Kütahya Dumlupınar University
Institute of Graduate Studies

Journal of Scientific
Reports-A
E-ISSN: 2687-6167
Number 48, March 2022

RESEARCH ARTICLE

MIXTURES of PHARMACEUTICALS and PERSONAL CARE PRODUCTS (PPCPs) EFFECT PLANT STRESS MARKERS and NUTRIENT UPTAKE in WHEAT and BARLEY

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Received Date:06.01.2021 *Accepted Date:02.03.2022*

ABSTRACT

Common drug active substances (gemfibrozil, β-estradiol, caffeine, and ciprofloxacin) were combined in simple mixtures and applied to soil containing wheat or barley. Mixtures in water (50 µg/mL) were applied twice during the growing period. Harvested plant samples were extracted and antioxidant enzyme activities, as indicated by CAT, POD, and SOD were compared in exposed and control plants. Lipid peroxidation markers were also determined. Finally, mineral element uptake in exposed and control plants was determined. The data indicated differences in several, if not all, of the plant biomarkers between control plants and those treated with simple mixtures of pharmaceutical substances; many were directly related to oxidative damage. It was determined that there were statistically significant differences in the element intake of 8 nutrients (magnesium, potassium, phosphorus, calcium, zinc, mangan, copper, and iron) in control plants compared with plants treated with PPCPs; we observed both increases and decreases in plant nutrients depending on the particular nutrient, pharmaceutical treatment, and plant species.

Kewwords: *Antioxidant Enzymes, Barley, Mineral Element, PPCPs, Wheat*

1. INTRODUCTION

Besides the numerous different chemicals produced today, an increase in population leads to increased consumption of chemicals such as pharmaceuticals and personal care products (PPCPs) [1,2]. It has been estimated that the consumption antibiotics is 100,000 – 200,000 tons on a global scale; almost 15,000 tons of antibiotics are released in Europe each year [3]. According to data from Turkey, pharmaceutical use increased 1.4 fold from 2010 to 2016 (1.62 billion units to 2.23 billion units). In addition, in 2016, about 440 new pharmaceuticals were released to the market worldwide [4]. Antibiotics, antimicrobials, painkillers, allergy medicines, caffeine etc. are just a few of the substances that make up PPCPs.

PPCPs have recently been shown to be among the most common contaminants in the environment [3]. The entry of pharmaceutical substances into an ecosystem can occur in many ways. PPCP waste, sewage sludge, pharmaceutical producers, food companies, and fish farms are some of the sources of PPCPs into the environment. The cycle begins with animal (including human) use, entrance into the

wastewater treatment system, discharge to surface water or water re-use, and eventually to compartments where exposure occurs. Research has shown that many PPCPs are excreted from humans without metabolism [1,2]. In addition, these micro-pollutants can often interfere with sewage treatment systems and be relatively resistant to degradation at wastewater treatment plants [2,5]. Pollution of the environment from PPCPs is an important problem and can have a negative impact on living organisms. Increased water shortages due to population growth, urbanization, and the climate change has brought about interest in the recycling of treated wastewater, particularly in many arid and semi-arid regions of the world. Today, in many countries, treated wastewater is applied to land for agricultural irrigation [6].

The potential impact of PPCPs in recycled water on soil processes and plants has not been fully described. The potential for biological degradation of these micro-pollutants or their interactions with each other is largely unknown. Studies conducted to date indicate that plants can uptake some PPCPs; the transport of PPCPs in the environment depends on their physical properties such as volatility, lipophilicity water solubility, and sorption potential. In addition, -properties of the soil are also important [7-10]. Several studies have shown that plants are affected by exposure to PPCPs; effects include impact on photosynthetic pigments, number and size of leaves, inhibition of root growth and development, and physiological functions. Any negative impact on plants may also have an effect on soil microorganisms, particularly those in the root zone or rhizosphere. This is a potential concern for the symbiotic relationship between plants and microorganisms and for nutrient cycling in the soil. Although not all PPCPs and plants have been evaluated, it appears that most PPCPs don't induce phytotoxic effects. PPCPs may be metabolized, detoxified, inactivated, and sequestered following uptake by plant roots. However, recent studies have shown that oxidative activities triggered by ROS overproduction are a potential adverse impact of PPCP exposure to plants; oxidative damage can be the phytotoxic response from prolonged exposure to pharmaceutical substances [11,12]. Generally, the potential effects of PPCPs on plants can be evaluated by monitoring ROS production and subsequent oxidative damage Osma et al. 2018 [13]; the responses of plants can vary according to plant species. Recent studies indicate that the impacts of individual PPCPs manifests at relatively high exposure concentrations. Plant responses can serve as a monitoring tool for evaluating the presence of PPCPs [3,14-17].

Data on the possible negative impacts to plants of PPCP mixtures are scarce. The types of interactions (additive, synergistic, or antagonistic) that PPCPs might have, even for relatively simple mixtures is in need of further evaluation. Our goal with this research was to determine the effects of simple (binary) mixtures of PPCPs on plant stress markers.

2. MATERIAL AND METHODS

In this study, simple mixtures of four medicinal active substances (gemfibrozil, β-estradiol, caffeine, and ciprofloxacin) were evaluated for impacts on wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). The cultivation process was carried out in the laboratory according to methods described by [13]. Briefly, for sowing, 5 g of barley and 7 g of wheat seeds were planted in 650 g of soil and covered with an additional 100 g of soil. Based on the field capacity, soil samples were moistened with deionized water. After germination of wheat and barley seeds, gemfibrozil and estradiol, gemfibrozil and caffeine, or gemfibrozil and ciprofloxacin mixtures (50 µg/mL) were applied twice during the experiment. Wheat was harvested at the end of 15 days growth; barley at the end of 11

days. Biomass of wheat and barley plants were determined gravimetrically. A portion of the plant samples was assigned for use in subsequent physiological and biochemical investigations.

Malondialdehyde is one of the peroxidation products of polyunsaturated fatty acids formed by the increase of free radicals. The amount of malondialdehyde (MDA) was measured using thiobarbituric acid (TBA) method with slight modifications [18]. Leaf samples were weighed approximately 0.5 g/each and homogenized by adding 5% trichloroacetic acid (TCA). The leaf homogenates were then centrifuged at 15,000 x g for 15 minute at 4 °C. To each 1 mL aliquot of the supernatant, 2 mL TBA reagent (0.5 % TBA in 20% TCA, w/v) was added. 1 mL of 0.1 % TCA and 2 mL TBA reagent were combined as a negative control. Test and negative control tubes were heated at 95 °C for 30 min and were then rapidly cooled in an ice bath. Chilled tubes were centrifuged at 15,000 xg for 15 minute at 4 °C. Following centrifugation the absorbance of the supernatant at 532 was determined. The absorbance of non-specific molecules is read at 600 nm and was subtracted from absorbance of samples. MDA amount was determined by its molar extinction coefficient at 532 nm (155 mmol/L^{-1}·cm^{-1}) [13].

First, 0.1 g of fresh plant sample harvested from plant leaves was put in each of 12 test tubes. 4 mL of distilled water was added into the tubes and kept at 4 °C for 24 h. Then, amounts of ions in distilled water from the samples collected to detect the damage to cells were measured by electrical conductivity meter [13, 19].

After 0.5 g tissue were weighed and put into the porcelain mortar, 5 mL of cold homogenate buffer was added to it (0.1 M KH_2PO_4 at pH 7.0 containing 1 % PVP and 1 mM EDTA). The mixture was transferred into a centrifuge tube and centrifuged at 15000 × g and at 4 °C for 15 min. Supernatant antioxidant obtained from centrifugation was used as a source for enzyme activity deaths [13]. Whether the plants were under stress physiologically was determined by measuring antioxidant enzyme (superoxide dismutase, catalase, peroxidase) activities through the increases in types of reactive oxygen species during watering and soil stress conditions. Chemicals and methods used for each antioxidant enzyme were different [13]. The method used for the specification of Catalase (CAT) activity was the method that of Havir and McHale (1987). Activity measurement with this method is based on the principle whereby a decrease in absorbance in a CAT activity measurement environment while H_2O_2 is converted into O_2 and H_2O is observed at 240 nm [13, 20]. In order to determine catalase activities in the extraction solution obtained from plant samples, 5 mM H_2O_2 solution was used. After 103.5 mM of KH_2PO_4 buffer and 40 mM of H_2O_2 substrate solution are mixed and put into 3 mL quartz vials, 20 µL of enzyme extract from leaves and 50 µL of enzyme extract from roots were added. After the vial was placed into the spectrophotometer, its absorbance against a blank was monitored at 240 nm for 3 min at 1 min intervals. Absorbance per minute from the point where absorbance decreased linearly was calculated. These average absorbance values were converted into µmol H_2O_2 through a standard curve. The amount of enzyme that decreased absorbance 1 µmol at 25 °C in 1 min was accepted as one enzyme unit, and the results are presented as enzyme units per gram of tissue (EU g^{-1} tissue) [13,20]. Plant leaves (0.5 g) were blended with 10 mM potassium phosphate buffer (pH = 7.0) which contains 4% (w/v) polyvinylpyrrolidone. The homogenized pulp was centrifuged at 12,000 x g for 30 minutes at 4° C. Then the extract was isolated to determine the type of enzyme. After adding the plant extract to 50 mM phosphate buffer (pH = 7.0) which contains 1 mM guaiacol and 0.5 mM H_2O_2, peroxidase (POX) was determined by monitoring the increase in absorbance at 470 nm. One unit of POX activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 per min [13,21].

Activity determination of superoxide dismutase (SOD) was performed using spectrophotometry based on the photochemical reduction of nitro blue tetrazolium (NBT) inhibition [13]. The composition of reaction mixture contained following concentrations: 50 mM sodium carbonate, 13 mM methionine, 2 µM riboflavin, 75 µM NBT, 50 mM phosphate buffer (pH = 7.8), 0.1 mM EDTA, and 0.1 mL of plant extract. The measurements for the mixture at 560 nm absorbance were realized thereafter preparation. As control and blank, a maximum color-producing reaction mixture having no enzyme and a non-irradiated complete reaction mixture were utilized, respectively. The activity as one unit in the tubes was measured in terms of reducing the absorbance by 50% compared to the tubes having no enzyme; hence, the determinations of the values were in EU g^{-1} tissue quantities [13].

At harvest, samples were collected by hand and packed into polyethylene bags. Only the shoots of each plant were analyzed. Plants were oven dried at 80 °C for 24 h. Plant samples were milled with a micro-hammer cutter, sieved through a 1.5-mm sieve, and transferred to a clean polyethylene bag. After each milling, the mortar was cleaned with ethyl alcohol and distilled water to prevent cross-contamination of samples [22,23]. The conditions for the samples subjected to digestion were as defined: the maximum power applied was 1200 W; the ramp setting was for 20 min.; the pressure used was 180 PSI; the temperature setting was 210° C; and the hold time applied was 10 min. Following digestion, the solutions were evaporated to near dryness in a beaker. After evaporation, the volume adjustments for the remaining material samples were done to 10 mL using 0.1 M HNO3. The Varian Inductively Coupled Plasma–Optical Emission Spectrometry (ICP–OES) was employed for the determinations of elements in the all samples [22,23].

Analysis of Variance (ANOVA) in SPSS 22 was used to determine treatment effects relative to control (untreated) plants. S-N-K ve Tukey's B were performed when significant treatment effects were observed. For all statistical analyses, a p value ≤ 0.05 was determined as being significant [23].

3. RESULTS AND DISCUSSION

The potential impacts of binary mixtures (gemfibrozil and estradiol, gemfibrozil and caffeine, and gemfibrozil and ciprofloxacin) on possible plant stress markers in wheat and barley were investigated. The data indicated differences in several, if not all, of the plant biomarkers between control plants and those treated with simple mixtures of pharmaceutical substances. Many of the plant biomarkers we evaluated were directly related to oxidative damage.

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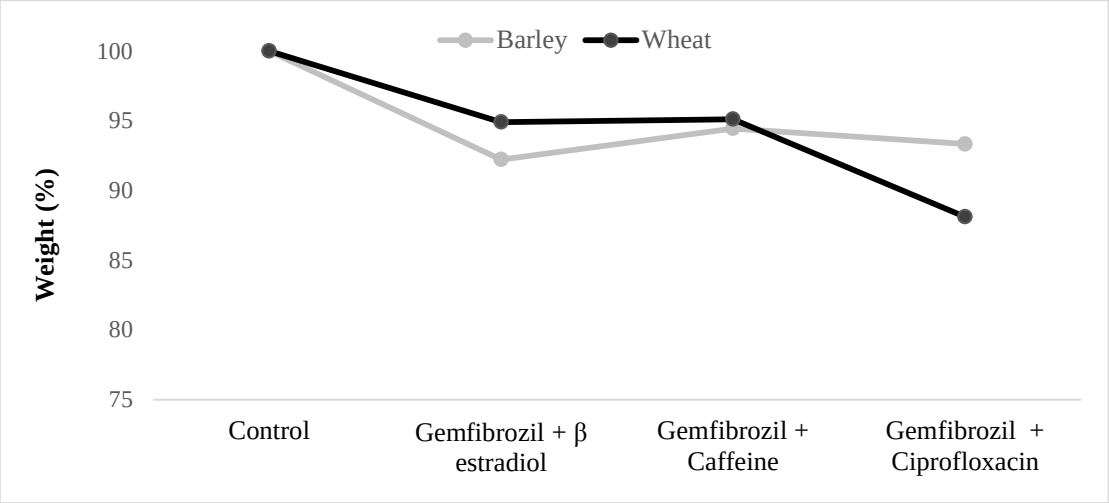

Figure 1. The leaf weights in wheat and barley treated with mixture PPCPs.

We observed slight decreases in plant biomass in both wheat and barley plants treated with the binary mixtures compared to control plants. However, the decrease in plant biomass was only significant for the gemfibrozil + ciprofloxacin treatment (Fig. 1).

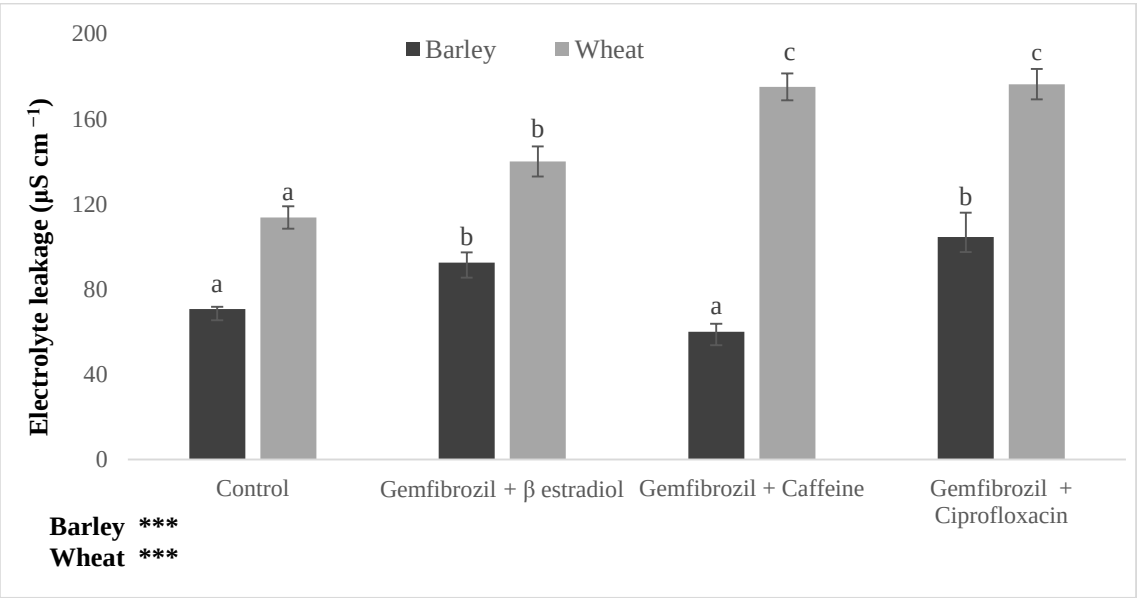

Figure 2. Electrolyte leakage concentrations in wheat and barley growth with mixture PPCPs. (*p<0,05; **p<0,01; ***p<0,001 significant).

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Average electrolyte leakage (mean ± standard error) was 71 ± 1.1 $\mu S \cdot cm^{-1}$ in control samples of barley. The highest electrolyte leakage in barley occurred in the gemfibrozil + ciprofloxacin treatment (104 ± 11 $\mu S \cdot cm^{-1}$). Control samples of wheat had an average electrolyte leakage of 114 ± 5.2 $\mu S \cdot cm^{-1}$ while gemfibrozil, in combination with either caffeine (175 ± 6.3 $\mu S \cdot cm^{-1}$) or ciprofloxacin (176 ± 5.2 $\mu S \cdot cm^{-1}$), produced the highest electrolyte leakage in wheat. Following statistical analysis of the electrolyte leakage data, it was concluded that there was a significant treatment effect of the binary mixtures in both barley and wheat (Figure 2).

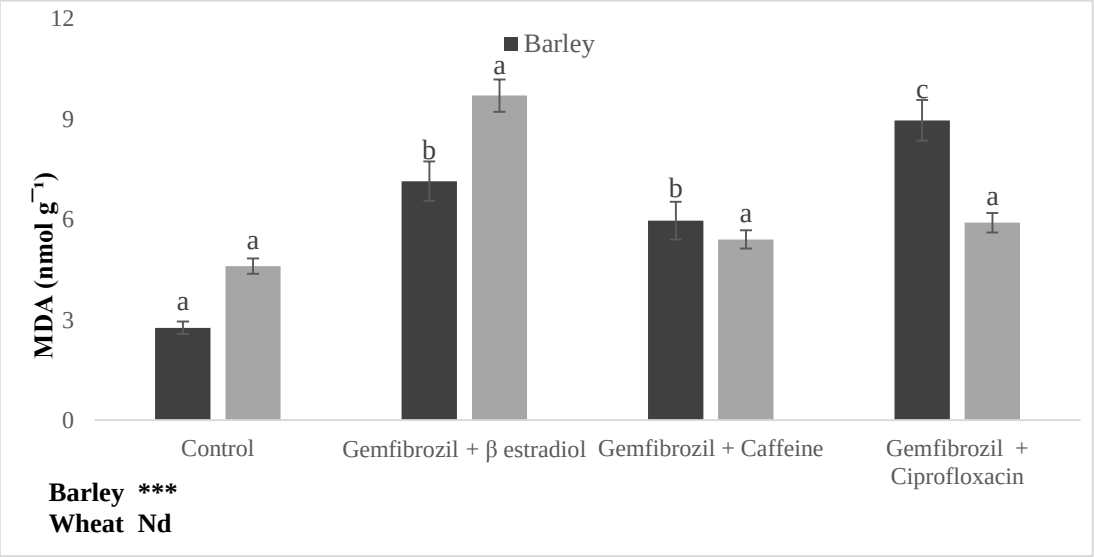

Figure 3. MDA concentrations in wheat and barley growth with mixture PPCPs.. (*p<0,05; **p<0,01; ***p<0,001 significant).

MDA levels were elevated in treated plants (both species), however, the treatment effect was only statistically significant for barley (Fig. 3). MDA was 2.8 ± 0.2 mmol/g in control samples of barley. The highest MDA levels in barley occurred in the gemfibrozil + ciprofloxacin treatment (9.0 ± 0.6 mmol/g). Control samples of wheat had an average MDA level of 4.6 ± 0.9 mmol/g, while the gemfibrozil + β-estradiol treatment produced the highest MDA levels in wheat (9.7 ± 2.0 mmol/g).

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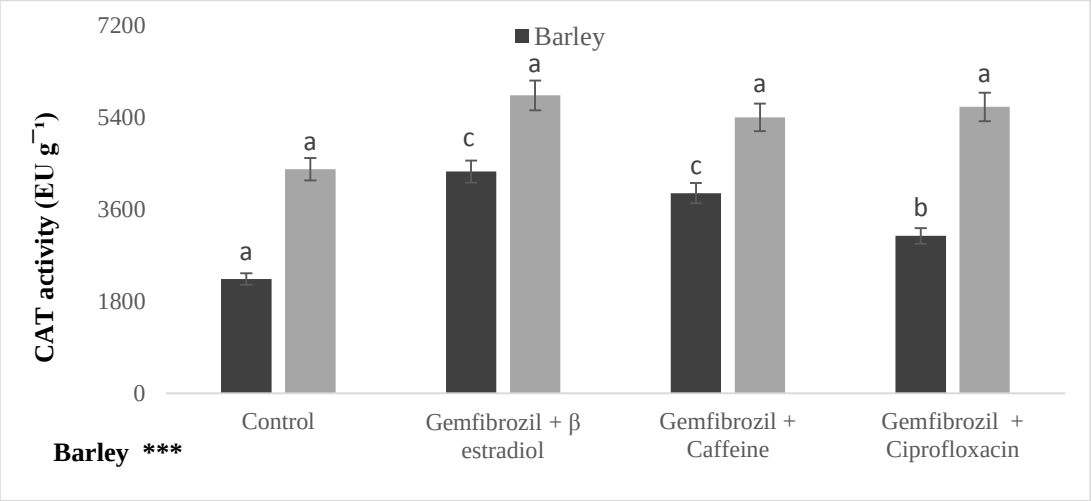

Figure 4. CAT activities in wheat and barley growth with mixture PPCPs. (*p<0,05; **p<0,01; ***p<0,001 significant).

When catalase (CAT) enzyme activity was examined in barley and wheat plants, there were significant treatment effects between the control samples and samples treated with pharmaceutical mixtures (Fig. 4). CAT activity in control barley plants was 2236 ± 145 EU/g, while treated plants had CAT activities ≥ 3082 EU/g. CAT activity in control wheat plants was 4397 ± 498 EU/g, while treated plants had CAT activities ≥ 5406 EU/g. For both plant species, the gemfibrozil + estradiol treatment produced the largest increase in CAT activity compared to control (untreated) plants.

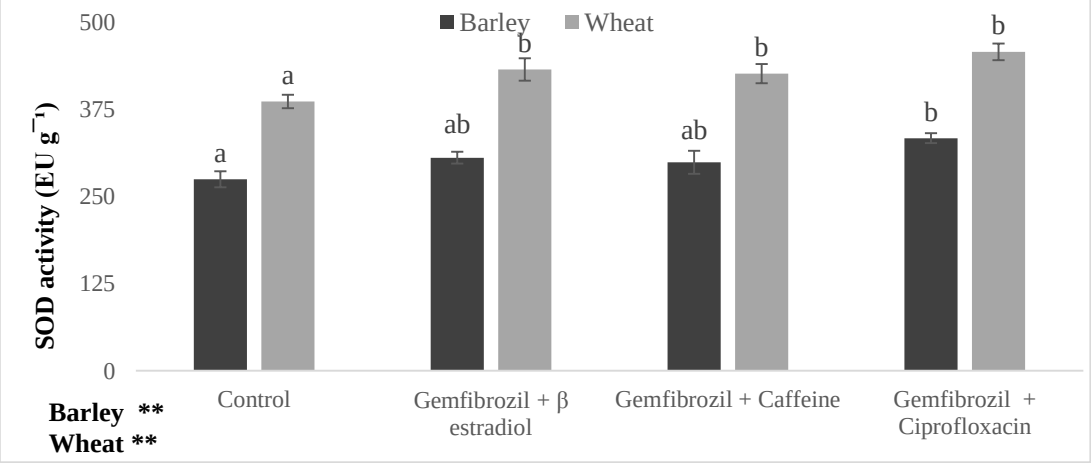

Figure 5. SOD activities in wheat and barley growth with mixture PPCPs. (*p<0,05; **p<0,01; ***p<0,001 significant).

When superoxide dismutase (SOD) enzyme activity was examined, a slight increase was observed in barley and wheat plants treated with pharmaceutical mixtures versus control (untreated) plants (Fig.

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5). SOD enzyme activity was 275 ± 12 and 386 ± 9.7 EU/g in control barley and wheat samples, respectively. The treatment of gemfibrozil + ciprofloxacin produced the highest increase in SOD activity over controls for both plant species (333 ± 7.1 EU/g for barley and 458 ± 12 EU/g for wheat).

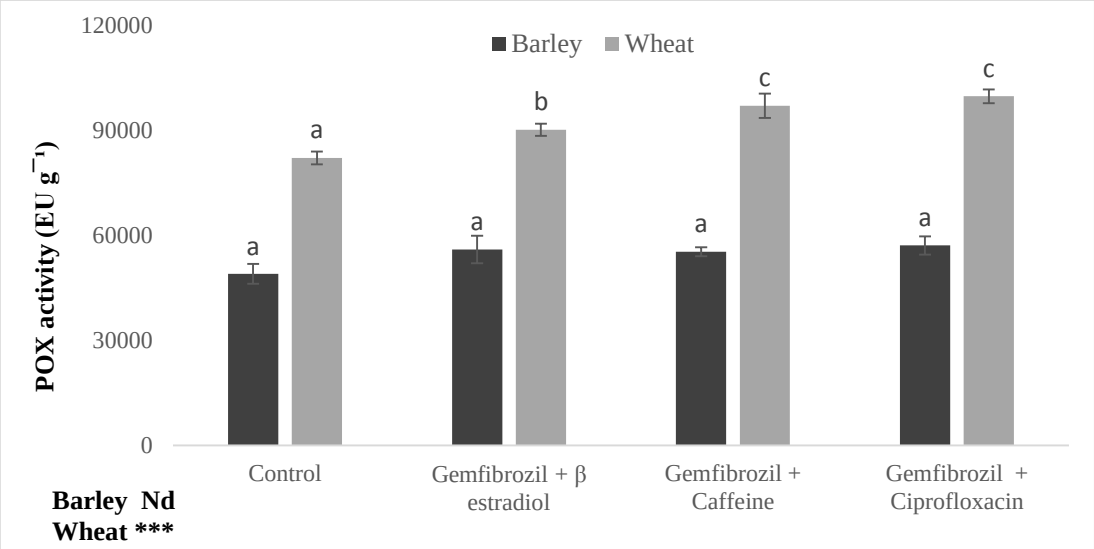

Figure 6. POX activities in wheat and barley growth with mixture PPCPs. (*p<0,05; **p<0,01; ***p<0,001 significant).

Peroxidase (POX) activity in barley was in the range of 48,936 ± 2824 EU/g (control) to 57,063 ± 2656 EU/g (gemfibrozil + ciprofloxacin). The gemfibrozil + ciprofloxacin had a similar effect on wheat (82,036 ± 1814 EU/g in controls and 100,750 + 1956 EU/g in treatment plants) (Fig. 6). When these differences were examined statistically, the slight increase in POX with treatment was not significant in barley, however, there was a treatment effect on POX for wheat plants.

Minerals are essential in the growth and development of all living organisms. Living organisms take up the elements necessary through the cycle of matter. It was determined that there were statistically significant differences in the element intake of 8 nutrients including Mg, K, P, Ca, Zn, Mn, Cu, and Fe in control plants compared with plants (both species) treated with pharmaceutical active substances (Table 1). We observed both increases and decreases in plant nutrients depending on the particular nutrient, pharmaceutical treatment, and plant species. Our data indicate that there are changes in electrolyte leakage and the activities of peroxidase, catalase and superoxide dismutase enzymes in both barley and wheat plants exposed to simple mixtures of pharmaceuticals. For both barley and wheat and for many of the plant biomarkers examined, exposure to gemfibrozil + ciprofloxacin produced the most dramatic and statistically significant effects on plants compared to control (untreated) plants.

There are many research reports that indicating that plants accumulate PPCPs [3,24-26]. However, research on the biochemical/physiological effects of PPCPs on plants is relatively new, especially mixtures of PPCPs. Dodgen et al. [27] applied individual PPCPs (bisphenol A, diclofenac, naproxen, nonylphenol) to cabbage and lettuce. They established that there was more accumulation in roots than

in leaves or stems. In addition, as the accumulation of PPCPs increased, the negative effects on plant development became more pronounced. An et al. [28] studied the ecotoxicologic effects of paracetamol on germination of wheat. They observed that the development of wheat seeds decreased in a concentration dependent manner. Paracetamol and diclofenac have also been shown to effect peroxidase activity, chlorophyll, and membrane activity of duckweed, *Lemna minor* [29] Chlorophyll and carotenoids in *Lemna gibba* were also negatively impacted by exposure to ibuprofen.[30] PPCP exposure to cucumbers produced a decrease in chlorophyll, but increased levels and activity of antioxidant enzymes in roots and leaves [12]. The authors speculated that the latter effect was a plant defense mechanism.

Our data indicate that there are changes in electrolyte leakage and the activities of peroxidase, catalase and superoxide dismutase enzymes in both barley and wheat plants exposed to simple mixtures of pharmaceuticals. For both barley and wheat and for many of the plant biomarkers examined, exposure to gemfibrozil + ciprofloxacin produced the most dramatic and statistically significant effects on plants compared to control (untreated) plants.

Table 1. Concentration of mineral elements in wheat and barley treated with mixture of PPCPs. (*p<0,05; **p<0,01; ***p<0,001 significant).

Element		**Wheat**			**Significant**	**Barley**			**Significant**
Mg	Control	2102,5	±	132,2		2623,5	±	196,4	
	Gemfibrozil + β estradiol	1863,8	±	53,5		1851,1	±	43,6	
	Gemfibrozil + Caffeine	2019,7	±	89,0		2087,0	±	10,4	
	Gemfibrozil + Ciprofloxacin	1721,6	±	50,0	*	1970,3	±	116,5	***
K	Control	57772,1	±	1011,8		42286,3	±	5361,4	
	Gemfibrozil + β estradiol	57434,8	±	1213,4		34367,9	±	481,0	
	Gemfibrozil + Caffeine	64124,1	±	1045,5		37165,7	±	208,1	
	Gemfibrozil + Ciprofloxacin	58105,0	±	1044,3	***	34423,2	±	1480,0	**nd**
Ca	Control	1154,9	±	128,1		1449,4	±	45,3	
	Gemfibrozil + β estradiol	980,3	±	26,8		894,2	±	16,2	
	Gemfibrozil + Caffeine	951,5	±	18,1		1030,4	±	15,5	
	Gemfibrozil + Ciprofloxacin	884,5	±	27,0	**nd**	926,2	±	42,9	***
P	Control	7662,4	±	267,3		7194,3	±	1090,8	
	Gemfibrozil + β estradiol	7363,6	±	179,2		4347,7	±	35,6	
	Gemfibrozil + Caffeine	8084,5	±	142,7		4616,5	±	17,3	
	Gemfibrozil + Ciprofloxacin	7054,3	±	151,4	*	4524,8	±	90,4	**
Zn	Control	33,8	±	1,0		47,5	±	2,9	
	Gemfibrozil + β estradiol	30,9	±	0,2		46,5	±	1,0	
	Gemfibrozil + Caffeine	29,9	±	0,1		51,2	±	0,6	
	Gemfibrozil + Ciprofloxacin	51,1	±	0,9	***	45,7	±	0,7	**nd**
Mn	Control	30,9	±	1,4		29,5	±	0,1	
	Gemfibrozil + β estradiol	27,3	±	0,5		26,3	±	0,6	
	Gemfibrozil + Caffeine	26,0	±	0,1		25,9	±	0,1	
	Gemfibrozil + Ciprofloxacin	25,1	±	0,3	***	24,5	±	0,4	***

Cu	Control	14,8	± 0,1		13,7	± 0,5	
	Gemfibrozil + β estradiol	14,9	± 0,5		17,1	± 0,2	
	Gemfibrozil + Caffeine	15,7	± 0,3		18,0	± 0,1	
	Gemfibrozil + Ciprofloxacin	15,2	± 0,1	**nd**	17,2	± 0,9	***
Fe	Control	69,1	± 5,2		236,4	± 58,2	
	Gemfibrozil + β estradiol	67,2	± 3,6		60,7	± 0,9	
	Gemfibrozil + Caffeine	69,6	± 1,4		80,4	± 2,2	
	Gemfibrozil + Ciprofloxacin	82,8	± 2,1	*	75,6	± 8,0	***

There are many research reports that indicating that plants accumulate PPCPs [3,24-26]. However, research on the biochemical/physiological effects of PPCPs on plants is relatively new, especially mixtures of PPCPs. Dodgen et al. [27] applied individual PPCPs (naproxen, bisphenol A, nonylphenol, diclofenac) to lettuce and cabbage. They established that there was more accumulation in roots than in leaves or stems. In addition, as the accumulation of PPCPs increased, the negative effects on plant development became more pronounced. An et al. [28] studied the ecotoxicologic effects of paracetamol on germination of wheat. They observed that the development of wheat seeds decreased in a concentration dependent manner. Paracetamol and diclofenac have also been shown to effect peroxidase activity, chlorophyll, and membrane activity of duckweed, *Lemna minor* [29] Chlorophyll and carotenoids in *Lemna gibba* were also negatively impacted by exposure to ibuprofen [30]. PPCPs exposure to cucumbers produced a decrease in chlorophyll, but increased levels and activity of antioxidant enzymes in roots and leaves [12]. The authors speculated that the latter effect was a plant defense mechanism. Christou et al. [31] studied the impact of diclofenac, sulfamethoxazole, trimethoprim, and 17α-ethinylestradiol to clover singly, and then as a mixture, on biochemical/physiological parameters; the mixture had a greater impact than individual PPCPs. A similar observation was made by Geiger et al [32] for algae exposed to a mixture of ibuprofen, ciprofloxacin and chlorophenols, although some of the negative effects of algal development likely came from the chlorophenols.

4. CONCLUSIONS

It appears that based on our data, pharmaceuticals and personal care products in mixtures can cause deleterious impacts to plants which could lead to agronomic costs. When we consider the number of potential combinations of PPCPs present in wastewater (even after treatment), additional consideration should be given to the potential impacts these PPCPs may have as treated wastewater is recycled and used for other purposes. The potential impact that PPCPs taken up by plants irrigated with recycled water could have on living things that feed on these plants is largely unknown. In order to minimize the effects of these substances, which have high polluting potential on the environment, importance should be emphasized to make legal arrangements related to the usage and disposal of drugs and to raise awareness of people as well.

ACKNOWLEDGMENT

This study was supported in part by Erzincan Binali Yıldırım University BAP, FBA-2017-404.

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