Copper sulfate and Cobalt chloride effect on total phenolics accumulation and antioxidant activity of *Artemisia annua* L. callus cultures

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ABSTRACT

This study was conducted to investigate the influence of Copper sulfate ($CuSO_4$) and Cobalt chloride ($CoCl_2$) on callus biomass production, total phenolics accumulation and antioxidant activity of *Artemisia annua*. The *in vitro* leaf explants were cultured on Murashige and Skoog (MS) medium contained 1 mg/l α-naphthalene acetic acid (NAA) and 0.5 mg/l 6-benzyl adenine (BA) for callus induction. $CuSO_4$ and $CoCl_2$ were added separately in four concentrations (1, 2, 5 and 10 mg/l) to MS medium. $CuSO_4$ at 2 mg/l was the most appropriate level for callus fresh and dry weights which recorded 1.29 g/explant and 0.122 g/explant, respectively. Application of $CoCl_2$ at different levels showed a positive impact on the growth of callus. Herein, the highest callus fresh weight (1.44 g/explant) and dry weight (0.139 g/explant) were achieved by 2 mg/l $CoCl_2$, when compared with control (0.97 g/explant for fresh weight and 0.092 g/explant for dry weight). Elicitation with $CuSO_4$ at 2 mg/l recorded the highest total phenolic production (8.75 mg/g dry weight (DW)). However, the highest significant percentage of antioxidant activity (90.85%) was recorded for 5 mg/l $CuSO_4$-elicited culture. For $CoCl_2$, the highest significant values of total phenolic content (8.40 mg/g DW) and antioxidant activity (91.46 %) were achieved by 2 mg/l $CoCl_2$ enriched medium compared to control (6.66 mg/g DW and 90.68%, respectively). The current study revealed the possibility of using callus cultures of *A. annua* as a promising tool for the *in vitro* production of phytochemicals.

Keywords: *Artemisia annua*; elicitors; Copper sulfate; Cobalt chloride; and callus.

INTRODUCTION

Artemisia annua L. is among the primary sources of natural drugs used in modern and traditional medicine (Jelodar *et al.,* 2014). *A. annua* is originated from China, which is mostly spread in the cool temperate, temperate, and Mediterranean zones (Ferreira and Janick, 1996). Artemisinin, a sesquiterpene lactone originally obtained from *A. annua*, was discovered as an antimalarial drug. Worldwide, drugs containing artemisinin derivatives are used to treat malaria. The medicinal properties of the extracts of *A. annua* allowed using it for the treatment of various diseases due to its anti-inflammatory, anti-malarial, anti-oxidative, immunosuppressive, anti-cancer, anti-parasitic, anti-microbial, and anti-viral properties. The therapeutic properties of *A. annua* extracts are not only due to the main phytoconstituents, but also related to the synergistic contribution of other constituents (Han *et al*., 2008). Flavonoids and phenolics have a wide biological activity, such as anti-inflammatory, anti-oxidant, anti-tumor, anti-bacterial, and anti-aging, and also have high industrial values (Patil *et al.,* 2018). In this connect, phenolic compounds of *A. annua* exhibited anti-inflammatory function in human intestinal cells (De Magalhães *et al*., 2012), resistance to the malaria parasites, *Plasmodium falciparum* (Suberu *et al*., 2013) and against cancer metastasis (Ko et al., 2016). However, the study of phenolic compounds derived from tissue culture of *A. annua* is still limited. The use of similar method using tissue culture for the improvement of artemisinin production, may be also effectively adopted to enhance medicinally important phenolics of the *A. annua* plant (Ali *et al*., 2013). Studies on tissue culture of *A. absinthium* (Ali and Abbasi, 2014) and *A. scoparia* (Yousaf et al., 2019) demonstrated the ability of callus cells to synthesis and accumulate phenolic compounds.

Over the last decades, biotechnology has been intensively investigated plant, specifically plant tissue culture as a possible technique for the commercial production of high-value secondary metabolites, particularly in the pharmaceutical industry. Various chemical compounds such as polyphenols, alkaloids, saponins, carotenoids, anthocyanins, etc. had been successfully biosynthesized and accumulated using plant tissue culture (Bosila *et al*., 2012; 2016; Toaima *et al*., 2017; Elateeq *et al*., 2020; 2021). This method could also be used to improve the accumulation of various secondary metabolites, which are naturally difficult to be produced in large amounts in plants (Ali *et al*., 2013).

The major limitation in using plant tissue culture system to produce bioactive

phytomolecules from medicinal plants is the extremely low accumulation of these ingredients compared to field cultivated plants. The biosynthesis and accumulation of secondary metabolites in plant cell is enhanced under stress factors; their production can be increased by elicitors and precursors (Zhao *et al*., 2010). Elicitors are compounds of mainly microbial origin (biotic) or nonbiological origin (abiotic), which upon contact with plant cell trigger the accumulation of various defense compounds, such as flavonoids, phenols, phytoalexins (Savitha *et al*., 2006). Elicitors were employed under *in vitro* conditions to stimulate the formation of various by-products in plant tissue cultures by reducing the time required to increase culture volumes and increasing the active substances content (Angelova *et al*., 2006; Namdeo, 2007; Elateeq *et al*., 2020). Using biotic and abiotic elicitors as tools for sustainable production of phytochemicals has been successfully reported in the formation of phenol in leaf callus of *Cicer spiroceras* (Kordi *et al*., 2014), phenol and flavonoids of *Cynara scolymus* callus cultures (Tanoori *et al*., 2015), alkaloids, flavonoids, phenolics, and saponins in *Centella asiatica* callus culture (Rao *et al*., 2015), and flavonoids and flavonolignans in callus cultures of *Silybum marianum* (Elateeq *et al*., 2020).

Iron, Cobalt, Copper, Manganese, and Zinc are essential metals for plant, however, a very small concentrations of them are required for plant cell and become toxic at higher amounts (Hussein *et al*., 2010). Metals may stimulate the production of bioactive ingredients by changing aspects of secondary metabolism (Verpoorte *et al*., 2002). Microelements are essential as catalysts for many biochemical reactions (Bhojwani and Razdan, 1996).

It was proved that modulation of microelements in culture media is an appropriate strategy to increase various phytoconstituents content in plant cell, tissue and organ cultures of different species, such as adventitious roots of *Panax ginseng* (Sivakumar *et al*., 2005), hairy roots of *Pumbago indica* (Gangopadhyay *et al*., 2011), callus cultures of *Silybum marianum* (Elateeq, 2013), shoot cultures of *Cryptostegia grandiflora* (Abd El-Mawla, 2014), cell suspension cultures of *A. annua* (Ghasemi *et al*., 2015), *Melissa officinalis* (Urdová *et al*., 2015) and *Panax quinquefolius* (Biswas *et al*., 2016). Cu^{2+} and Co^{2+} have received more attention because of their positive effects on enhancing the accumulation of plant secondary metabolites. Therefore, the present study aims to enhance the accumulation of total phenolics in *in vitro* callus cultures of *A. annua* by Copper sulfate and Cobalt chloride as abiotic elicitors to provide a suitable plant material for the efficient production of phenolics with a high capacity of antioxidant activity.

MATERIALS AND METHODS

This investigation was carried out in the Laboratory of Biotechnology, Horticulture Department, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt.

Plant material:

Seeds of *A. annua*, used in this study were obtained from Sekem Holding Company, Belbeis Rd, Belbeis, Sharkia, Egypt**.**

Explant preparation:

A. annua seeds were washed several times with commercial detergent and tap water and surface sterilized with 70% ethanol for 1 min followed by 20% commercial Clorox solution (5.25% sodium hypochlorite) containing few drops of Tween 20 for 20 min. The seeds were subsequently rinsed five times with sterile distilled water to remove the residual sodium hypochlorite.

The sterile seeds were sown on 350 ml jars containing 25 ml of MS basal medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and solidified with 8 g/l agar and incubated for 7 days in the dark for germination. Afterwards, seedlings were transferred to 16-h light/8-h dark cycle with illumination from cool white fluorescence lights at 40 μmol-2 s-1 for 20 days at 25±2°C.

Establishment of callus culture:

Callus was established from leaf segments (0.5 cm^2 excised from *in vitro* seedling, Figure 1A) cultured on MS medium with 30 g/l sucrose, solidified with 2 g/l phytagel and supplemented with 1 mg/l α-naphthalene acetic acid (NAA) + 0.5 mg/l 6-benzyl adenine (BA) after 5 weeks of incubation in 16-h light/8-h dark cycle at 25±2°C.

Effect of Copper sulfate and Cobalt chloride:

To test the effect of elicitors (Copper sulfate and Cobalt chloride) on callus biomass, total phenolics productions and antioxidant activity of *A. annua*, callus pieces (0.25 - 0.30 g fresh weight) taken from leaf-derived callus in the age of 5 weeks Figure (1B) were transferred to MS medium containing 30 g/l sucrose, 1 mg/l NAA + 0.5 mg/l BA, and supplemented with Copper sulfate ($CuSO_4.5H_2O$) or Cobalt

chloride ($CoCl_2.6H_2O$) each at concentration of 0, 1, 2, 5 and 10 mg/l.

Culture conditions:

The pH value of all tested media was adjusted to 5.8 with NaOH or HCl (1 N) prior to the addition of solidified agents. The different treatments of all experiments were planted under Laminar Airflow Hood and incubated for 5 weeks in a growth chamber under 25 ± 2°C and 16-h light/8-h dark cycle with illumination from cool white fluorescence lights at 40 $\mu mol^{-2}\ s^{-1}$.

Measurements:

Determination of callus biomass

Fresh weight (FW) of callus (g/explant) was determined after 5 weeks of incubation period by weighing callus immediately after removing the residual phytagel and the values were expressed in grams. Dry weight (DW) of callus (g/explant) was determined after drying in an oven at 65°C until constant weight and the values were expressed in grams.

Determinations of total phenolics content

Approximately 100 mg of callus dry matter was placed in 5 ml of 95% ethanol at 0°C for 48 h. Each sample was then homogenized and centrifuged at for 8 to 10 min to remove particulate matter. Total phenolic levels were determined using a slight modification of the Chandler and Dodds (1983) method that was originally based on the method of Singleton and Rossi (1965). One ml of the extract supernatant was mixed with 1 ml of 95% ethanol and 5 ml of distilled water. To each sample, 0.5 ml of 50% Folin-Ciocalteu reagent was added. After 5 min, 1 ml of 5% Na_2CO_3 was added with thorough mixing to stabilize color development. The mixture was allowed to stand for 60 min under room temperature and the absorbance of the solution was read spectrophotometrically (JENWAY 6800 UV/Vis. spectrophotometer) at 725 nm against blank prepared as above but without sample. The different gallic acid dilutions were prepared and determined by the same technique to draw the standard gallic acid concentration curve. Total phenolics content was expressed as mg gallic acid equivalents per g DW of callus tissue.

Determination of free radical scavenging activity

Callus tissue (100 mg DW) was extracted with 95% ethanol (5 ml) and incubated at 25°C for 24 h, then filtered. The DPPH (2,2-diphenyl-1-picrylhydrazyl) test was carried out as described by Wu *et al*., (2018) with slight modification. About 0.7 ml extracts were mixed with 3 ml DPPH solution (200 μM) and shaken thoroughly. The reaction mixture was incubated in darkness for 30 min at room temperature. The absorbance was read at 517 nm (JENWAY 6800 UV/Vis. spectrophotometer). The percentage of DPPH radical scavenging activity was calculated using the following formula:

DPPH activity %= A(control)-A(sample) /A(control) ×100

Where A(control) is the absorbance of the DPPH solution with 0.7 mL of 95% Ethanol free sample, and A(sample) is the absorbance of callus extract mixed with DPPH solution.

The statistical analysis:

All experiments were conducted in a complete randomized design (CRD). The data were subjected to Analysis of Variance (ANOVA) using COSTAT computer package ver. 6.4 (CoHort software Monterey, California, USA). The significance of differences among means was carried out using Duncan's Multiple Range Test (DMRT) (Duncan, 1955) at $P<0.05$.

RESULTS AND DISCUSSION

Effect of Copper sulfate on callus biomass production in *A. annua*

Data presented in Table (1) and Figure (2) illustrate the variations in callus fresh weight. Results indicated that by increasing the levels of Copper sulfate ($CuSO_4$) added to MS medium from 1 to 2 mg/l, callus FW increased from 0.92 to 1.29 g/explant. Higher levels of Copper sulfate (5 and 10 mg/l) led to an inhibition in callus biomass FW (1.16 and 1.07 g/explant), but it still higher than control treatment (0.97 g/explant). Similar observation was recorded in *Beta vulgaris* by Trejo-Tapia *et al*. (2001). Maximum production of callus FW (1.29 g/explant) was obtained by 2 mg/l of Copper sulfate which represented 134.10% of control.

Also, increasing the levels of Copper sulfate from 1 to 2 mg/l, increased callus dry weight from 0.095 to 0.122 g/explant (Table 1 and Figure 3). However, further increase in Copper sulfate to 5 and 10 mg/l decreased callus DW to 0.110 and 0.111 g/explant, without significant differences between them. In this concern, George and Klerk (2008) reported that high concentrations of Copper can be toxic to plant cells. Adding Copper sulfate at 2 mg/l to MS medium recorded the best value of callus

dry matter (0.122 g/explant, 1.33-fold increase of control) compared to control MS medium (0.092 g/explant). Also, there were no significant differences between the lowest concentration of Copper sulfate (1 mg/l) and the control treatment.

Effect of Copper sulfate on total phenolic content in *A. annua* callus

Although heavy metals are a serious problem in the environment and affect plant growth and crop productivity (Shakir Ullah *et al*., 2020), some of them can be employed as abiotic elicitors to stimulate the productivity of bioactive phytoconstituents in *in vitro* cultures of medicinal plants (Bota and Deliu, 2011; Elateeq, 2013 and Elateeq *et al*., 2020). Regarding the effect of Copper sulfate, as an abiotic elicitor, on total phenolic content (mg/g DW) in *A. annua* callus cultures, data of Table (2) and Figure (4) show that supplementing MS medium with Copper sulfate at a level higher than that standard one in the normal medium resulted in a significant enhancement in total phenolic content in callus cultures, which could be related to its effect as an abiotic elicitor or an abiotic stress. This observation was also noted by Bhuiyan and Adachi (2003), as they found that $CuSO_4$ produced positive responses for the enhancement of betacyanin accumulation when added to the callus medium of *Portulaca*.

In the current study, elicitation with Copper sulfate at 2 mg/l was the most appropriate treatment for total phenolic production (8.75 mg/g DW; 1.31-fold increase of control) followed by 1 mg/l of Copper sulfate (8.27 mg/g DW; 1.24-fold increase of control). Total phenolic content was decreased by increasing Copper sulfate levels from 5 to 10 mg/l recording 7.55 and 7.87 mg/g DW, respectively, but it still better than control treatment (6.66 mg/g DW). These results are in harmony with Trejo-Tapia et al. (2001) on *Beta vulgaris*, Ali *et al*. (2006) on *Panax ginseng* and Bota and Deliu (2011) on *Digitalis lanata*. Copper is believed to be necessary in energy conversions and is a component of enzymes (Sathyanarayana and Varghese, 2007). Copper plays an essential role in the biosynthesis of phenols in the plant and a lack of it can lead to a reduction of phenolic compounds (Dicko *et al*., 2006).

Effect of Copper sulfate on antioxidant activity of *A. annua* callus

DPPH free radical scavenging activity was determined for callus extracts of cultures elicited with Copper sulfate. Data presented in Figure (5) prove that Copper sulfate is a suitable elicitor in *A. annua* callus cultures to stimulate the antioxidant activity. In this concern, the highest significant value of antioxidant activity percentage (90.85%) was recorded for 5 mg/l $CuSO_4$-elicited culture followed by MS normal medium (90.68%). Elateeq (2013) on *Silybum marianum* callus cultures found that $CuSO_4$ at 5 mg/l was the most appropriate treatment for active substances production. Also, Fang *et al*. (1999) found that elicitation of *Vaccinium pahalae* cells with 5 mg/l $CuS0_4$ enhanced the accumulation of anthocyanin rather than other concentrations.

Antioxidant activity percentage was increased gradually by increasing Copper sulfate levels from 1 to 2 and 5 mg/l recording 88.99, 90.09 and 90.85%, respectively. However, Copper sulfate at 1, 2 and 10 mg/l recorded lower antioxidant activities than control. Hence, the lowest significant value of antioxidant activity percentage (88.99 %) was recorded for the lower supplemented level of $CuSO_4$ (1 mg/l). This observation is in line with the results recorded with Kordi *et al*. (2014) who reported that an inhibition in secondary and primary metabolites production, and antioxidant activity in *Cicer spiroceras* was recorded when Copper sulfate was used at a concentration less than 1 mg/l in callus culture.

Bidchol *et al.* (2011) reported that antioxidant activity could also be caused by nonphenolic compound. Many other metabolite compounds may also contribute to the antioxidant activity. This is consistent with the results of many studies that other phytocompounds, such as terpenoids, alkaloids, carotenoids, vitamins, proteins influence the antioxidant activity in plants (Hasshimoto *et al.,* 2005 and Basma *et al.,* 2011).

Copper is among heavy metals that are important for plant growth. Additionally, it can work as abiotic agent, stimulating the production of reactive oxygen species (ROS) (Schützendübel and Polle, 2002). On the other side, the level of Cu^{2+} in the culture media affects mitochondrial respiration through incorporation of Copper into the cytochromes. Therefore, the participation of Copper in the establishment of the mitochondrial electron transport system may be responsible for the effect of copper on the production of plant secondary metabolites (Morimoto *et al*., 1988). Moreover, being a redox-active transition metal, Copper has many functions, e.g., it is a cofactor for many enzymes, it participates in the signalling of transcription and hormones,

protein trafficking machinery, carbohydrate biosynthesis, lignin formation in cell walls and in oxidative stress responses (Purnhauser and Gyulai, 1993; Hirayama *et al*., 1999 and Yruela, 2005).

Effect of Cobalt chloride on callus biomass production in *A. annua*

Data displayed in Table (3) and Figure (6) illustrate the variations in callus fresh weight of *A. annua* due to the effect of various concentrations of Cobalt chloride ($CoCl_2$). Application of Cobalt chloride at different levels showed a positive effect on callus FW of *A. annua* compared to control. Cobalt chloride increased the growth of callus on the scope of the fresh weight as follows: 1.11, 1.44, 1.20 and 1.04 g/explant for 1, 2, 5 and 10 mg/l of Cobalt chloride, respectively, when compared with control (0.97 g/explant). The highest callus FW (1.44 g/explant) was achieved by 2 mg/l Cobalt chloride, followed by 5 mg/l (1.20 g/explant), which represent 1.47- and 1.24-fold higher than control, respectively.

In accordance with these results, Elateeq (2013) reported that a significant enhancement in callus fresh weights of *Silybum marianum* were recorded at all supplemented levels of Cobalt chloride (1, 5, 10 and 20 mg/l). Certain elements, such as Cobalt (Co) can inhibit ethylene synthesis (Bhojwani and Razdan, 1996). So, the positive effect of Cobalt chloride on biomass accumulation may be attributed to reducing the accumulation of ethylene in the media as suggested by Sung and Huang (2000).

Regarding the effect of Cobalt chloride ($CoCl_2$) on callus dry weight, Table (3) and Figure (7) show that the highest DW of callus was obtained with 2 mg/l Cobalt chloride (0.139 g/explant; 1.50-fold increase of control) followed by 1 and 5 mg/l (0.107 and 0.110 g/explant, respectively). When the concentration of Cobalt chloride exceeded 5 mg/l, the biomass of callus showed a decrease in DW. In this context, insignificant differences were recorded in calli dry weight harvested from MS standard medium (0.092 g/explant) and the treatments of 10 mg/l $CoCl_2$ (0.093 g/explant).

The proliferation of callus cells is an energy-consuming activity. Therefore, the respiration rate in cells is usually high during cell division and callus proliferation to provide the required energy. Microelements are the efficacious parts of some oxidative enzymes. So, these micronutrients trigger the respiration rate in plant cell (Amarasinghe, 2009). Furthermore, Cobalt is a component of vitamin B12 molecule which is concerned with nucleic acid synthesis and is essential for nitrogen fixation (Sathyanarayana and Varghese, 2007).

Effect of Cobalt chloride on total phenolic content in *A. annua* callus

Concerning the effect of the abiotic elicitor Cobalt chloride on total phenolic content (mg/g DW) in *A. annua* callus culture, data of Table (4) and Figure (8) indicate that a significant increase in total phenolic content in callus cultures was noticed when MS medium contained Cobalt chloride at a level higher than that found in the normal one. A similar observation was found in cell suspension of *Vitis vinifera* by Cai *et al*. (2013). They found that all tested concentrations of Cobalt ($CoCl_2$) (5, 25 and 50 μM) stimulated the phenolic acid production. Zhang and Wu (2003) on cell suspension cultures of *Taxus* spp. found that $CoCl_2$ at 10, 20, 50 and 100 μM had a positive effect on paclitaxel yield versus the control.

In general, callus grown on $CoCl_2$ media at all tested concentrations stimulated the accumulation of total phenolic. In this concern, the highest significant value of total phenolic content (8.40 mg/g DW; 126.13% in comparison to control) was occurred by 2 mg/l $CoCl_2$ enriched medium, followed by 1 mg/l $CoCl_2$ (7.88 mg/g DW). Increasing $CoCl_2$ level to 10 mg/l inhibited the biosynthesis of phenolics but remained higher than that reported in MS standard medium (6.97 and 6.66 mg/g DW, respectively). Similarly, Taha (2016) reported that the concentrations of 1 and 2.5 mg/l Cobalt chloride increased certain secondary metabolites hesperidin and rubinin to the highest rates significantly at 1 mg/l concentration, while datiscosid compound and rutin reached to the highest rates significantly at 2.5 mg/l of Cobalt chloride in callus tissue of *Cordia myxa*.

Effect of Cobalt chloride on antioxidant activity of *A. annua* callus

With regard to the influence of different levels of Cobalt chloride on the antioxidant activity (%) in *A. annua* callus culture, the data in Figure (9) show that the antioxidant activity was a significant response to the tested levels of Cobalt chloride.

The highest significant percentage of antioxidant activity (91.46 %) was recorded when 2 mg/l $CoCl_2$ was incorporated in the callus medium. An inhibition in the antioxidant activity percentage was recorded (90.36%) when the callus had grown on media

contained higher concentrations of $CoCl_2$ (10 mg/l), which represented the lowest significant value. It was noticed that low and moderate concentrations of $CoCl_2$ (1, 2 and 5 mg/l) augmented to standard MS medium promoted the antioxidant activity in callus cultures versus MS normal medium. In this respect, the antioxidant activity was significantly increased from 90.68% to 90.92 and 91.46 % by increasing $CoCl_2$ level from standard level in MS medium to 1 and 2 mg/l, respectively. However, further increase in $CoCl_2$ level at 5 mg/l decreased the content of antioxidant activity but kept its superior to MS normal medium (91.19 versus 90.68 %). Similar findings were reported by Huang *et al*. (1995) on cell suspension of *Stizolobium hassjoo* who found that when Cobalt ($CoCl_2.6H_2O$) level was increased in the culture medium to 25-fold, L-DOPA content was enhanced. Moreover, Trejo-Tapia *et al*. (2001) revealed that the highest accumulation of betalains in *Beta vulgaris* was achieved with $CoCl_2$ at 1.2 mg/l among other microelements. This treatment recorded a 60% increase over the value reached with the standard medium. Haque *et al*. (2011) stated that the biosynthesis of neuro-excitatory β-ODAP (β-N-oxalyl-L-α, β-diamino propionic acid) was significantly increased by oversupply of Co^{2+} ($CoCl_2.6H_2O$) in callus cultures of *Lathyrus sativus*.

Heavy metals may trigger the production of bioactive ingredients by changing aspects of secondary metabolism (Verpoorte *et al*., 2002). Heavy metals are known to affect plant cells *via* the induction of signaling pathways related at least partially to jasmonic acid. This is generally caused by the synthesis of proteins similar to pathogen-related and other hypersensitive responses (Santiago *et al*., 2000 and Maksymiec *et al*., 2005). Palit *et al*. (1994) reported that Cobalt is a transition metal which is an important factor in many enzymes and coenzymes. It affects the cell growth and metabolism of plants to varying degrees, according to its level in the culture medium.

Manipulating the microelement levels in the culture media represents a promising strategy to increase the productivity of bioactive metabolites in plant tissue cultures (Elateeq, 2013; Abd El-Mawla, 2014; Urdová *et al*., 2015; Biswas *et al*., 2016 and Zarad, 2017). This effect may be related to their effects as nutritional factors to increase biomass (Zhong and Wang, 1996) or as prosthetic groups of enzymes to trigger secondary metabolism (Huang *et al*., 1995; Trejo-Tapia *et al*., 2001), or as elicitors to stimulate the biosynthesis of a target product (Suvarnalatha *et al*., 1994; Elateeq *et al*., 2020). In the present work, the higher percentages of the antioxidant activity assayed for microelements-elicited calli of *A. annua* may be due to the higher contents of the total phenolics.

CONCLUSIONS

Phenolics are valuable bioactive phytochemicals accumulated in the medicinal plant *A. annua*. Using tissue cultures system to improve the production of high-value pharmaceuticals is a promising approach to overcome the various limitations facing conventional production. Elicitation strategy had gained more attention due to its role in enhancing the accumulation of various plant secondary metabolites. Thus, it is essential to provide appropriate and accessible elicitors that facilitate the sustainable production of important plant compounds. In this report, abiotic elicitation with Copper sulfate ($CuSO_4$) and Cobalt chloride ($CoCl_2$) in *A. annua* callus cultures represented an appropriate approach for the production of callus biomass having abundant of total phenolics. Moreover, antioxidant capacity was enhanced in the callus extracts of Copper sulfate and Cobalt chloride elicited cultures, which would expand its application in the fields of nutraceuticals and pharmaceuticals. Further studies should explore the genes involved in the biosynthetic pathway of artemisinin and phenolics in *A. annua* under microelements elicitation.

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Table 1 Effect of Copper sulfate levels on callus fresh and dry weight of *A. annua* after 5 weeks incubation period

Copper sulfate levels (mg/l)	Callus fresh weight (g/explant)	Callus dry weight (g/explant)
Control	0.97 cd	0.092 b
1	0.92 d	0.095 b
2	1.29 a	0.122 a
5	1.16 b	0.110 ab
10	1.07 bc	0.111 ab

Mean values followed by different letters in the column are significantly different according to DMRT at $P<0.05$.

Table 2: Effect of Copper sulfate levels on total phenolic content in callus cultures of *A. annua* after 5 weeks incubation period.

Copper sulfate levels (mg/l)		Total phenolic content (mg/g DW)
Control		6.66 e
$CuSO_4$	1	8.27 b
	2	8.75 a
	5	7.55 d
	10	7.87 c

Mean values followed by different letters in the column are significantly different according to DMRT at $P<0.05$.

Table 3: Effect of Cobalt chloride levels on callus fresh and dry weight of *A. annua* after 5 weeks incubation period.

Cobalt chloride levels (mg/l)	Callus fresh weight (g/explant)	Callus dry weight (g/explant)
Control	0.97 d	0.092 c
1	1.11 c	0.107 b
2	1.44 a	0.139 a
5	1.20 b	0.110 b
10	1.04 cd	0.093 c

Mean values followed by different letters in the column are significantly different according to DMRT at $P<0.05$

Table 4: Effect of Cobalt chloride levels on total phenolic content in callus cultures of *A. annua* after 5 weeks incubation period.

Cobalt chloride levels (mg/l)		Total phenolic content (mg/g DW)
Control		6.66 e
$CoCl_2$	1	7.88 b
	2	8.40 a
	5	7.21 c
	10	6.97 d

Mean values followed by different letters in the column are significantly different according to DMRT at $P<0.05$

Figure 1: (A) *in vitro* seedling (4 weeks age) of *A. annua,* **(B)** leaf callus established on MS medium supplemented with 1 mg/l NAA + 0.5 mg/l BA after 5 weeks incubation periods in *A. annua*.

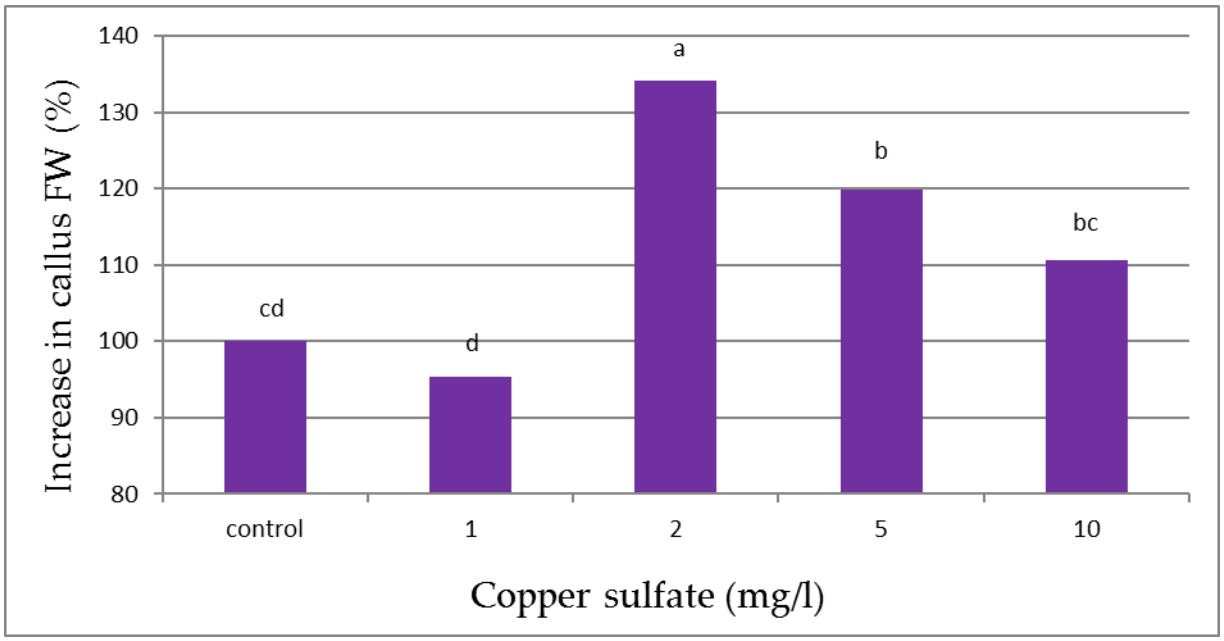

Figure 2: The percentage increase (%) in fresh weight of *A. annua* callus compared to control after five weeks of culture on MS medium contained different concentrations of Copper sulfate. Columns with different letters are statistically different according to DMRT (P<0.05).

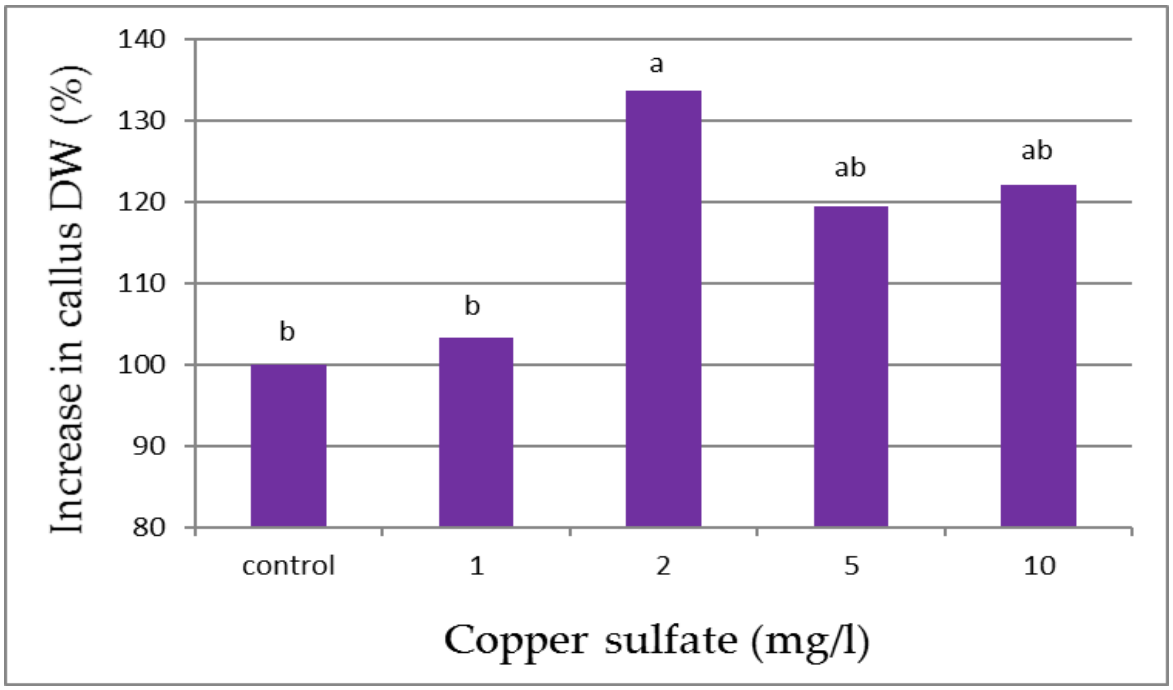

Figure 3: The percentage increase (%) in dry weight of *A. annua* callus compared to control after five weeks of culture on MS medium contained different concentrations of Copper sulfate. Columns with different letters are statistically different according to DMRT (P<0.05).

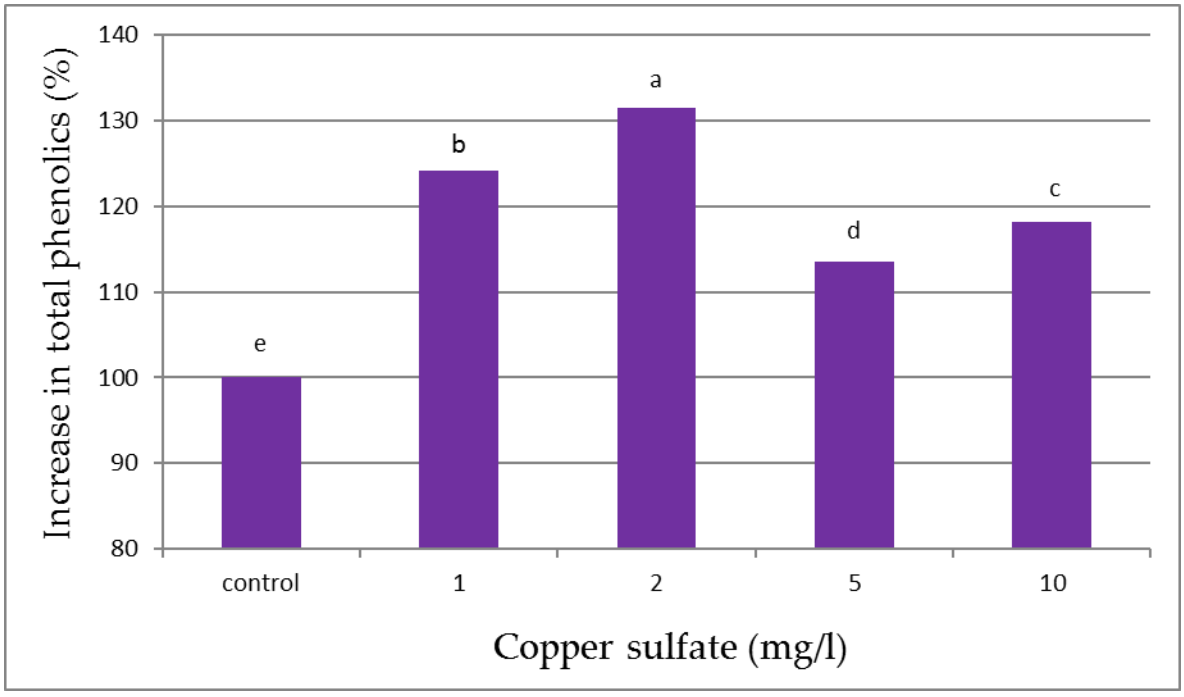

Figure 4: The percentage increase (%) in total phenolic content of *A. annua* callus compared to control after five weeks of culture on MS medium contained different concentrations of Copper sulfate. Columns with different letters are statistically different according to DMRT (P<0.05).

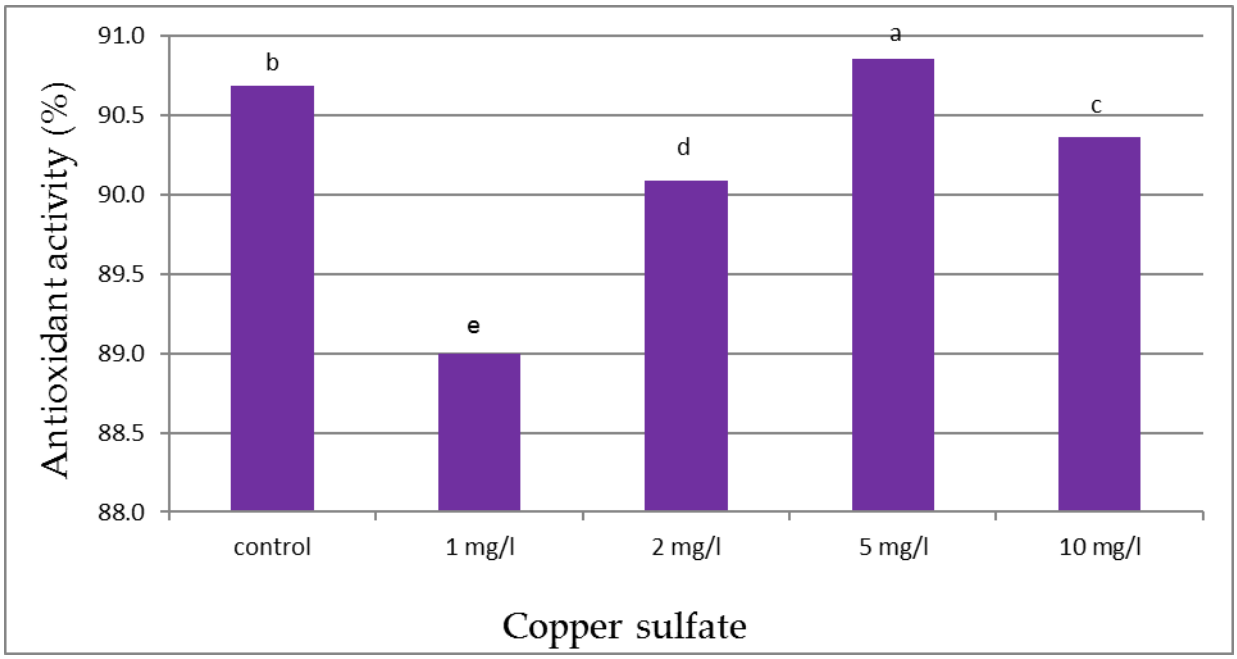

Figure 5: Effect of Copper sulfate on antioxidant activity (%) in callus of *A. annua* after five weeks incubation periods. Columns with different letters are statistically different according to DMRT (P<0.05).

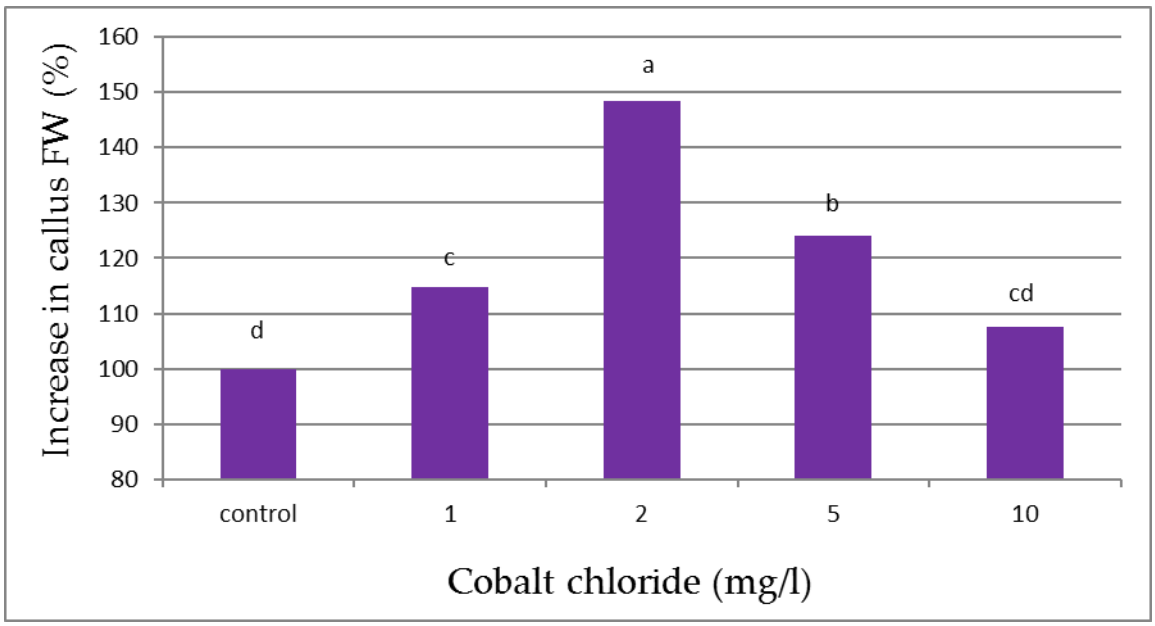

Figure 6: The percentage increase (%) in fresh weight of *A. annua* callus compared to control after five weeks of culture on MS medium contained different concentrations of Cobalt chloride. Columns with different letters are statistically different according to DMRT (P<0.05).

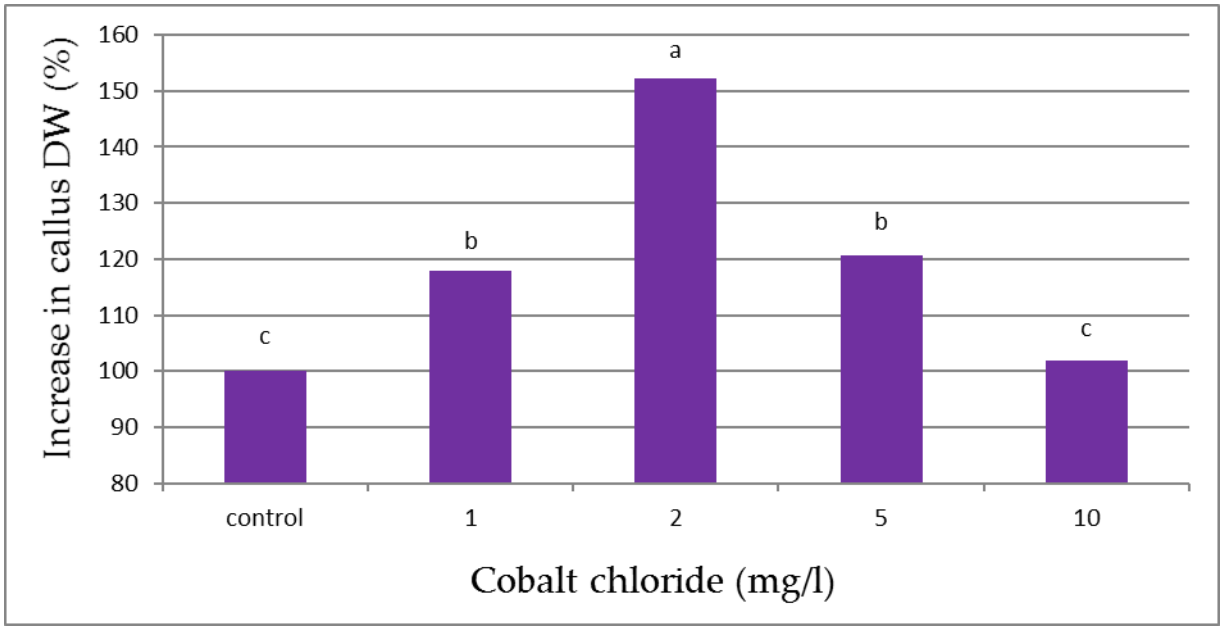

Figure 7: The percentage increase (%) in dry weight of *A. annua* callus compared to control after five weeks of culture on MS medium contained different concentrations of Cobalt chloride. Columns with different letters are statistically different according to DMRT (P<0.05).

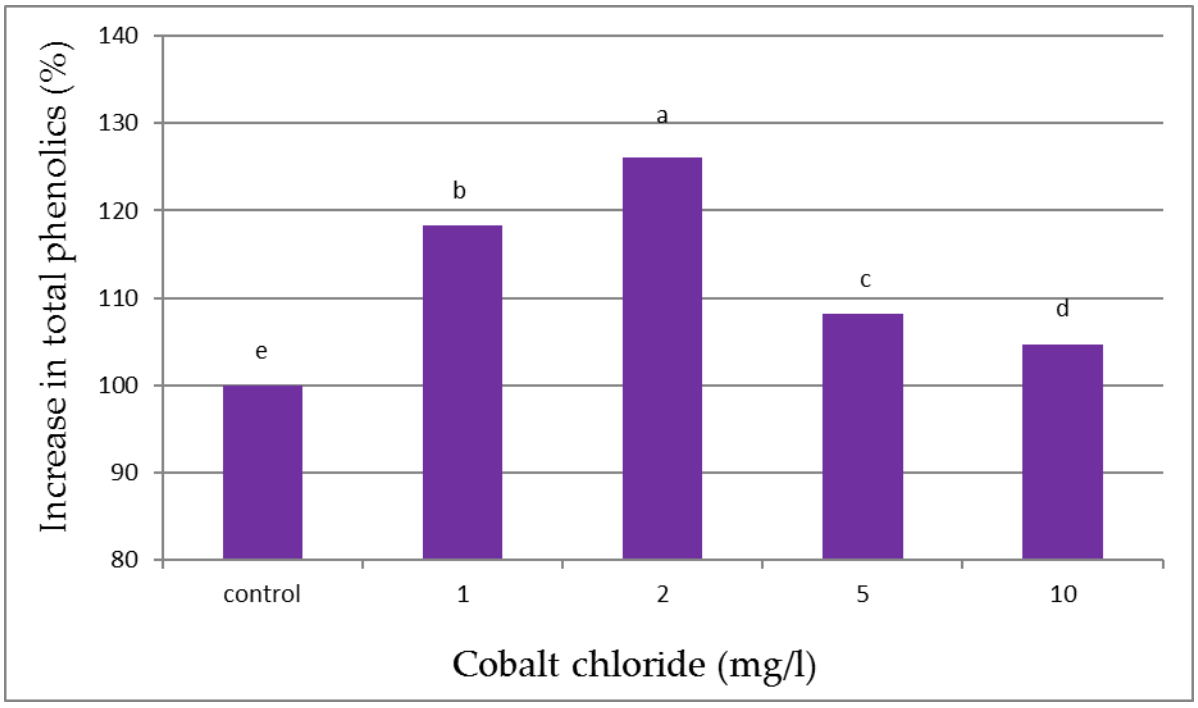

Figure 8: The percentage increase (%) in total phenolic content of *A. annua* callus compared to control after five weeks of culture on MS medium contained different concentrations of Cobalt chloride. Columns with different letters are statistically different according to DMRT (P<0.05).

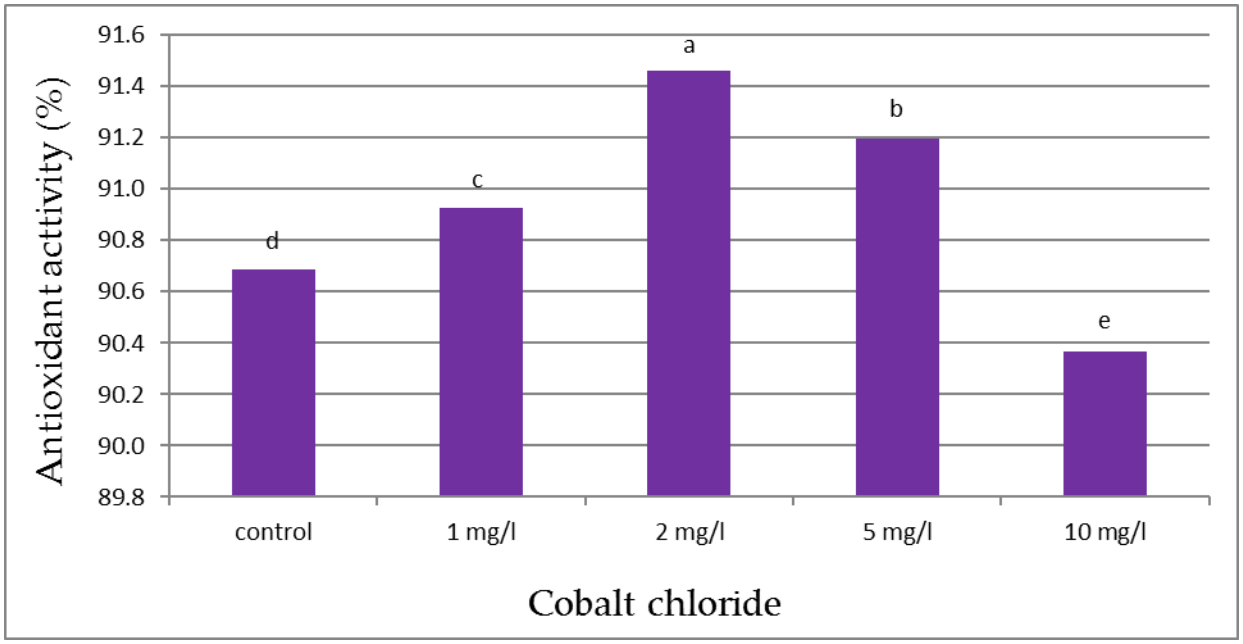

Figure 9: Effect of Cobalt chloride on antioxidant activity (%) in callus of *A. annua* after five weeks incubation periods. Columns with different letters are statistically different according to DMRT (P<0.05).

تأثير كبريتات النحاس وكلوريد الكوبالت على تراكم الفينولات الكلية ونشاط مضادات الأكسدة فى مزارع كالس نبات أرتيميزيا أنوا

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الملخص العربى

أجريت هذه الدراسة لمعرفة تأثير كبريتات النحاس ($CuSO_4$) وكلوريد الكوبالت ($CoCl_2$) على انتاج الكتلة الحيوية للكالس، والفينولات الكلية ، والنشاط المضاد للأكسدة في نبات أرتيميزيا أنوا .لإنشاء الكالس ، تمت زراعة الورقة كمنفصل نباتي على بيئة موراشيجي وسكوج (MS) مع إضافة 1 مللي جرام /لتر من نفتالين حامض الخليك (NAA) و 0.5 مللي جرام /لتر 6-بنزيل أدينين (BA). تمت إضافة $CuSO_4$ و $CoCl_2$ بشكل منفصل في أربعة تركيزات (1 ، 2 ، 5 و 10 مللي جرام /لتر) إلى بيئة MS. كان $CuSO_4$ عند 2 مللي جرام /لتر هو التركيز الأنسب لإنتاج الوزن الطازج والجاف للكالس والتي سجلت 1.29 جرام /منفصل نباتي. و 0.122 جرام /منفصل نباتي على التوالي. أظهر تطبيق $CoCl_2$ بمستويات مختلفة تأثيرًا إيجابيًا على نمو الكالس. ومن هنا ، تم الحصول على أعلى وزن طازج من الكالس (1.44 جرام /منفصل نباتي) ووزن جاف (0.139 جرام /منفصل نباتي) عند 2 مللي جرام /لتر من كلوريد الكوبالت، مقارنةً بالكنترول (0.97 جرام وزن طازج /منفصل نباتي و 0.092 جرام وزن جاف /منفصل نباتي). التحفيز باستخدام كبريتات النحاس $CuSO_4$ عند 2 مللي جرام / لتر سجل أعلى إنتاج لإجمالي الفينولات (8.75 مللي جرام /جرام وزن جاف). ومع ذلك ، تم تسجيل أعلى نسبة معنوية من النشاط المضاد للأكسدة (90.85٪) عند 5 مللي جرام /لتر من CuSO4. وبالنسبة إلى CoCl2 ، تم تحقيق أعلى القيم المعنوية للمحتوى الفينولي الكلي (8.40 مللي جرام /جرام وزن جاف) والنشاط المضاد للأكسدة (91.46٪) عندما تم دمج 2 مللي جرام / لتر من CoCl2 في بيئة الكالس مقارنةً بالكنترول (6.66 مللي جرام /جرام وزن جاف و 90.68٪ ، على التوالي) . كشفت الدراسة الحالية عن إمكانية استخدام مزارع الكالس كطريقة واعدة لإنتاج المواد الكيميائية النباتية من نبات الأرتيميزيا أنوا.

الكلمات الاسترشادية: أرتيميزيا أنوا، المستحثات، كبريتات النحاس، كلوريد الكوبالت، الكالس