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INFLUENCE OF LEAD, CADMIUM, AND ZINC ON PHENOLS, FLAVONOIDS AND ANTIOXIDANT ACTIVITY IN CAULIFLOWER (*BRASSICA OLERACEA*. VAR. *BOTYRIS*)

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Abstract

This study was conducted to assess the impact of toxic heavy metals (lead, cadmium, and zinc) present in polluted waste-water on some phytochemical attributes (phenolic, flavoniods and antioxidant activity) in cauliflower plant. For this purpose, fresh and waste-water irrigating cauliflower samples were collected from different industrial sites of districts Narowal, Lahore and Kasur, Punjab, Pakistan. These samples were used for the estimation of Pb^{2+}, Cd^{2+} and Zn^{2+}, phenols, flavonoids and antioxidant activities, to find out their accumulation and effects on cauliflower plants. The average concentration of Zn in sewage-irrigated samples was the highest (164.53 $mgkg^{-1}$), followed by Cd^{2+} (81.91 $mgkg^{-1}$) and Pb (72.81 $mgkg^{-1}$). Fresh-water Irrigated Cauliflower Samples (FICS) showed overall maximum (337.33 mgg^{-1}) total Phenolic Contents (TPC) from Narowal followed by Kasur region. In the current study, heavy metals generally reduced the TPC in WICS compared to FICS. The maximum flavonoid contents (179.33 mgg^{-1}) were observed in wastewater irrigated Cauliflower samples from Narowal followed by Lahore. It was observed that antioxidant activity was decreased in WICS or in heavy metal stress samples, while an increase in flavonoids was recorded in the plant that were irrigated with wastewater.

Key words: Cauliflower; Industrial wastewater; Heavy metals; Phytochemicals

Introduction

Vegetables are the most important food and highly supportive for prevention from various chronic diseases, healing of body organs, alkaline reserve for body maintenance and are good source of carbohydrates, minerals and vitamins (Ulgar *et al*., 2018). *Brassica oleracea*. var. botyris, commonly known as "Cauliflower" is a member of family *Brassicaceae*. The *Brassica* genus comprises of over 30 species with many varieties and hybrids. These are the source of animal fodder, human consumption, condiments, biofuel, oil production (Sharma *et al*., 2014; Schmidt & Bancrft, 2010). Many *Brassica* spp. are also known for metal accumulators and are considered as potential phyto-extraction plants (Gall & Rajakaruna, 2013). The pertinent concern is relatively high proportions of toxic metal absorption in these plants with no visible symptoms. Moreover, it was observed that heavy metals diffuse not only into surface but also to underground waters and ultimately enter the food chain, and are considered as bio accumulate (Grazuleviciene *et al*., 2009; Gashi *et al*., 2017). Heavy metal in vegetables due to some contaminated soils and waters has now become a global issue. These heavy metals easily taken up and accumulated into the edible parts of vegetables (Mohammadi *et al*., 2020). Once these vegetables containing high levels of heavy metals are consumed by human beings, these may cause many medical and physical complications (Jaishankar *et al*., 2014).

With the accumulation of heavy metals, plants use different defense mechanisms especially enzymatic and non-enzymatic antioxidant systems. Free radical-induced oxidative stress, antioxidants are the important species which can protect organisms from this stress (Mathew *et al*., 2011). The stress of heavy metal is also associated with the enhanced production of Reactive Oxygen Species (ROS), free radicals, for instance; superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), or non-free radical species like molecular oxygen [O] and hydrogen peroxide (H_2O_2) as well as cell poisoning compounds like methylglyoxal (Imran *et al*., 2015). The plants primarily produce several ROS under the heavy metal stress conditions. These ROS result into a number of diseases, like neurodegenerative, cancer and aging etc. which commonly aappear in the mitochondrial respiratory chain (Ercan *et al*., 2018).

Wastewater irrigation in soil due to heavy metals has become an ecological issue in Pakistan (Bhutto *et al*., 2009). It is an alternative supply to low quality water and to enhance the vegetable crop yield in urban and semi-urban agricultural lands of Kasur, Lahore and Narowal districts. Such effluents are one of the main reasons of heavy metals stress in modified crops and soils in these cities (Rehman *et al*., 2018; Mahmood & Malik, 2014). Less attention on vegetables contamination due to heavy metal has been paid in Pakistan (Sabeen *et al*., 2020). Therefore, present study was aimed to evaluate the heavy metal uptake and its impact on some phytochemicals and antioxidants in *B. oleracea* plants grown by using polluted water in various areas of Punjab, Pakistan.

Materials and Methods

The experimental work was carried out in Pakistan Council for Scientific and Industrial Research (PCSIR) Lahore and University of Punjab Lahore, Pakistan. Samples of Cauliflower were collected from different industrial sites from districts Narowal, Lahore and Kasur, Pakistan. They were labeled as irrigated and waste-watered (Fig. 1). From each site, total 6 samples were collected, 3-fresh and 3-wastewater irrigated, respectively (Fig. 1; A-F). Samples were dried under sun for 12-15 days and stored in sterilized seal bags for further analysis of phytochemicals.

Fig. 1. (A-I); A: Photograph showing waste-water irrigation cauliflower sample from Narowal, B: Lahore and C: Kasur, D: Fresh water irrigation cauliflower sample from Narowal, E: Lahore and F: Kasur. G-I: Wastewater site of Narowal, Lahore and Kasur, respectively.

Digestion of Samples: All the glass and plasticware used in this study were carefully washed by using detergent, rinsing with tap water followed by soaking in HCl (2:1) and dipped in metal-free water. From each dried cauliflower sample, 1 gm grounded fine powder was added to a flask containing concentrated HNO_3 (4 mL) and HCl (12 mL). Sample and acid mixture was allowed to stand for at least 12 hours for completion of reaction. Later mixture was boiled for 2 hours, cooled, rinsed with 15 mL of deionized water. Digested mixture was filtered through pre-washed Whatman No. 540. Each filtrate volume of upto 100 mL was made by using ultra 2M HNO_3. The prepared samples were stored at 4°C in acid-washed polyethylene bottles (Mapanda *et al*., 2005). The assessment of heavy metals concentrations were carried out by a flame atomic absorption spectrophotometer (AAS, Model-A, Shimadzhu Analyst-800, Japan) using standard solutions of metals with help of hallow cathode lamp. The concentration of heavy metals i.e., Cadmium (Cd), Lead (Pb), and Zinc (Zn) (Perkin Elmer; 2000) were expressed as mg Kg^{-1}.

$$\frac{Concentration\ \left(\frac{mg}{L}\right) Dilution\ \times\ Factor\ (mL)}{weight\ of\ the\ Sample\ (g)}$$

Phytochemical analysis

Estimation of phenols, flavonoids and antioxidant activities in each cauliflower sample was done by the following methods.

Preparation of plant extract: Dried cauliflower sample (0.50 g) was put into ethanol and acetone (in 6 mL solution). After 24 hours at 25°C temperature, the sample was placed on an Orbital Shaker (Lab-line 3520 at 1.5 RPM) along with a stoppered flask with occasional shaking. After that, filtration of sample's suspension was carried out by Whatmann filter paper No. 1. Filtrated ethanol was evaporated in pre-weighed petri-dish; sample extract was mixed in ethanol (2-3 mL) or DMSO (Dimethyl Sulphoxide) as per requirement and was stored in amber colored bottles at 4°C in refrigerator. This extract was used to estimate phenols, flavonoids and antioxidant activitiy.

Determination of phenolic contents: For the determination of total phenols, Folin-Ciocalteu reagent was utilized (Folin & Ciocalteu, 1927). A mixture of 0.2 mL volume of sample extract with Folin-Ciocalteu reagent (1:10 diluted, 1.5 mL) and 3.75 mL volume of 20% Na_2CO_3 was prepared. After 15 minutes, the total phenols were measured at 760 nm against blank using UV-spectrophotometer and Gallic acid utilized as a standardized scale. Total phenol values of the corresponding extract were drawn w.r.t. dry mass of the extract using Gallic Acid Equivalent (GAE; mgg^{-1}).

Determination of flavonoid contents: The mixture of 0.5 mL sample extract with 0.25 mL of 5% aluminum chloride, 3.25 mL methanol, 0.25 mL of 1M potassium acetate were separately mixed. Then, volume was made up to 25 mL in the volumetric flask and kept at 25°C for half an hour. $AlCl_3$ calorimetric process was employed for the determination of flavonoids activity (Chang *et al*., 2002).The absorbance of reaction mixture was measured by using UV-spectrophotometer at 415 nm against blank and Quercetin solutions at concentrations 50-100 μgmL^{-1} in methanol. Calibration curve was prepared??

Antioxidant activity assay: The free radical scavenging using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method of Manzooco *et al*., (1998) was followed for the analysis of antioxidant enzymes activity by minor changes. Plant extract was used for making dilutions by adding 0.2 mL sample extract with 0.8 mL ethanol and 3 mL DPPH solution. After 30 minutes of incubation at 25 °C, the absorbance was measured at 517 nm. The DPPH radical scavenging percentage was measured from the calculated absorbance data and as a reference or standard antioxidant in this assay method, Butylated Hydroxy-Toluene (BHT) was used.

$$\text{DPPH (\% inhibition)} = \frac{(\text{Abs.of blank} - \text{Abs.of sample})}{\text{Abs of blank}} \times 100$$

Different percentages (20, 60, and 100) of each sample have been evaluated for antioxidant activity in DPPH assay.

Statistical Analysis

Duncan Multiple range test at significance level of $p<0.05$ was used to anlyse the data by using SPSS version 26.0.0 wherever required.

Results and Discussion

Heavy metal concentration in vegetables: The results revealed that Zn^{2+}, $Pb^{2+}$and Cd^{2+} concentration was significantly higher in cauliflower in WW than those grown in FW. However, $Pb^{2+}$and Cd^{2+} concentration from cauliflower grown on WW, surpassed the approved limits (European Union, 2002), while it was in the Indian safe limits (Awashthi, 2000). Concentration of Cd^{2+} in all cauliflower samples, exceeded to the EU safe limits (European Union, 2002), irrigated with FW. Cauliflower samples from Lahore region showed maximum Cd^{2+} concentration (81.91 $mgkg^{-1}$), (Table 1) followed by Narowal (61.66 $mgkg^{-1}$). Whereas Kasur samples grown on WW showed the maximum Pb^{2+} concentration (65.79 $mgkg^{-1}$). However, Pb^{2+} concentration (0.0001 $mgkg^{-1}$) was not considerable in FW samples from Narowal. Among heavy metal concentration in cauliflower grown on WW the trend appeared as Zn > Cd > Pb while in samples grown at FW the trend appeared as Zn > Pb > Cd (Table 1). Heavy metals are generally considered very dangerous because they are normally accumulated in the living systems thereby causing not only injurious effects but also accumulate in food chain. Wastewater irrigation may lead to the high risk to consumer's health and cause of various chronic diseases. Heavy metal concentration in cauliflower grown in waste-water (WW) and freshwater (FW) was also compared in conjunction with the acceptable limits in literature and in this investigation (European Union, 2002; Awashthi, 2000). In a study Khilji *et al*., (2021) investigated the toxic effects of heavy metals by irrigating the plants with waste water from paper sludge and observed that their accumulation caused sever stress in plants and hence retarded the growth. The presence of toxic metals in the wastewater of various industries in Pakistan might be the reason of chemicals or raw materials used for the processing and finshing of various product. It was further observed that type of metals also varies from industry to industry (Khilji & Sajid, 2020).

Total phenolic contents (TPC): It was observed that ethanolic extract of Freshwater Irrigated Cauliflower Samples (FICS) showed maximum (337.33 mgg^{-1}) TPC collected from Narowal followed by Kasur region. While it's minimum value (47.3 mgg^{-1}) was recorded in ethanolic extract of Wastewater Irrigated Cauliflower Samples (WICS) from Lahore. However, in case of acetonic extract, this result was opposite in WICS where minimum phenolic (19.334 mgg^{-1}) contents were found in FICS, collected from Lahore followed by Kasur (Fig. 2). It was observed that phenolic compounds were generally greater in the leaves of control groups compared to CS leaves planted in heavy metals. In the current study, heavy metals have generally reduced the TPC in WICS

compared to FICS. Our results were inline with previous observation of Elguera *et al*., 2013 who reported that total phenolics were likely to be reduced by means of enhancing metal concentration in *Lepidium sativum* leaves. Heavy metal stress in plant is a leading cause of some biochemical, physiological changes and production of free radical has been well reported in literature (Zhushan & Shuhua, 2020). In plants, the generation of superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (HO•) which are mutually termed as Reactive Oxygen Species (ROS), is due to oxidative stress prompted by heavy metals present in polluted water (Georgiadou *et al*., 2018). So, these ROS are the chief cause of death in plants by specifically damaging membrane proteins, phenolic contents, plants lipids, nucleic acids and pigments related to photosynthesis on account of high reactivity. Plant cells have their own inclusive, sound and unified internal antioxidant defense system made up of both non-enzymatic and enzymatic constituents to mitigate the damages caused by the ROS. The detoxification of these ROS by the induction of specific enzymes is significant in the defense against oxidative stress due to toxic metal concentrations (Unal *et al*., 2015).

Total flavonoid contents (TFC): Flavonoids possess numerous protecting functions like enhancing the antioxidative activity (Rusak *et al.*, 2005). Hence, they are also considered as defender contrary to heavy metal stress. The maximum flavonoid contents (179.33 mgg^{-1}) were recorded in ethanolic extract of WICS collected from Narowal followed by FICS from Lahore (Fig. 3). Whereas minimum flavonoids (31.30 mgg^{-1}) were evaluated in ethanolic extract of WICS from Lahore followed by Kasur. However, in case of acetonic extracts, there were an opposite relationship of FICS and WICS. The maximum flavonoid (143.15 mgg^{-1}) contents were found in acetonic extract in FICS, collected from Narowal and Lahore. It was also observed that ethanol extract exhibited maximum value of flavonoids than that of acetone except cauliflower sample from Kasur. Cadmium and some other metals inhibit the action of antioxidative enzymes, specifically glutathione reductase (Adinuta *et al*., 2015). As a result, substitute antioxidants, like flavonoids, could be over-produced (Michalak, 2006). Heavy metals make complexes with these recognized compounds, and thus might be an excellent plants self-protective response to heavy metal stress (Korkina, 2018).

Antioxidant activity: FICS showed the highest antioxidant activity treated with ethanol and collected from Narowal followed by Kasur region. Whereas, in case of acetone, the minimum range has been shown in FICS compared to WICS. In this study, results indicated that antioxidant activity increased under the heavy metal stress (Fig. 4). This increase in antioxidant activity under heavy metal stress was also observed by Sajid & Khilji, (2020). Further, it was also noticed that with the increase of the phenolic contents, antioxidant activity was also increased. These results are in agreement with those of Kisa (2018), his findings indicated that generally by increasing the total phenols antioxidative activity also increased significantly. Another study carried out by (Márquez-García *et al*., 2012) also highlighted a linear correlation between phenolic content and antioxidative activity. Increase in phenolics contents, mediates the lignin biosynthesis a sign of the distinctive anatomical change induced by all type of stressors. It leads to an increase in the endurance of cell wall and the physical barriers establishment preventing callus formation against heavy metals drastic effects (Diaz *et al*., 2001). Therefore, heavy metals exposure too many plant roots release high phenolics level (Ghori *et al*., 2019).

Conclusions

Plants growing in polluted/contaminated areas have the ability to gather heavy metals at higher contents. Heavy metals are generally very dangerous because they normally accumulated in the living systems thereby causing not only injurious effects but accumulate in food chain. Wastewater irrigation vegetable could possibly result into the high risk to consumer's health and cause of various chronic diseases. The antioxidant activity was enhanced with the rise in total phenolic contents due to heavy metal toxicity, while a reduction in flavonoid activity was recorded in the plants irrigated with wastewater. An increase in phenolics is an indication of protection of plant cells against harmful action of heavy metals. The present study suggests that serious attention should be given for regular monitoring and regulating the municipal and industrial effluents.

Table 1. The heavy metals averaged concentrations ($mgkg^{-1}$) in Cauliflower Samples (CS) irrigated by both waste and freshwater in three districts of Punjab, Pakistan.

Region	Sample	Cd	Pb	Zn
NRL	FICS	5.497 ± 0.45	-	59.12 ± 2.41
	WICS	61.66 ± 2.41	30.27 ± 1.42	82.49 ± 3.45
KSR	FICS	6.452 ± 0.75	19.91 ± 1.25	90.41 ± 2.22
	WICS	39.76 ± 1.45	72.81 ± 2.95	164.53 ± 2.45
LHR	FICS	7.455 ± 1.40	10.51 ± 1.40	49.66 ± 1.45
	WICS	81.91 ± 2.15	32.82 ± 1.45	89.21 ± 2.11
EU*	NA	3	100	300
Ind.St*	NA	3-6	250-500	300-600

EU*= European Union standard (2002); Ind. St*= Indian standard, Awashthi (2000); FICS= Freshwater Irrigated Cauliflower Samples; WICS = Wastewater Irrigated Cauliflower Samples

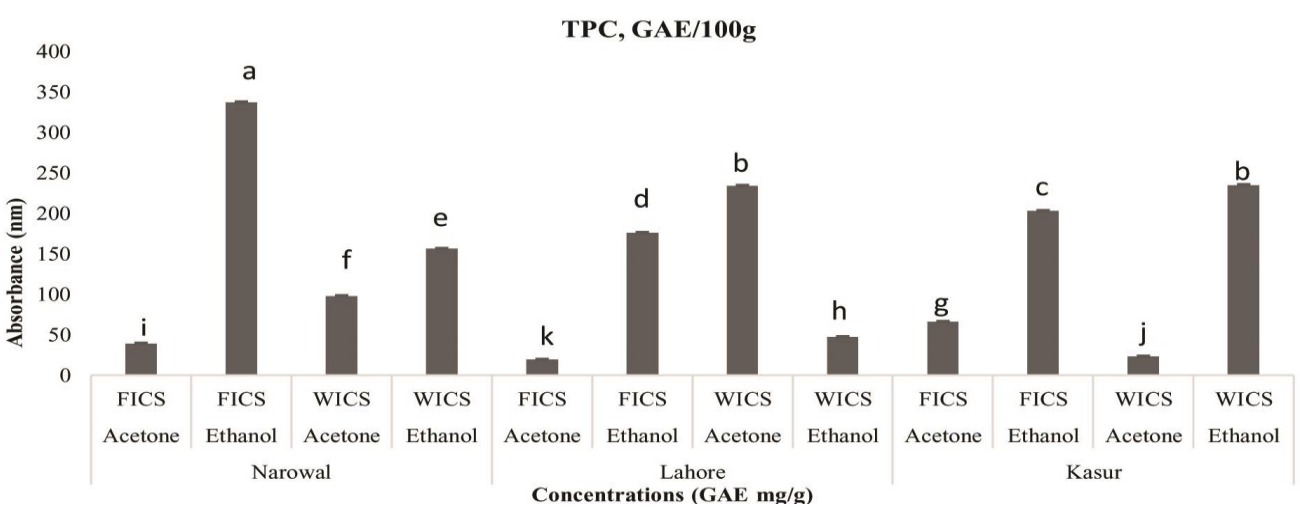


Fig. 2. Determination of total phenolic contents of Cauliflower.

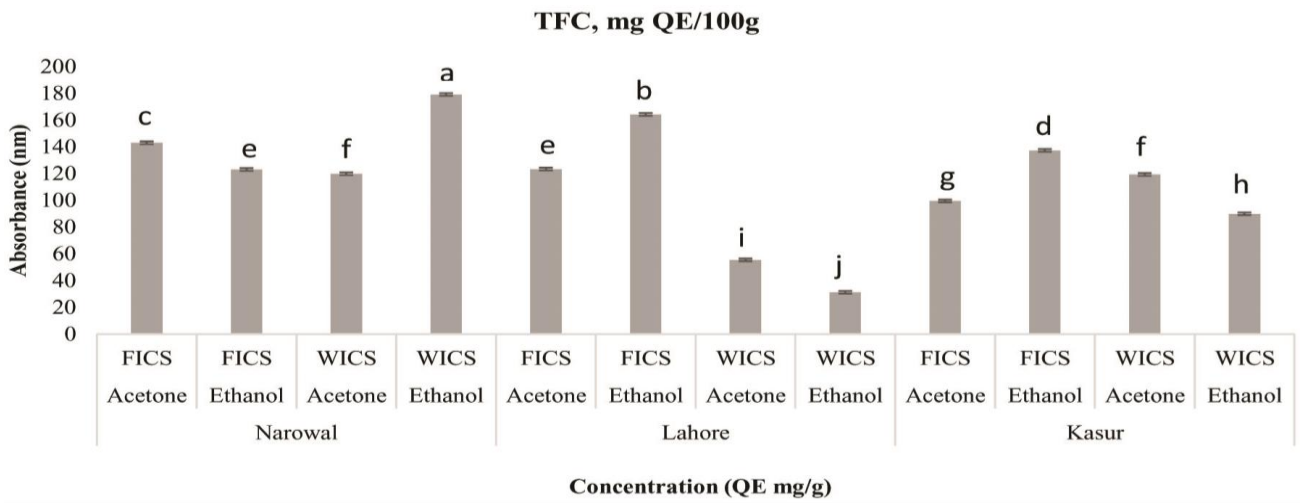
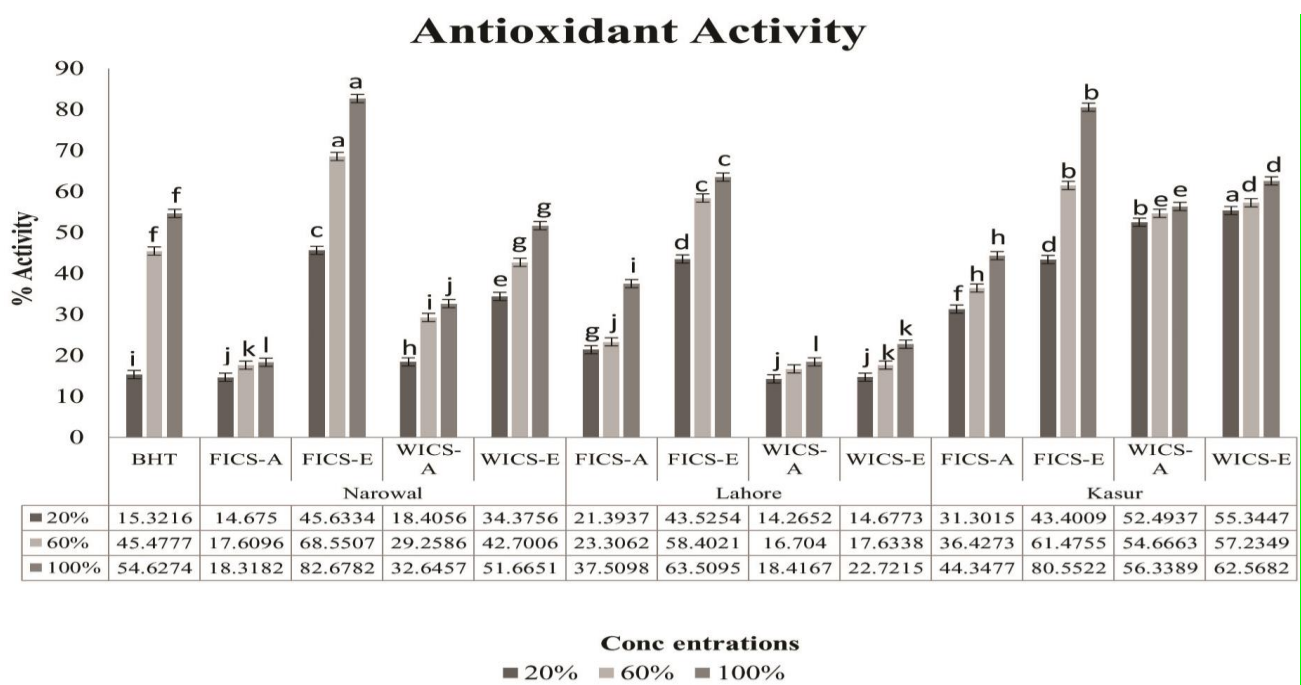


Fig. 3. Determination of total flavonoid contents of Cauliflower.



	BHT	FICS-A	FICS-E	WICS-A	WICS-E	FICS-A	FICS-E	WICS-A	WICS-E	FICS-A	FICS-E	WICS-A	WICS-E
	Narowal					Lahore				Kasur			
20%	15.3216	14.675	45.6334	18.4056	34.3756	21.3937	43.5254	14.2652	14.6773	31.3015	43.4009	52.4937	55.3447
60%	45.4777	17.6096	68.5507	29.2586	42.7006	23.3062	58.4021	16.704	17.6338	36.4273	61.4755	54.6663	57.2349
100%	54.6274	18.3182	82.6782	32.6457	51.6651	37.5098	63.5095	18.4167	22.7215	44.3477	80.5522	56.3389	62.5682

Fig. 4. Percentage Antioxidant activity of Cauliflower in comparison with BHT as standard reference by DPPH.

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Conflict of Interest: No conflict of interest is declared by the authors.

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