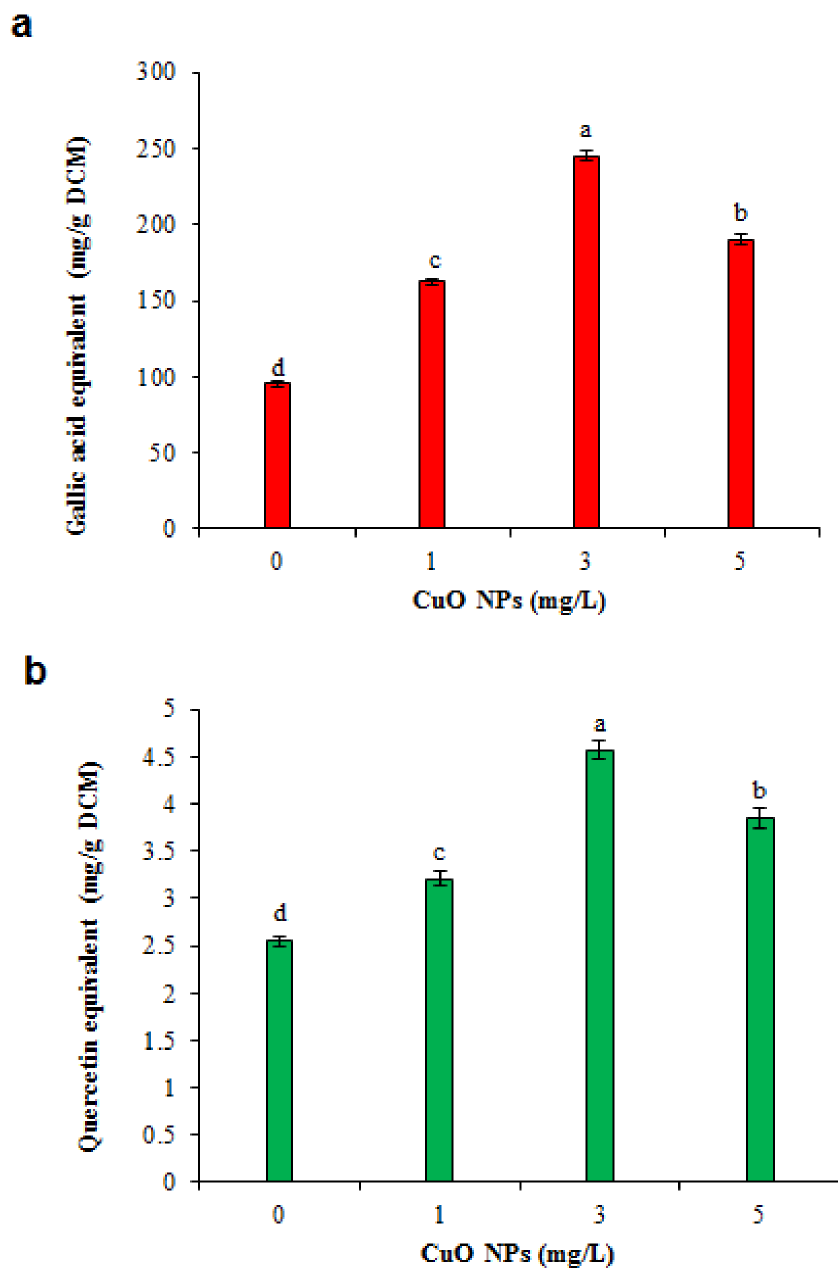

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536 **Fig. 3** Effect of CuO NPs on total phenolic and flavonoid contents (TPC and TFC) in cell
537 suspension cultures of *G. sylvestre*. **a**. TPC, **b**. TFC. Different letters indicate a significant
538 difference at $P \leq 0.05$.

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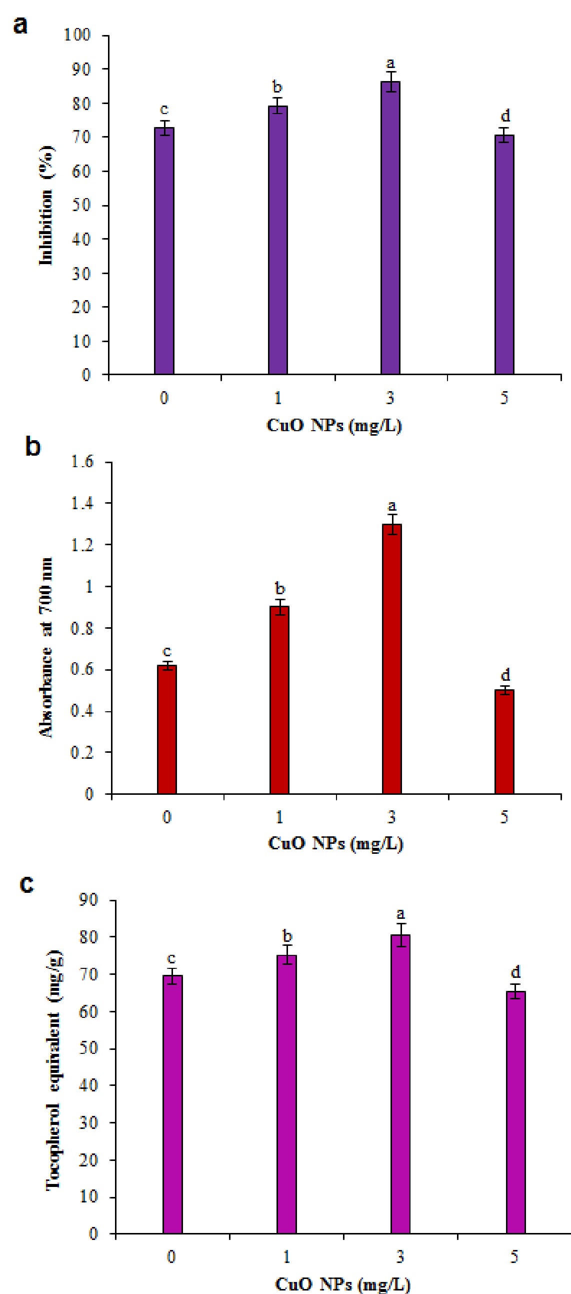

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542 **Fig. 4.** Effect of CuO NPs on antioxidant activities in cell suspension culture of *G. sylvestre*. **a**.
543 Free radical-scavenging activity by DPPH method, **b** Total Fe^{3+}– Fe^{2+} reductive potential
544 reference antioxidants (butylated hydroxytoluene), **c** Total antioxidant capacity (TAC) by
545 phosphomolybdenum method [TAC was expressed as equivalents of α-tocopherol (µg/g of
546 extract)]. Different letters indicate a significant difference at $P \leq 0.05$.

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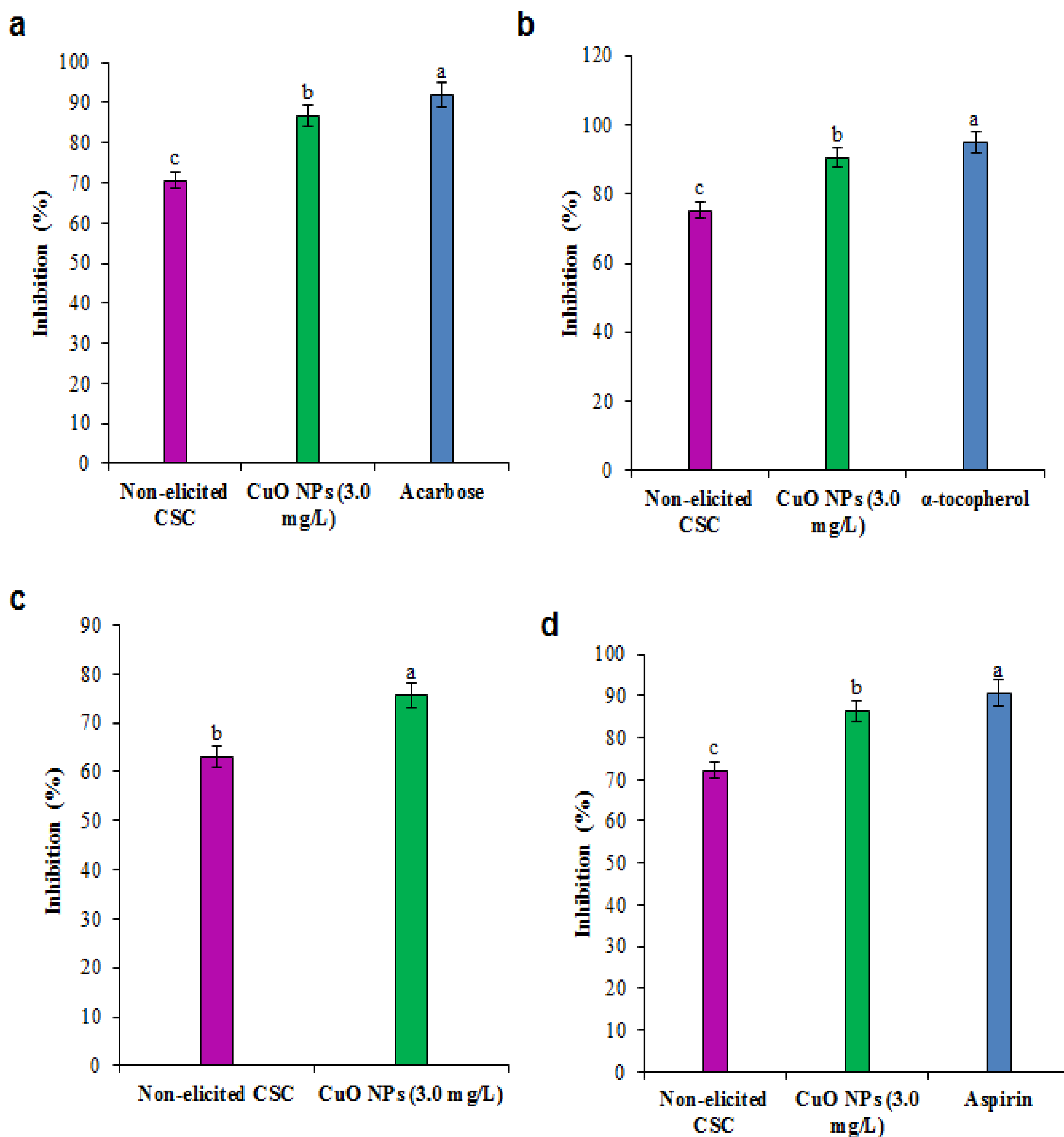

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551 **Fig. 5** Effect of CuO NPs on antidiabetic and anti-inflammatory activities cell suspension
552 cultures of *G. sylvestre*. **a**. In vitro α-amylase activity, **b**. Non-enzymatic glycosylation of
553 hemoglobin activity, **c**. Lipoxygenase inhibition activity, **d**. Albumin denaturation inhibition
554 assay. Different letters indicate a significant difference at $P \leq 0.05$.

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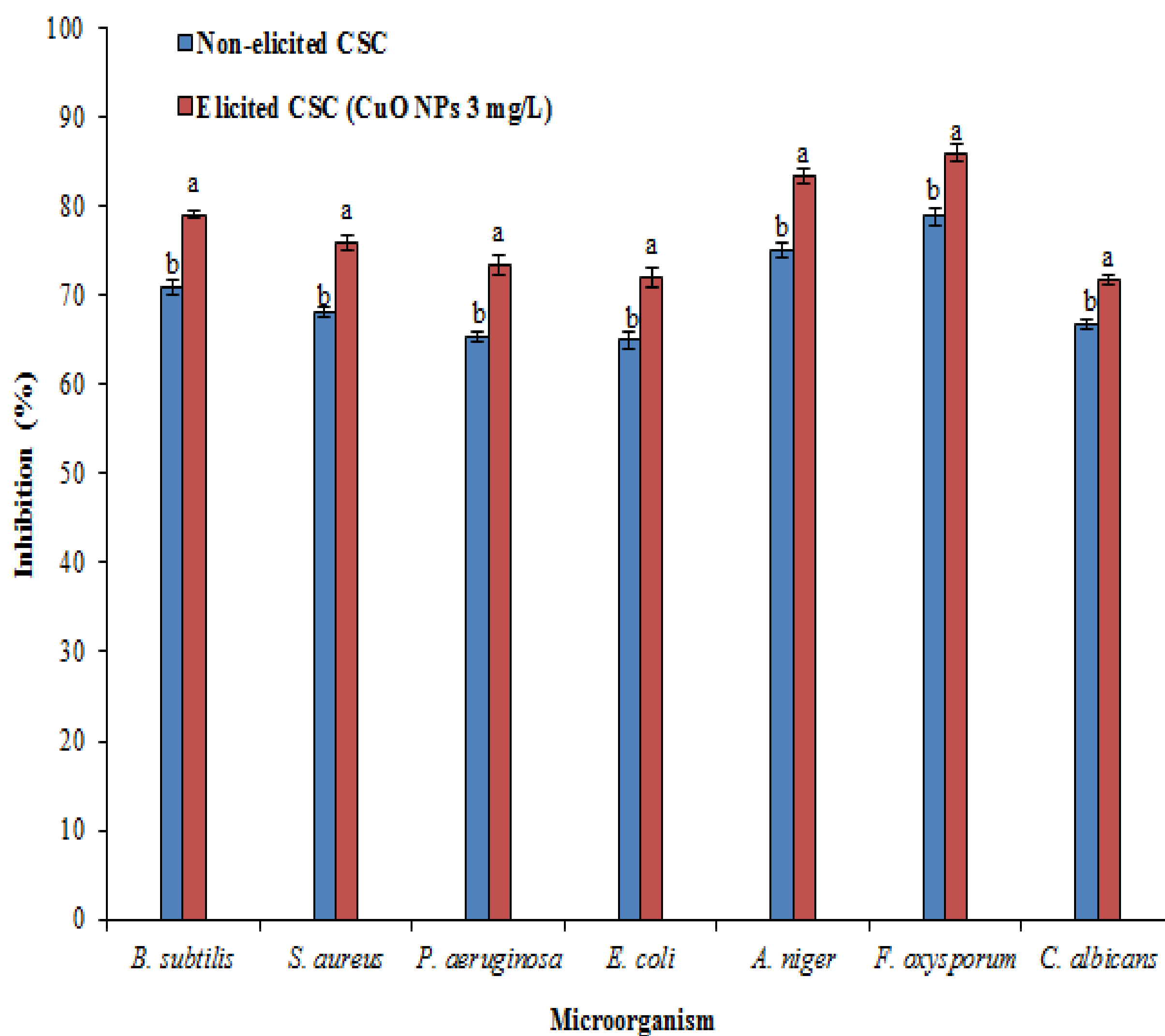

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559 **Fig. 6** Effect of CuO NPs on antimicrobial activity in cell suspension cultures of *G. sylvestre*
560 using disc diffusion method. Different letters indicate a significant difference at $P \leq 0.05$.
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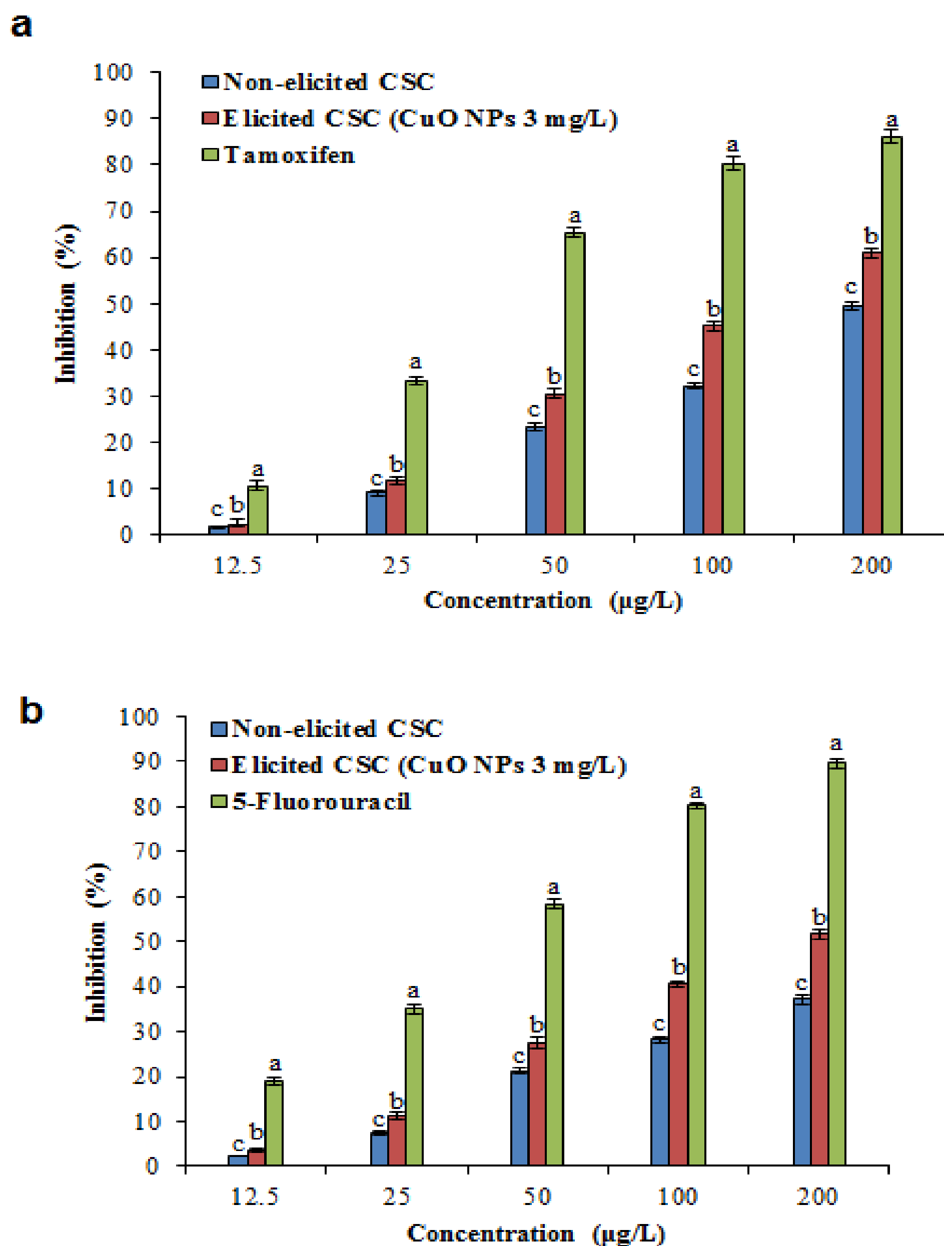

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567

568 **Fig. 7** Effect of CuO NPs on cell viability of MCF-7 and HT-29 cell lines in cell suspension
569 cultures of *G. sylvestre*. **a.** MCF-7, **b.** HT-29. Different letters indicate a significant difference at
570 $P \leq 0.05$.

571