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Original Article

The antioxidant and anti-tumor activities of the Lebanese *Centranthus Longiflorus* L.

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ABSTRACT

This study aimed to examine both the impact of growth period on the chemical composition and the *in vitro* anti-oxidant and anti-proliferative properties for *Centranthus longiflorus*. Aqueous and ethanolic extracts from leaves and stems, collected from two harvests, were tested to diagnose their different secondary metabolites content. This was conducted through classical phytochemical screening tests. In addition, a test for their antioxidant (DPPH and H_2O_2 radical scavenging) and for cell viability (Neutral red assay on HeLa cells), were conducted. The results have shown that both aqueous and ethanolic extracts from the different parts of the two *C. longiflorus* harvests contain alkaloid, coumarin, saponin, flavonoid, polyphenols, volatile oils and reducing sugars in different concentrations. Moreover, an antioxidant activity was observed by this plant where it inhibited the viability of HeLa cell line in a time-dependent (0–72 h) and dose-dependent (0–250 µM) manner. As such, the *in vitro* anti-oxidant and antiproliferative effects that were revealed from the two harvests of *C. longiflorus* reflect the significant potential use of this plant in the development of a new anti-cancer agent.

Keywords: antioxidant activity, antiproliferative activity, *Centranthus longiflorus*, phytochemical screening

INTRODUCTION

Cancer is considered a leading cause of death worldwide, accounting for 8 million deaths in 2010 [1]. It is caused by numerous factors ranging from environmental factors to hereditary genetics. Current treatment of cancer can be done by surgery, chemotherapy, and radiation therapy [2-4]. In addition, there is widespread use of Complementary and Alternative Medicine (CAM) in developed countries [5, 6]. A recent study estimated the overall prevalence for the use of herbal products to be 13% to 63% among cancer patients [6]. In Canada, the investigators found that 67% of the respondents in a randomized survey reported using CAM [7]. Thus, many investigations are now being carried out to discover naturally occurring compounds that can be used to suppress or prevent the process of carcinogenesis [8, 9]. Lebanon is among the countries that are highly rich in medicinal plants in the Mediterranean region. In this study, we are interested in a Lebanese plant, *Centranthus longiflorus* L., an endemic plant to the Mediterranean (Lebanon, Syria, Turkey, Italy and Palestine). It is known as red valerian. In the literature, there is no study on the phytochemical screening, antioxidant and anti-tumor activities of this plant. For that, our study aimed, for the first time, to determine the phytochemical screening for aqueous and ethanolic extracts of two harvest periods of *C. longiflorus* leaves and stems, and to evaluate their antioxidant capacity using two *in vitro* tests, hydrogen peroxide radical and DPPH radicals and also to determine their anti-proliferative activity against cervical cancer cell line (HeLa) by using neutral red cytotoxicity assay.

MATERIALS AND METHODS

Plant Material: *C. longiflorus* plant was collected from Mount Lebanon during 2013. The collection process covered two different periods, the first was on May (premature plant) and the second was on June (mature plant).

Preparation of Extracts: *C. longiflorus* leaves and stems were placed in the selected solvent (100 g of each part of the plant in 500 mL of distilled water or ethanol) after being well washed and divided into small pieces. Then, the paste was macerated

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and stirred for 8 hours at room temperature and then at 37 °C. After that, the macerate was filtered to remove insoluble residues. Subsequently, the filtrate was condensed by evaporating half of the solvent using a rotary evaporator. Finally, the filtrate was frozen before being lyophilized powder to be processed.

Phytochemical screening tests: To diagnose the phytochemical composition (alkaloids, tannins, resins, coumarins, saponins, phenols, terpenoids, flavonoids, volatile oils and carbohydrates) of the aqueous and ethanolic extracts of the leaves and stems of *C. longiflorus*, a qualitative detection was performed according to Muanda [10].

Antioxidant activity: To evaluate the antioxidant activity for each of the prepared samples, two *in vitro* tests (the free radical 2,2 -diphenyl- 1 - picrylhydrazyl (DPPH) and the H_2O_2 test) were performed according to Rammal et al. [11].

***DPPH radical scavenging*:** Increasing concentrations of each sample (0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) have been prepared. To 1 mL of each dilution of the part extract prepared, 1 mL of the DPPH reagent was added. The solution was incubated for 30 minutes at room temperature in the dark, and then the absorbance was measured at 517 nm. The control was prepared by mixing 1 mL of DPPH with 1 mL of used solvent (distilled water or ethanol) and the blank was composed of 1 mL of the selected solvent.

Scavenging with hydrogen peroxide (H_2O_2): A solution of H_2O_2 (40 mM) was prepared in PBS (pH 7.4). Various concentrations of plant part extracts were added to a solution of H_2O_2 (0.6 mL, 40 mM), and the absorbance was measured at 230 nm after 10 minutes against a blank containing PBS without H_2O_2.

Cells and cell culture: Cervical cancer (HeLa) cell line were grown in 25 cm² tissue culture flasks (Nunclon TM, Nunc) at 37°C, 5% CO_2 in DMEM: Dulbecco's Modified Eagle Medium (Sigma Chemical Company), containing 10% fetal bovine serum, penicillin 1% antibiotic solution (penicillin 50 U/mL and streptomycin 0.5 mg/mL). Once cell confluence had been reached, HeLa cells were transferred, under sterile conditions, into 48-multiwell plates (10.000 cells/well) for the toxicity tests.

Neutral Red Analysis: Cell viability was performed using Neutral Red assay based on the initial protocol as described earlier [12, 13]. Neutral Red, a chromogenic dye, is an indicator of lysosomal activity. Live cells demonstrate a chromogenic change with Neutral Red that is detected spectrophotometrically. Briefly, cells were detached from the tissue culture flask with 2 ml of trypsin solution. The cell pellet was obtained by centrifugation at 1.000 rpm for 5 minutes. The density of the viable cells was counted by the trypan blue exclusion in a haemocytometer. Cells were then plated in 96-well microtiter plate, at a concentration of 1×10^4 cells/well and incubated in a humidified 37°C, 5% CO_2 incubator that allows the cells to adhere. After 24 hours, the cells were treated with five different concentrations of aqueous and ethanolic extracts: 50, 100, 150, 200, and 250 μg/mL each being tested in three replicates. The plates were incubated for 24, 48 and 72 hours at 37°C in a 5% CO_2 incubator. The untreated cells were regarded as a negative control, whilst cells incubated only with ethanol (0.5%, v/v) were used as a vehicle control. No effect due to the ethanol was observed. Arsenic was used as the positive control. At 24, 48 and 72 hours, the old medium was replaced with 100 μL of fresh medium containing 40 μg/mL neutral red and incubated for 3 hours. This is to allow the uptake of the vital dye into the lysosomes of viable and undamaged cells. Then, the media was discarded and cells were washed twice with 100 μL of 1X PBS. The intracellular accumulation of neutral red dye was extracted in 200 µL of a 50% ethanol-1% acetic acid lysing solution. The optical density (OD) of the eluted dye was read at 490 nm using a microplate reader. The experiments were conducted in triplicates. The average of three replicates was then obtained.

Statistical analysis: Data are presented as mean ± standard deviation (SD) of three independent experiments and statistical significance was determined using the Independent Student's t-test and Prism 6.0 software (GraghPad Software, Inc.). A significant difference was considered if $P < 0.05$.

RESULTS AND DISCUSSION

Phytochemical screening: The results of the phytochemical screening represented in Tables 1 and 2 show that the different parts of *C. longiflorus* are rich in various secondary metabolites at different concentrations depending on the solvent used. Indeed, we note the presence of saponins, flavonoids, volatile oils, carbohydrates and coumarins in the aqueous extract from the different studied parts of this plant. On the other hand, the ethanolic extract was rich in secondary metabolites including: phenols, flavonoids, alkaloids, carbohydrates and volatile oils. Thus, there is a difference in the distribution of metabolites extracted using the same solvent between the leaves and stems of this plant. Stems are richer in

metabolites compared to leaves, which assigns them greater biological availability. Consequently, *C. longiflorus* by its richness in different secondary metabolites may have several medical importances. In addition, comparing the first and second harvest we see a difference in the distribution of metabolites for the second harvest which contains more metabolites than the first one mainly for the aqueous extracts. This can be attributed to the effect of the maturation of the plant, wherein in the first harvest (in May, the plant is at the beginning of the maturation) it scored less water and less temperature as the stems are still small, whilst in the second harvest (in June, the plant is mature) it had received less water and more temperature, and this can in turn influence the distribution of metabolites between the two harvests which may play important roles in various biological activities.

Antioxidant activity: The activity of chemical compounds draws our attention because of their potential role in the prevention of certain human diseases. A number of studies indicated that flavonoids, polyphenols and triterpenoids have an antioxidant activity and ability to scavenge free radicals (14). These phytoconstituents can have multiple biological effects against tumors, heart disease and various diseases due to their trapping free radicals. Hence our study was to evaluate the antioxidant power of the extracts through two different *in vitro* methods: DPPH and H_2O_2.

DPPH: Figure 1 show the increase in the antioxidant activity of the aqueous extract of leaves and stems of *C. longiflorus* with increasing concentrations. This increase reached 94 % and 87 % at the concentration of 0.5 mg/mL of leaves and stems respectively for the first harvest, while for the second one, it reached 87 % and 74 % at the same concentration of leaves and stems respectively. The ethanolic extract shows an antioxidant activity of 87 % and 79 % for leaves and stems respectively at a concentration of 0.5 mg/mL for the first harvest whilst for the second harvest this activity was about 88 % and 66 % at the same concentration for the leaves and stems respectively (figure 2). The obtained results show a difference in the antioxidant activity between the aqueous and the ethanolic extract in favor of the aqueous which agree with the results obtained in the phytochemical screening presented in table 1 and table 2. Indeed this extract was richer in flavonoids and polyphenols, which have an important role in the antioxidant activity. Furthermore, comparing the antioxidant activity between the first and second harvest indicates that it is higher in the first one. In order to confirm the antioxidant power of the two extracts of this plant, another *in vitro* method, H_2O_2 test has been used. The results show that aqueous extract has exerted an antioxidant potential up to approximately 93 % and 83 % for leaves and stems respectively at a concentration of 0.5 mg/mL for the first harvest whilst for the second harvest this activity was about 72 % and 64 % at the same concentration for the leaves and stems respectively (figure 3). Figure 4 shows an increase of the antioxidant activity of the ethanolic extracts of *C. longiflorus* in a concentration dependant manner. It has reached a percentage of 86 % and 75 % at the concentration of 0.5 mg/mL of leaves and stems for the first harvest whilst for the second harvest this activity was about 70 % and 79 % for the leaves and stems respectively. Hydrogen peroxide is an important reactive species of oxygen because of its ability to penetrate biological membranes. But, it can be toxic if it's converted into hydroxyl radical in the cell. Scavenging of H_2O_2 by the plant extracts can be attributed to the phenolic compounds giving an electron to H_2O_2, and therefore reduce the H_2O. Our results showed that extracts from the plant are capable of scavenging H_2O_2 depending on the concentrations used. These results showed that *C. longiflorus* has an important antioxidant activity and therefore it can be considered a good natural source that could be used in the treatment of diseases associated to the oxidative stress.

Cell viability effect of crude aqueous and ethanolic extract of *C. longiflorus* in HeLa cancer cells: An evaluation of the antiproliferative activity of each of the extracts prepared from leaves and stems of *C. longiflorus* was done by measuring the viability of the HeLa cell line using the Neutral Red Cytotoxicity/ Viability Assay after the treatment of this cancerous cell line for 24, 48 and 72 hours with increasing concentrations (50, 100, 150, 200, and 250 µg/mL) of these extracts. The effect of inhibition by these extracts from the first and second harvest was dose and time-dependent (figures 5, 6). The percentage of inhibition was calculated according to the formula mentioned above. The results of the Neutral Red assay for *C. longiflorus* showed that it has in its two studied parts (leaves and stems) an important antiproliferative activity. For the two extracts (aqueous and ethanolic), those of the second harvest possess greater antiproliferative activity than the first. Strongest inhibition percentages are those obtained from the aqueous extracts of stems and leaves of the second harvest. This inhibition was about of 96%, 98% and 99% after 24, 48 and 72 hours respectively at a concentration of 250 µg/mL for these two extracts (figures 5, 6).

DISCUSSION

Plant kingdom provides a rich source of potential cancer chemopreventive and therapeutic due to

some of its chemical ingredients alkaloids, taxanes, camptothecins and epipodophyllotoxins [15]. Researchers of anticancer natural products focused their research on a wide variety of natural compounds, especially on phenolic compounds. The antitumor effects of plant compounds have been associated with the induction of free radicals, anti-inflammatory activity, induction of cell cycle arrest or apoptosis, inhibition of tumor angiogenesis and metastasis [16, 17]. Several studies have shown that saponins, flavonoids, phenols, and tannins have an important role in the anticancer and in the antioxidant activity [18-21]. The presence of tannins, flavonoids, phenolics, and saponins, among others in the extracts used in the present study, may be responsible for the antiproliferative activities on the HeLa cell line. In our study, and for the first time, the antioxidant and antiproliferative properties of *C. longiflorus* have been evaluated. The obtained results indicated that, during the two harvest period, different concentrations of both aqueous and ethanolic extracts from the leaves and stems of this plant have exerted an important antioxidant potential using two in vitro tests, DPPH and H_2O_2 with a significant antiproliferative capacity. On the other hand, both ethanolic and aqueous extracts of the second harvest might be a significant source of novel promising anticancer compounds in view of their pronounced antiproliferative activity against HeLa cells. This activity may be at least in part due to the phenolic and flavonoids compounds. It is also important to note that second harvest extracts is found to be more effective than first harvest.

Conclusion: In conclusion, for the first time, the results of this study suggest that aqueous and ethanolic extracts of first and second harvest of *C. longiflorus* has a promising anti-oxidant, antiproliferative on HeLa cervical carcinoma cells. Further investigations are needed to determine the entire anti-cancer molecular mechanism of extracts. Also, it is very important to determine the cancer-suppressive effect of the tested extracts in *in vivo* experiments.

Acknowledgements: We thank the Lebanese University for financial support.

Table 1: Comparison of the phytochemical composition of crude aqueous extracts from leaves and stems of *C. longiflorus*

	First harvest		Second harvest	
	Leaves	**Stems**	**Leaves**	**Stems**
Alkaloids	+++	-	-	++
Tannins	-	-	-	-
Resins	-	+	-	-
Coumarins	+	+	+	+
Saponin	++	++	+	+
Phenols	-	-	++	+
Terpenoids	-	-	+	-
Volatile oils	+	+	+	+
Flavonoids	+	++	+	+++
Carbohydrates	-	+	+++	+++
Glycosides	-	-	-	-

Table 2: Comparison for the phytochemical composition of crude ethanolic extracts from leaves and stems of *C. longiflorus*

	First harvest		Second harvest	
	Leaves	**Stems**	**Leaves**	**Stems**
Alkaloids	+	+	-	+
Tannins	-	-	-	-
Resins	-	+	-	+
Coumarins	+	++	+	+
Saponin	+	+	-	-
Phenols	+	-	+++	-
Terpenoids	-	-	+	-
Volatile oils	+	+	+	+
Flavonoids	++	+++	+	+++
Carbohydrates	+	++	+	++
Glycosides	-	-	-	-

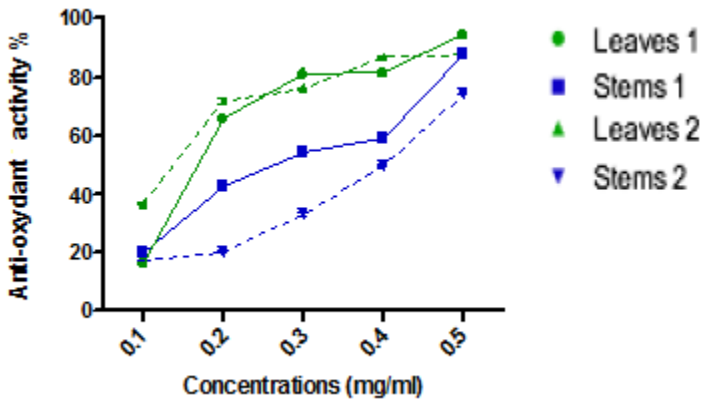

Figure 1: Antioxidant activity of aqueous extracts of *C. longiflorus* (leaves and stems) from the two harvest period (1= first harvest & 2 = second harvest) measured by the DPPH radical scavenging activity

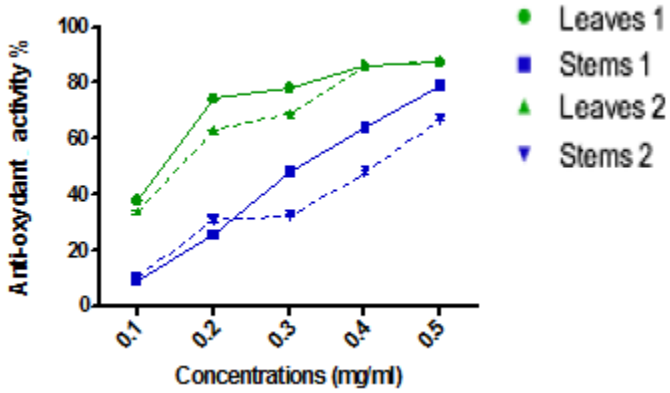

Figure 2: Antioxidant activity of ethanolic extracts of *C. longiflorus* (leaves and stems) from the two harvest period (1= first harvest & 2 = second harvest) measured by the DPPH radical scavenging activity.

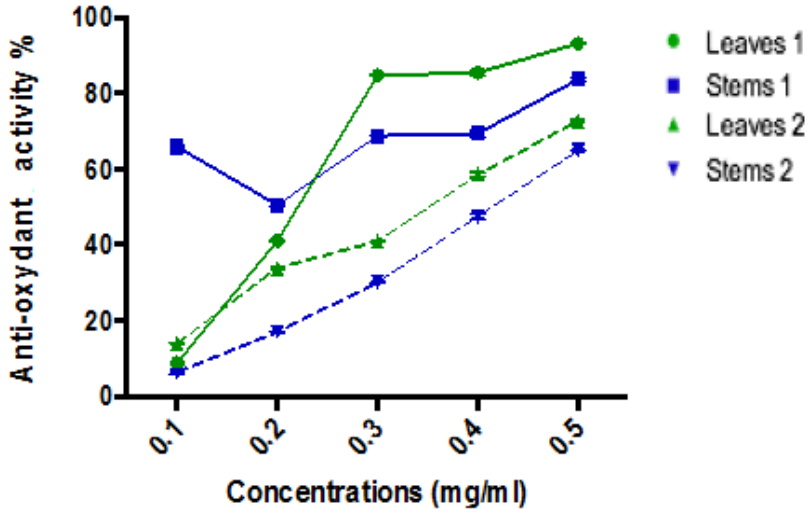

Figure 3: Antioxidant activity of aqueous extracts of *C. longiflorus* (leaves and stems) for the two harvests (1= first harvest & 2 = second harvest)

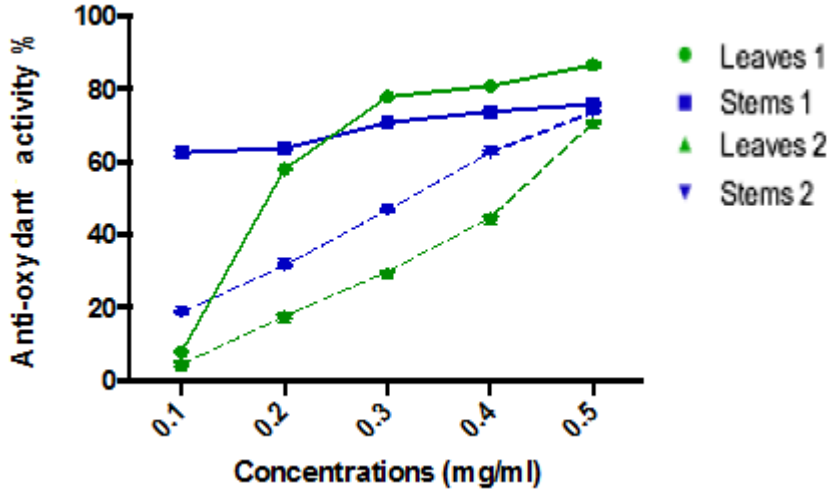

Figure 4: Antioxidant activity of ethanolic extracts of *C. longiflorus* (leaves and stems) foe the two harvests (1= first harvest & 2 = second harvest)

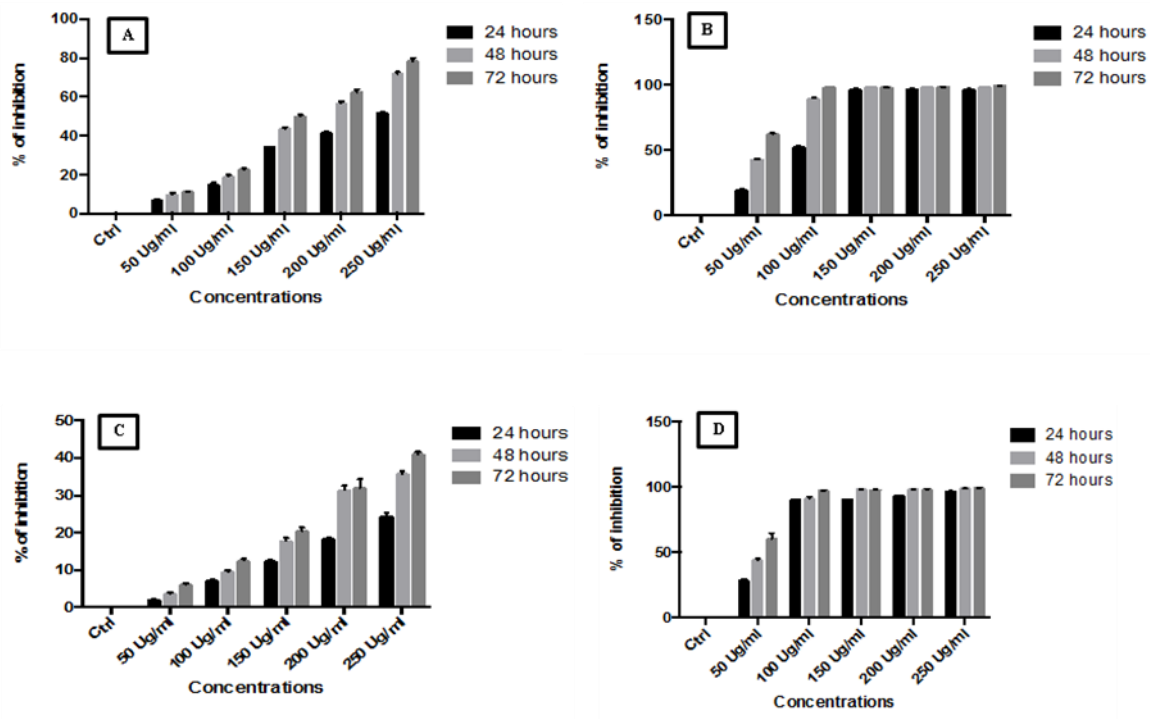

Figure 5. Effect of crude aqueous extract of *C. longiflorus* plant on viability (% of inhibition) of HeLa cancer cells as determined by Neutral Red assay. (A-B) HeLa cells were treated with (0-250 µg/ml) of aqueous extract from the first (A) and second harvest (B) of *C. longiflorus* leaf for 24-72 h. (C-D) HeLa cells were treated with (0-250 µg/ml) of aqueous extract from the first (C) and second harvest (D) of the stems of *C. longiflorus* for 24-72 h. Each experiment was done in triplicate. Data are expressed as mean ± SD. *$p < 0.05$ was considered to be statistically significant, **$p < 0.01$ very significant, and ***$p < 0.001$ highly significant.

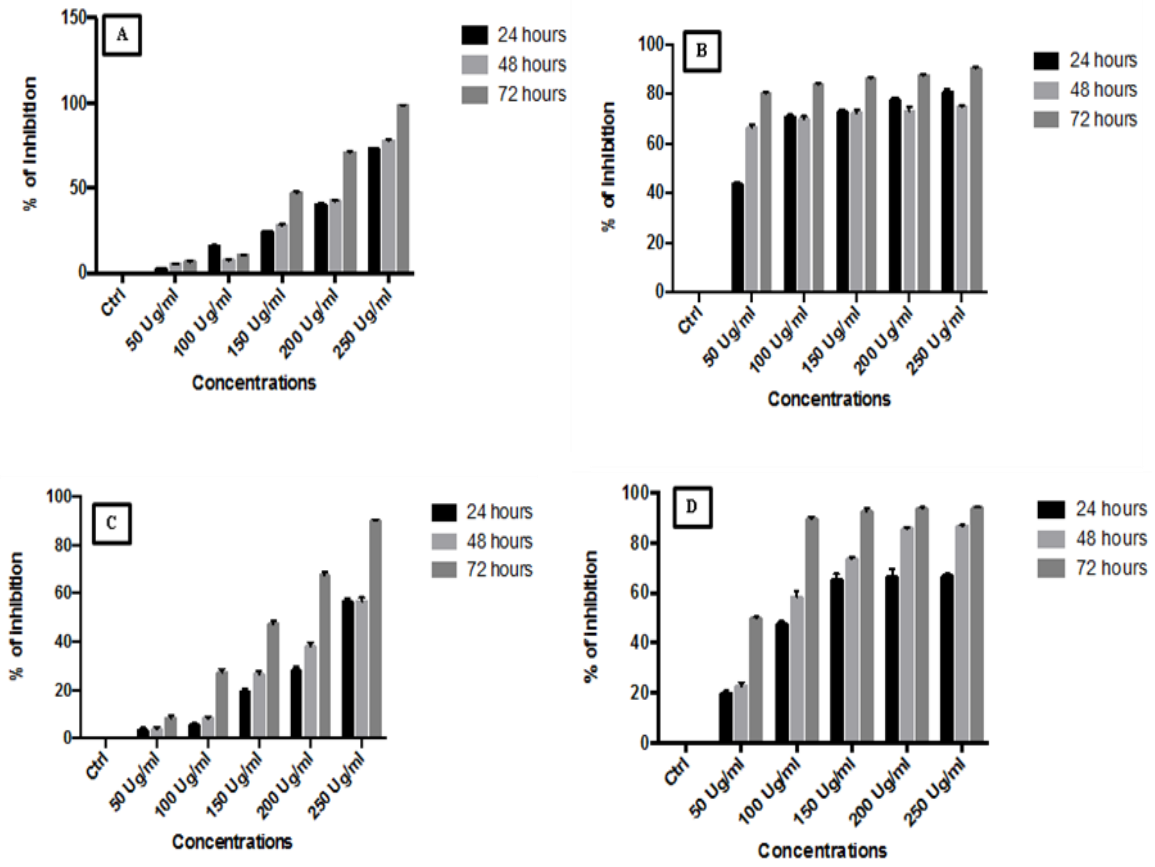

Figure 6. Effect of ethanolic extract of *C. longiflorus* plant on viability of HeLa cancer cells as determined by Neutral Red assay. (A-B) HeLa cells were treated with (0-250 µg/ml) of ethanolic extract from the first (A) and second harvest (B) of *C. longiflorus* leaf for 24-72 h. (C-D) HeLa cells were treated with (0-250 µg/ml) of ethanolic extract from the first (C) and second harvest (D) of the stems of *C. longiflorus* for 24-72 h. Proliferation was measured by Neutral Red assay. Each experiment was done in triplicate. Data are expressed as mean ± SD. *p < 0.05 was considered to be statistically significant, **p < 0.01 very significant, and ***p < 0.001 highly significant

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