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The effect of Pharmaceuticals and Personal Care Products (PPCPs) on Human Embryonic Kidney (HEK 293) cells proliferation

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Abstract

Background: Pharmaceuticals and personal care products (PPCPs) considered an environmental issue that needs to be managed ,especially, PPCPs are not removed by conventional waste water treatment. Therefore, the aim of current study was to analyses the toxicity of PPCPs, as single and mixed exposures, on human embryonic (HEK 293) cells proliferation.

Methods:The culture medium RPMI Medium 1640 was used for growing the HEK293 cells. The PPCPs stocks were prepared with Dimethyl Sulfoxide (DMSO) and diluted into three concentrations (minimum, middle and maximum) as the working stocks for the toxicity study. The cell proliferation was studied using hemocytometer test.

Results: The human embryonic kidney (HEK293) cella were very sensitive to the effects of pharmaceuticals and personal care products (PPCPs). The highest reduction in cells proliferation (90%) was seen upon exposure to a mixture of the maximum concentrations of PPCPs (750µg/L for IBU and HHCB, 75µg/L for KET and 5µg/L for EE_2) used in current study. In addition, for exposure to a single PPCP, the results showed that the highest reduction in cells proliferation (46%) was seen upon exposure to the synthetic hormone EE_2 (5µg/L).

Conclusion: The human embryonic kidney cells (HEK293) used in this study were verry sensitive to the toxic effects of pharmaceuticals and personal care products (PPCPs). The latter can reflect the human sensitivity to the daily exposure to the PPCPs.

Keywords*:* PPCPs, HEK293 cells, Ibuprofen, Ketoprofen, 17α-ethinylestradiol, Galaxolide, Ozonation, cell culture.

I. INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) during the current decades have been recognized as emerging contaminants because of their high persistence in aquatic life. The term "PPCPs" broadly refers to any product with healthcare or medical purposes for humans and/or animals [1]. In addition to pharmaceuticals found in the water stream, a large amount of personal care products, such as fragrances, skin and hair products and sun block products are also used worldwide [2]. One of the main groups is synthetic musks. They are divided into two categories; nitro and polycyclic musks. They are used as fragrance additives and to fix the fragrance for longer times in detergents, cleaning agents, soaps, shampoo and deodorants. During the last two decades, synthetic musks have been detected in aquatic life like fish and mussels, marine, surface and sewage water as well as in human adipose tissue, breast milk and blood [3]. Over the past 30 years there have been an interest in the toxicity issue of PPCPs [4]. PPCPs are known to be released into the aquatic environments through many multiple pathways including domestic wastewater, hospital discharges, improper manufacturer disposal, sewage treatment plants (STPs) and water treatment plants [5]. Hospital effluents are generally exhibit higher detection frequencies and concentrations of pharmaceuticals [6]. The excreted PPCPs may either retain in their original concentrations and structures or mobilized and converted into other active (or inactive) compounds during their lifespan in aquatic matrices [7]. Till day, there is only several acute toxicity and chronic toxicity studies conducted to investigate the ecotoxicity of PPCPs toward aquatic organisms and human health. Most of the data collected on the ecological risk of most PPCPs are limited by studies of acute toxicity using three organisms and measured or predicted the environmental concentrations (i.e. fish, daphnia, and alga) [8,9]. However, the chronic toxicity is thought to be a major concern, and this type of toxicity is limited [10,11]. Till present days, no regulation of PPCPs discharge has been strictly enforced and implemented to minimize their effects on aquatic life and human [12]. However, data on their metabolites, by-products, and degradation products are very limited so far and only a few studies showed their on humans' health. The fates and removal mechanisms of PPCPs in STPs and waste treatment plants (WTPs) have not been fully understood [13]. Thus, numerous analytical methods have been developed to assess the profiles and occurrence patterns of PPCPs during the last decade [14]. Several review articles have reported the ecotoxic effects of PPCPs and their occurrences in various water bodies including groundwater, surface water, waste water and STPs [5,14,15,16]. There are many studies and approaches to apply different toxicity tests using the freshwater invertebrates such as daphnids, fish, algae, mussels and also human embryonic cells [17,18,19,20,21]. The latter are considered very sensitive to the toxic effects of PPCPs since these cells are derived from the human kidney cells and have direct contact to the fluids absorbents by human which may contain PPCPs and others micropollutants that may exist in tap water or even drinking water. Thus, [21] indicated that a mixture of 13 drugs can inhibit cell proliferation of human embryonic cells HEK 293 and affect their physiology and morphology. In addition, the study suggested that water-borne pharmaceuticals can be potential effectors on aquatic life. Also, there was 50% decline in cell viability of fish liver cell line RTL-W1 when exposed to a mixture of pharmaceuticals and personal care products [3]. Since PPCPs can not be removed by ordinary water treatment, advanced treatments, like ozonation, can be used to remove the PPCPs from water stream.

Therefore, the aim of current study was to analyses the toxicity of PPCPs, as single and mixed exposures, on human embryonic (HEK 293) cells proliferation.

2. MATERIALS AND METHODS

2.1 Medium preparation for cell culture

A culture medium is used in cell culture to maintain the pH and osmolality essential for cell viability and to provide the nutrients and energy needed for cell growth and multiplication. The medium used was RPMI Medium 1640 (Gibco, USA) which is basal media in powder form. To use this media, 0.4g of this medium together with 2g of sodium hydrogen carbonate $NaHCO_3$ (Sigma, China) were mixed in 1L of autoclaved distilled water, and adjusted pH between 6.8-7.2 by adding either 1M HCL or 1 M of NaOH while stirring. Then, the medium is filtered and then stored at 2-8 ºC in the refrigerator and protected from direct light. The most important step is to make a sterilized step by using nutrient agar (Oxoid, England), this agar was prepared prior to

medium preparation, by mixing 28g of nutrient agar in 1L distilled water and then the mixture was boiled and stirred continuously until it is totally dissolved. Later, the mixture was autoclaved for 15 min, then poured in Petri dish to the half inside the laminar flow cabinet to reduce the contamination and left for 24h in the refrigerator to come into solid form. Following this, it became ready to use in the sterilization step by putting 3 drops of the prepared cell culture medium and incubated at 37ºC and 5% CO_2 for 24h. A control cell culture, containing cells and medium only, was used for comparison with the cells plus PPCPs.

2.2 Cell culture preparation for control

The control cell culture was prepared starting from thawing the cells stock which was frozen at -80 ºC by placing the vial containing the freezing cells into water bath at 37ºC. When the cells were totally thawed, the cells vial were taken out from the water bath and sterilized with 70% ethanol (SIGMA, China). Meanwhile, a volume of 10mL RPMI medium supplemented with 10% FBS purchased all from (Gibco, USA) was also warmed in the water bath at 37ºC. From this medium, 1mL was taken and added to the cells then this mixture was all transferred to 15mL tube. The next process was to add the rest of the 4.5mL medium each time to ensure the total mixture was well-mixed and the final volume of 2mL cells and 10mL medium was 12mL. Then this tube was placed in the centrifuge (KUBOTA, 5220, Japan) for 5 min to pellet the cells.

Later, the tube was taken out of the centrifuge and sterilized with 70% ethanol and the supernatant was removed. The cells were re-suspended gently with 10mL of medium and transferred to five of T25 cm^2 culture flasks purchased from (NEST, USA. Each flask contained 2mL from the mixture and the size of 5mL was completed with another 3mL of the medium only and the flask then placed inside the tissue culture incubator (NUAIRE, HEPA, USA) at 37ºC and 5% CO_2.

After 24 hours, the medium of cells was replaced to ensure good growth of cells and they were not exceed 90 % confluence which is a term referred to the estimated number of adherent cells in a culture dish or flask. Replacement medium was done by taking out the flasks from the incubator, the medium removed and the attached cells were washed with the sterilized Phosphate-buffered saline (PBS). Later, 1-2mL of trypsin-EDTA solution were added and treated the cells with it for 1-3 min in the incubator. The cells must not be exposed to the trypsin for so long, once the cells are de-attached, 5-10mL of the medium was added to stop the trypsinization and then the cells were re-suspended gently and thoroughly, and the cells were transferred to the T25 cm^2 flasks. This procedure was done every 24h until the confluence reached 80-90% measured by hemocytometer. Once the 80-90% confluence reached, cells were transferred to a 6-well cell culture purchased from NEST (USA) to study the growth curve instead of using T25 m^2.

2.3 Preparation of PPCPs solution

The PPCPs stock was prepared with Dimethyl Sulfoxide (DMSO) (Nacalai Tesque, Japan) with a concentration of 1g/L. The stock concentration was diluted into three concentrations (minimum, middle and maximum) as the working stocks for our toxicity study (Figure 1). These stocks were kept in refrigerator with the working solution stock. The concentration used for this study depends on previous studies and reviews, for IBU (25-600µg/L), HHCB (100-600µg/L) [22,23], KET (15-60µg/L [24] and, finally, EE_2 (1-2µg/L) [25,26], where these maximum concentrations were found in the environment in the range of 600µg/L, for both Ibuprofen and Galaxolide, 60µg/L, for Ketoprofen, and 2µg/L, for the hormone. In addition, to study the toxic effects on cells from a wide range, another two concentrations were taken. Collectively, each PPCP havs three concentrations: 300, 600 and 750µg/L for HHCB and IBU, 1, 2 and 5µg/L for EE_2 and 25, 60 and 75µg/L for KET.

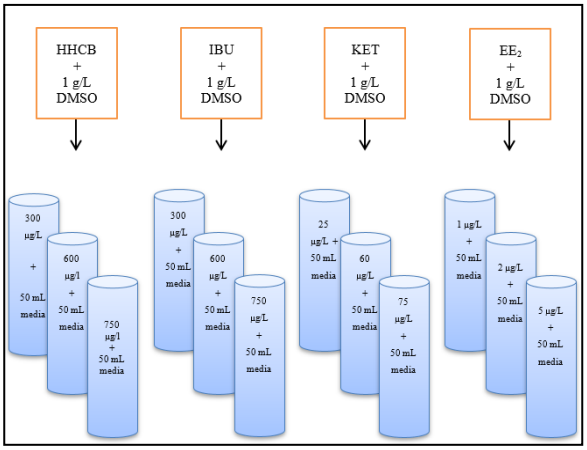

Figure 1 Preparation of PPCPs stocks

2.4. Determination of cell growth

The growth curve was conducted for the control without any PPCPs addition and for the PPCPs as single and mixture components. Each well used was cultured on each day of the 5-day cell culturing experiment. The steps were done in the same procedure for all samples and compared it with the control without PPCPs. From the T25 cm^2 flask which was considered the working stock, an equal amount of about 2×10^5 cells/mL was taken. The latter was calculated by measuring the total cells number per mL from the working stock by Equation (1) and substituted in Equation (2) to determine the volume to be taken from the working cells stock [27]:

$$\text{Total cells (per mL)} = \frac{\text{Total cells counted} \times 10^4}{\text{No. of squares}} \times \text{dilution factor} \quad (1)$$

$$M_1V_{1=}M_2V_2 \quad (2)$$

Where, M_1 is total number of cells/mL, V_1 is the wanted volume taken from the working cells stock, M_2 is the standard cells concentration for HEK293 which is equal to 2×10^5 cell/mL and V_2 is the total volume of cells and medium mixture used for 6 wells equal to 3mL.

The calculated cells volume was poured into the 6-well flask for monitoring cell growth and completing the volume to 3mL with the medium. The samples were triplicate and later the average was taken to reduce experimental errors. All growth curves were studied for 5 days. The cell proliferation was studied using hemocytometer test, which is considered the simplest and most direct method of cell counting in a suspension. The hemocytometer is a modified microscope slide that bears two polished surfaces each with display ruled and sub-divided grid, consisting of nine primary squares each measuring 1mm on a side (area $1mm^2$). This test followed Protocol 4 [27] and it was done by de-attaching the cells with trypsin, after that, centrifuged for 5min to concentrate the cell pallets and , then, re-suspended with 3mL cell culture medium. From the 6 wells of cell growth, 20µL were taken and well mixed with 20µL of trypan blue (GIBCO, USA).

3. Results and Discussion

3.1. Proliferation of HEK293 cells for control

The control cells proliferation and growth for 5 days using hemocytometer and the cells morphology on hemocytometer on 0 day are shown in Figure 2 that depicts the different phases of cell growth with the lag phase was conducted from zero day to the

first day, the log phase where the cells started to divide and increase from the first day until the second day. Later, the cells will stay constant and this stage is called plateau and occurred on third day of cell culture. Finally, the last stage of the cell culture where the dead cells are more than the live ones, called decline stage which happens in the last two days of cultured cells.

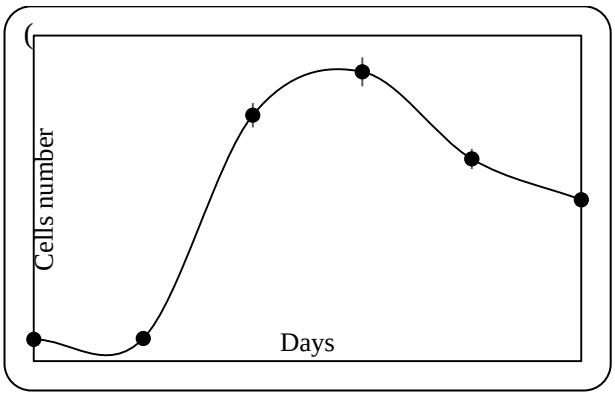

Figure 2 HEK293 cells proliferation for control

3.2. Proliferation of HEK293 cells for single and mixture exposure

3.2.1. Single exposure to IBU

Figure 3 illustrates the reduction in cell proliferation recorded for exposure to IBU. The highest average reductions, compared with the control, were 20.0, 26.0 and 32.0% for IBU concentrations of 300, 600 and 750µg/L, respectively.

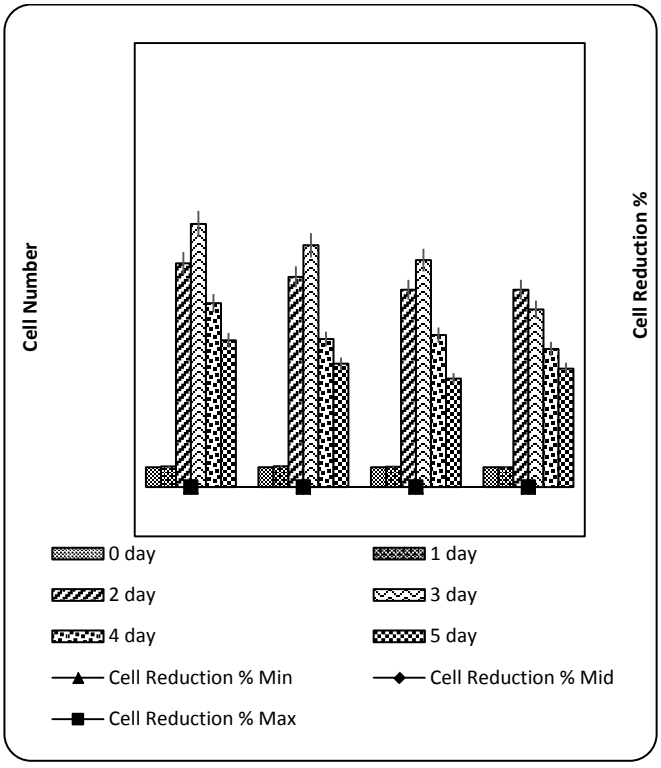

Figure 3 Reduction in cells proliferation upon exposure to IBU

3.2.2. Single exposure of KET

Figure 4 depicts the reduction in cells proliferation, compared with control, for different concentrations of KET and culturing day. The highest reductions were 10.0, 26.0 and 22.0% for KET concentrations of 30, 60 and 75µg/L, respectively. This reduction was due to the effect of ketoprofen on HEK293 cells. Ketoprofen was the fastest to remove and to degrade to less toxic compounds.

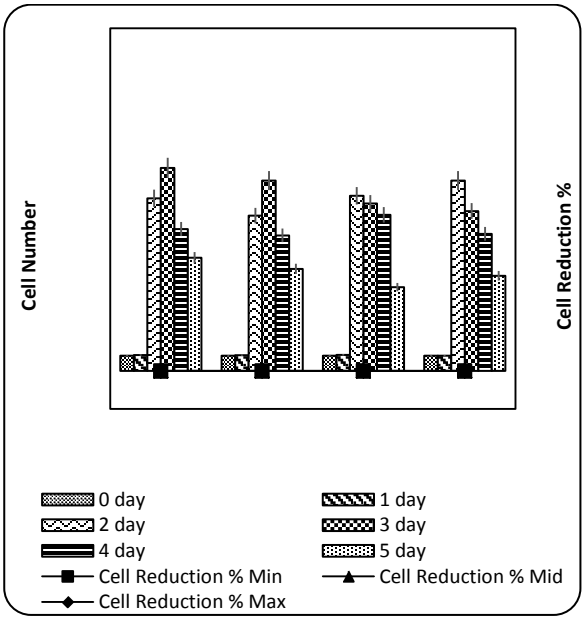

Figure 4 Reduction in cells proliferation upon exposure to KET

3.2.3. Single exposure of EE_2

The reduction in cells proliferation, compared with the control, upon treatment with EE_2 and with its ozonation by-products is shown in Figure 5. The highest averages of reduction were 32.0, 36.0 and 46.3% for EE2 concentrations of 1, 2 and 5µg/L, respectively. The reduction in cells proliferation for EE_2 was the highest among the PPCPs used in this study.

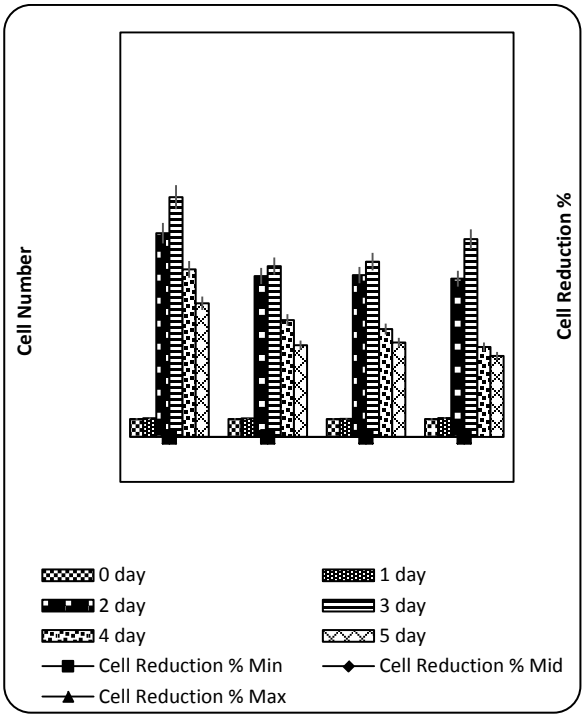

Figure 5 Reduction in cells proliferation upon exposure to EE2

3.2.4. Single exposure to HHCB

Figure 6 shows the reduction in cells proliferation compared with the control. For the cells treated with HHCB, the highest reductions in cells proliferation compared with the control were 14.0, 25.0, and 42.0% for HHCB concentrations of 300, 600 and 750µg/L, respectively.

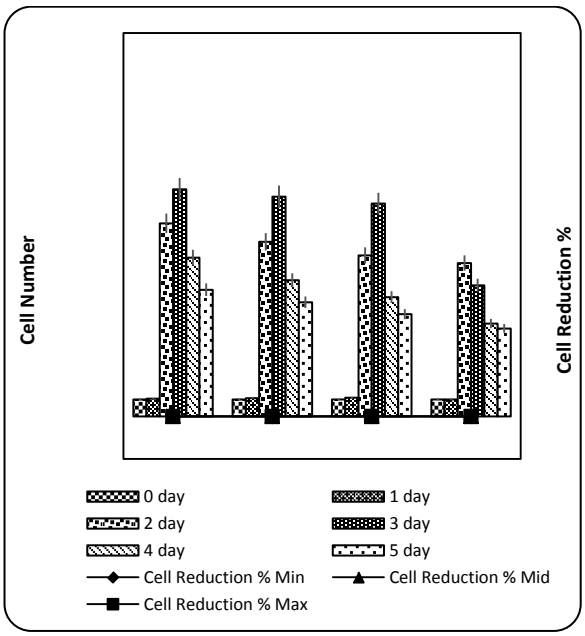

Figure 6 Reduction in cells proliferation upon exposure to HHCB

3.2.5. Mixed PPCPs exposure

Figure 7 shows the reductions in cells proliferation, compared with the control, for mixed PPCPs exposure. The highest averages of reduction in cells were 88.0, 92.0 and 94.0% upon exposure to minimum, middle and maximum concentrations of mixed PPCPs, respectively. This highest reduction in cells proliferation was due to the combined effect of PPCPs before treatment which is very close to the real world of daily human exposure to mixed PPCPs. The latter finding gives an indicator of the effects of the PPCPs on human cells.

It is very clear that the highest reduction, before ozonation, was upon exposure to the maximum concentration of mixed PPCPs in which the reduction reached 94.0% on the fifth day (Figure 7). The maximum reduction upon exposure to a single component was to the maximum concentration of EE_2 (5 µg/L; Figure 5) where the reduction reached 46.0% on the fourth day of cell growth that had given the highest impact on cells.

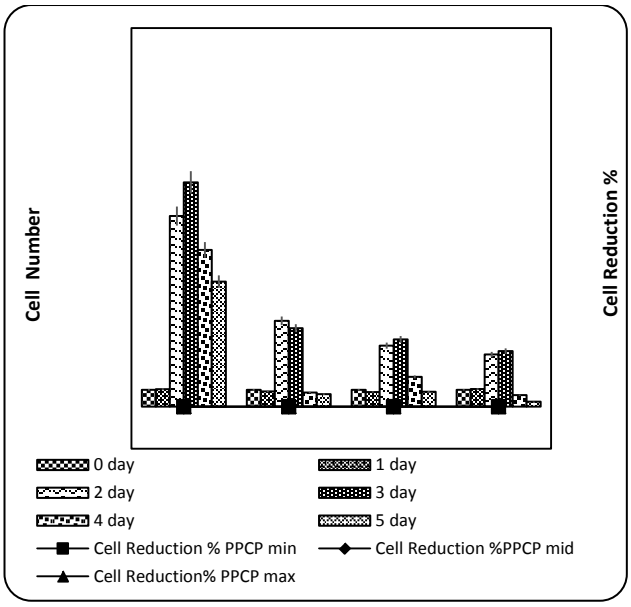

Figure 7 Reduction in cells proliferation upon exposure to mixed PPCPs

4. CONCLUSION

The human embryonic kidney cells (HEK293) were used in this study to monitor the effects of pharmaceuticals and personal care products (PPCPs) on the cells since they are considered very sensitive and can reflect the human sensitivity to the daily exposure to the PPCPs. From this study, PPCPs were applied with different concentrations and the reductions in cells proliferation compared with the control were between 1-46%. As a single component, the synthetic hormone EE_2 (5 µg/L) recorded the highest reduction (46%) in cells proliferation. The PPCPs mixture (750µg/L for IBU and HHCB, 75µg/L for KET and 5µg/L for $EE_{2)}$ gave the highest reduction (90%) in cells proliferation. The cells were very sensitive to the PPCPs which produced the highest reduction in cells proliferation.

Ethical Clearance: It was obtained from the Scientific Research Committee at Faculty of Engineering and Built Environment/ Universiti Kebangsaan Malaysia, Selangor, Malaysia.

Financial Disclosure: There is no financial disclosure.

Conflict of Interest: None to declare.

REFERENCES

1- Yin L Wang B, Yuan H, Deng S, Huang J, Wang Y and Yu G. Pay special attention to the transformation products of PPCPs in environment. *Emerging Contaminants.* 2007; 3(2): 69–75. https://www.sciencedirect.com/science/article/pii/S2405665017300045 [14 August 2018].

2- Al-Terehi MN, Al-Ganimi YK, AlAmeri KMA, Al- Saadi AH, Zaidan HK, Ewadh MJ. Effect of Pollutant Water on Some Organs and Blood Parameters in Rats. *Research in Pharmacy.* 2012; 2: 5. file:///C:/Users/X452E/Downloads/338-Article Text-528-1-10-20151029.pdf [29 August 2018].

3- Schnell S, Bols NC, Barata C and Porte C. Single and combined toxicity of pharmaceuticals and personal care products (PPCPs) on the rainbow trout liver cell line RTL-W1. *Aquatic toxicology (Amsterdam, Netherlands).* 2009; 93(4): 244–52. http://www.ncbi.nlm.nih.gov/pubmed/19500862 [30 May 2014].

4- Doloresco F, and Vermeulen L. Pharmacist labor market View project. https://www.researchgate.net/publication/235777417. 2014. Accessed 27, August 2018..

5- Liu JL. and Wong MH. Pharmaceuticals and personal care products (PPCPs): a review on environmental contamination in China. *Environment international.* 2013; 59: 208–24. http://www.ncbi.nlm.nih.gov/pubmed/23838081 [30 May 2014].

6- Kosma CI, Lambropoulou DA and Albanis TA. Investigation of PPCPs in wastewater treatment plants in Greece: Occurrence, removal and environmental risk assessment. *Science of the Total Environment.*2014; 466–467.

7- Oliveira TS, Murphy M, Mendola N, Wong V, Carlson D and Waring L. Characterization of Pharmaceuticals and Personal Care products in hospital effluent and waste water influent/effluent by direct-injection LC-MS-MS. *Science of The Total Environment. 2015*; 518–519: 459–478. ttp://linkinghub.elsevier.com/retrieve/pii/S0048969715002648

8- Stuer-lauridsen F. Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. *Chemosphere*. 2000; 40: 783–793. file:///C:/Users/hggf/Downloads/0c96052b5af05b6d6d000000.pdf [14 December 2014].

9- Tamura I, Yasuda Y, Kagota K, Yoneda S, Nakada N, Kumar V, Kameda Y, Kimura K, Tatarazako N and Yamamoto H. Contribution of pharmaceuticals and personal care products (PPCPs) to whole toxicity of water samples collected in effluent-dominated urban streams. *Ecotoxicology and Environmental Safety. 2017;* 144.

10- Brausch JM and Rand GM. A review of personal care products in the aquatic environment: environmental concentrations and toxicity. *Chemosphere*. 2011; 82(11): 1518–32. http://www.ncbi.nlm.nih.gov/pubmed/21185057 [19 October 2014].

11- Caminada D, Escher C and Fent K. Cytotoxicity of pharmaceuticals found in aquatic systems: comparison of PLHC-1 and RTG-2 fish

cell lines. *Aquatic toxicology (Amsterdam, Netherlands). 2006;* 79(2): 114–23. http://www.ncbi.nlm.nih.gov/pubmed/16828891 [24 December 2014].

12- Daughton CG. Daughton CG and Scuderi MST, 2014. "Pharmaceuticals and Personal Care Products (PPCPs): Bibliographic Database of Relevant Published Literature," U.S. Environmental Protection Agency, Las Vegas, Nevada (a comprehensive database of literature references co. *Environmental toxicology and chemistry*. 2014; / *SETAC* 28(12): 2490–4. http://www.ncbi.nlm.nih.gov/pubmed/19681645 [9 January 2015].

13- Blair B, Nikolaus A, Hedman C, Klaper R and Grundl T. valuating the degradation, sorption, and negative mass balances of pharmaceuticals and personal care products during wastewater treatment. *Chemosphere*. 2015; 134: 395–401. http://linkinghub.elsevier.com/retrieve/pii/S0045653515004245 [5 March 2017].

14- Evgenidou EN, Konstantinou IK and Lambropoulou DA. Occurrence and removal of transformation products of PPCPs and illicit drugs in wastewaters: A review. *Science of The Total Environment*. 2015; 505: 905–926.

15- Brausch JM and Rand GM. A review of personal care products in the aquatic environment: environmental concentrations and toxicity. *Chemosphere*. 2011; 82(11): 1518–32. http://www.ncbi.nlm.nih.gov/pubmed/21185057 [19 October 2014].

16- Luo Y, Guo W, Ngo HH, Nghiem LD, Hai FI, Zhang J, Liang S and Wang XC. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of the Total Environment*. 2014; 473–474: 619–641.

17- Carlsson C, Johansson AK, Alvan G, Bergman K and Kühler T. Are pharmaceuticals potent environmental pollutants? Part I: environmental risk assessments of selected active pharmaceutical ingredients. *The Science of the total environment*. 2006; 364(1–3): 67–87. http://www.ncbi.nlm.nih.gov/pubmed/16257037 [2 June 2014].

18- Hoeger B, Köllner B, Dietrich DR and Hitzfeld B. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (Salmo trutta f. fario). *Aquatic toxicology (Amsterdam, Netherlands). 2005;* 75(1): 53–64. http://www.ncbi.nlm.nih.gov/pubmed/16139376 [19 February 2016].

19- Li Z, Yin N, Liu Q, Wang C, Wang T, Wang Y, Qu G, Liu J, Cai Y, Zhou Q and Jiang G. Effects of polycyclic musks HHCB and AHTN on steroidogenesis in H295R cells. *Chemosphere*. 2013; 90(3): 1227–1235. http://dx.doi.org/10.1016/j.chemosphere.2012.09.056

20 - Lyssimachou A and Arukwe A. Alteration of Brain and Interrenal StAR Protein, P450 scc , and Cyp11β mRNA Levels in Atlantic Salmon after Nominal Waterborne Exposure to the Synthetic Pharmaceutical Estrogen Ethynylestradiol. *Journal of Toxicology and Environmental Health, Part A*. 2007; 70(7): 606–613. http://www.ncbi.nlm.nih.gov/pubmed/17365614 [9 January 2016].

21- Pomati F, Castiglioni S, Zuccato E, Fanelli R, Vigetti D, Rossetti C, Calamari D, Fanelli R, Vigetti D, Rossetti C and Calamari D. Effects of a Complex Mixture of Therapeutic Drugs at Environmental Levels on Human Embryonic Cells. *Environmental Science & Technology*. 2006; 40(7): 2442–2447. http://dx.doi.org/10.1021/es051715a

22- Luo Y, Guo W, Ngo HH, Nghiem LD, Hai FI, Zhang J, Liang S and Wang XC. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of the Total Environment*. 2014; 473–474: 619–641.

23- Li Z, Yin N, Liu Q, Wang C, Wang T, Wang Y, Qu G, Liu J, Cai Y, Zhou Q and Jiang G. Effects of polycyclic musks HHCB and AHTN on steroidogenesis in H295R cells. *Chemosphere*. 2013; 90(3): 1227–1235. http://dx.doi.org/10.1016/j.chemosphere.2012.09.056

24- F, J and C, C, J. 2012. Pharmaceutical products in the environment: sources, effects and risks.

25- Al-Odaini NA, Zakaria MP, Yaziz MI, Surif S and Kannan N. Occurrence of synthetic hormones in sewage effluents and Langat River and its tributaries, Malaysia. *International Journal of Environmental Analytical Chemistry*. 2013; 93(14): 1457–1469. http://www.tandfonline.com/doi/abs/10.1080/03067319.2012.727810

26- Al-Qaim FF, Abdullah MP, Othman MR, Latip J and Afiq W. A validation method development for simultaneous LC-ESI-TOF/MS analysis of some pharmaceuticals in tangkas river-Malaysia. *Journal of the Brazilian Chemical Society*. 2014; 25(2): 271–281.

27- Davis JM, John M. *Basic cell culture : a practical approach*. s.l.: Oxford University Press; 2002. https://global.oup.com/academic/product/basic-cell-culture-9780199638536?cc=my&lang=en& [25 March 2017].