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Potentiation of Cell Death and DNA Damage Through 5-Fluorouracil and Ferulic acid Coadministration in p53 Mutant HT-29 Cell Lines

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Abstract: When the Mediterranean diet is set in focus, scientific studies report a strong statistical correlation between human nutrition, diet, and cancer incidence. Considering the anticancer effects of a fiber-rich diet, it is understood that the anticancer effect is not only due to the bulk cellulosic material load but also related to the increased bioavailability of cellulose-bound bioactive (anticancer) compounds released due to intestinal microflora activities. Ferulic acid (FA) is one of the components found ubiquitously in the fiber fraction of plant food. Because of its effects on cancer cell viability and its association with a low incidence of cancer concerning a fiber-rich diet, FA can be considered an anticancer agent. In this work, it was investigated whether FA can potentiate the effects of anticancer drugs at lower doses. For this, a general anticancer drug named 5-Fluorouracil (5-FU) was used, and potentiation tests were performed on two cancer cell lines, namely A2780 besides HT-29, which has the homozygous mutation for p53. According to the results, it was interpreted that the anticancer effect of 5-FU was readily potentiated with 200 µM FA in both cancer cell lines, and DNA damage induced by 5-FU was potentiated by co-administration of FA. When cell viability and DNA damage of A2780 and HT-29 lines are evaluated together, we think it is most probable that 5-FU and FA administered jointly show its anticancer effect, especially by strengthening the apoptosis pathway triggered by DNA damage. If it might be possible to uncover the mechanism that drove DNA damage mediated apoptosis in p53 mutant HT-29 cells we may shed light on the treatment of chemotherapy-resistant cancer incidences. © 2023 NTMS.

Keywords: Cancer, HT-29; Chemotherapy; 5-Fluorouracil; Ferulic Acid.

1. Introduction

The famous Mediterranean Diet is a nutritional pyramid that rises on the shoulders of lightly to moderate cooked plant foods, fresh fruits, and vegetables. The second layer of this nutritional pyramid holds seafood and fish that provide saturated oils and minerals, while poultry, fermented products, meat products, and sweets make up the minor parts of this balanced diet. Various bioactive phytochemicals obtained from different plant sources have disease preventive effects, and some are proven to provide effective support in treatments against diseases such as atherosclerosis [1], thrombosis [2], cholesterolemia [3-5], diabetes [4-6], cancer [2, 5-7]. etc. On the contrary to Mediterranean Diet, an unbalanced diet progresses in

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the form of visceral fat, liver dysfunction, and obesity and results in various health problems [7]. Among these health problems, cancer cases hold a pretty significant fraction, and about one-third of cancer types are related to diet. In addition, it has been determined that there is a direct link between 13 types of cancer and obesity [8].
Although cancer therapy planning requires evaluation of cancer type, state of malignancy-metastasis, patient health status, age, etc., treatment is usually combined with chemotherapy and or radiotherapy due to lack of early diagnosis. In both chemotherapy and radiotherapy, the nontarget effects of treatment derive a general negative health status and exert negative pressure, especially on bodily essential organs like the liver, cardiovascular system, and kidneys [9-11]. The ultimate method of alleviating or even eliminating off-target effects in chemotherapy is targeted drug delivery [12, 13]. The basic idea behind targeted drug delivery stems from the problem that the dosage of the drug applied readily exceeds the tolerances of normal cells while barely killing cancer. A general pharmacologic trick in circumventing the overdosage of chemotherapeutics' is through drug interactions, i.e., administration with another bioactive compound and potentiating the anticancer effect at lower doses [14, 15].
5-Fluorouracil (Figure 1-A) is the reference cancer drug frequently used in cancer therapy studies where the context is determined as DNA damage and p53-apoptosis pathway [16, 17]. This drug interferes with nucleoside synthetic pathways and its metabolites are incorporated into nucleic acid chains during RNA and DNA synthesis, ending up with nucleic acid damage. One of its metabolites also complexes with Thymidylate Synthase (TS) and inactivates the enzyme, resulting in deoxy-thymidine depletion and consequently cytotoxicity and cell death [17]. 5-FU exerts its' cytotoxic effects on cancer cells through the tumor suppressor p53 regulated apoptosis pathway. The much-mentioned major disadvantage of this p53 apoptotic pathway approach is met with the apoptosis-resistant p53 mutant cancer cell lines. To eradicate cancer, drug combinations, dosage increment, or potentiating the 5-FU is usually considered at this stage.
Ferulic acid (Figure 1-B) is a phenolic acid, and it is described as one of the derivatives of cinnamic acid. This phenolic acid is a ubiquitous component of fiber material in the plant kingdom, and it is released in bulk amounts during processing of plant food [2, 18, 19]. This phenolic acid has low toxicity as its' glucuronic acid conjugates are readily absorbable and thoroughly excreted. The unconjugated portion of Ferulic acid is conjugated with glucuronide, and absorption can be achieved when the hindgut microbiota processes the fibrous plant parts. Ferulic acid is tested as a pharmacological agent in infection, inflammation, and cancer indications. It is frequently added to food and cosmetics products [2].

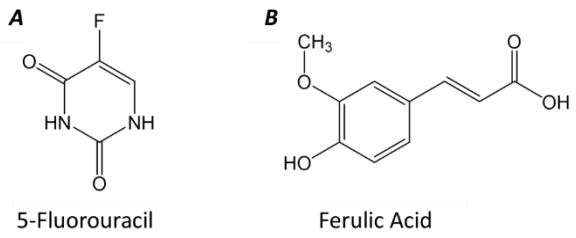

Figure 1. Chemical structure of *5-Fluorouracil* (A) and *Ferulic Acid* (B).

In this study Ferulic acid along with 5-Fluorouracil was applied on two cancer cell lines namely HT-29 and A2780 to test if 5-FU triggered p53 mediated apoptotic pathway could be potentiated at lower doses of 5-FU. Our aimed to test whether the anticancer effect of 5-Fluorouracil (a general anticancer drug) could be potentiated at lower doses on the HT-29 and A2780 cells when co-administered with Ferulic acid.

2. Material and Methods

2.1. Preparation of Cells Lines, Application of Ferulic Acid and 5-FU

HT-29 and *A2780* cell lines (ATCC, USA) were retrieved from liquid nitrogen and cell cultures were initiated in 75 cm^2 culture flasks with RPMI-1640 medium. Additives used in this media were 10 % *fetal bovine serum*, 1 % *penicillin*/ *streptomycin*, and 1 % non-essential amino acids. Cell cultures were maintained at 5 % CO_2 density at 37 °C throughout the experiment in Thermo Forma II CO_2 Incubator [USA]. After maintaining a proper confluency rate (min 90 %), cells were plucked out using *trypsin-EDTA* solution. Following the tests, cell vitality scores were measured with 0.4 % trypan blue. Cells with viability scores at least 90 % were used in the experiment. *5-FU* and *FA* test were started with a cell concentration of approximately 5×10^3 cells per well within 96-well plates. The test incubation durations were 24 hrs.
Working solutions of *Ferulic acid* and *5-FU* were prepared in DMSO. Cells were treated for 24 hours with different concentrations of compounds (25-400 μM for FU and 1-100 μM for 5-FU).

2.2. MTT Assay

MTT assay is the method of assessing cell viabilities through evaluation of colorimetric measure of metabolic activity. Measurements made in *MTT* assay depends on conversion of the tetrazolium salt (*MTT*: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to its insoluble purple colored formazan form. This conversion is handled with oxidoreductase enzymes in actively transpiring, metabolically active cells [20]. Highly condensed purple color designates cell viability whereas there exists no color development when cells are dead. Although *MTT* assay has many uses, it is utilized to test cytotoxicity of certain chemicals on cancer cells. In our work *MTT* assay was

performed as follows. First, whole liquid media was aspirated from the well, then 50 µl of 0.5 mg/ml *MTT* working solution was added into each well. After 3 hours of incubation at 37 °C in the incubator, *MTT* solution was aspirated out. Finally, 100 µl DMSO was added into the wells to release the *MTT* color developments, and scores were recorded in an *ELISA* reader (Thermo Multiscan Go, USA) at 570 nm wavelength [21].

The control group *MTT* scores (100 % viable) were taken as reference, and the test groups' viability scores were calculated concerning the control group [22, 23]. MTT scores were interpreted from at least cumulative of five independent experiment scores.

2.3. Alkaline Comet Assay

Following *5-Fluorouracil* (*5-FU*) and *Ferulic acid (FA*) applications, cancer cell lines' DNA damage tests were performed according to Alkaline Comet Assay (24). Depending on the initial viability test evaluations, concentrations of *5-FU* and *FA* used in Comet Assays were determined as 50 µM and 200 µM for *A2780* cells whereas it was determined as 20 µM and 200 µM for *HT-29* cells respectively. Test was performed on cells cultured in 6-well plates for 24 h culture period. First, whole media was aspirated, then cells were rinsed (2X) with PBS. Following this step, approximately 500 µl PBS was added to the well, and cells were scraped off the surface. Final cell suspension volume was adjusted to 1 ml with PBS.

10 µL of these suspended cells (approximately 10^4 cells/ µL) were added into 80 µL 1 % low melting agarose (LMA). The cell-LMA mix was overlaid on top of 1 % standard agarose-coated slides, and the surface leveling was done with lamella laid on top of the microscopy slides. Following cooling and gel solidification lamella were gently removed and cell lysis were performed. For lysis, slides were treated in lysis solution for 1 h at 4 °C. Lysis solution composition was 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Triton-X, and 10 % DMSO where pH 10 was maintained. Following lysis slides were electrophoresed at 25 V (5V/ cm, max. 300 mA) for 30 min. Finally, the slides were washed in neutralization solution three times for 5 min; neutralization solution was composed of 0.4 M Tris at pH 7.5. Staining was performed with ethidium bromide and the DNA damages were examined under fluorescence microscope (Zeiss Axio-scope, Germany). Tail DNA (%), evaluations were performed on randomly selected 250 cells from each group and performed with TriTek Comet Score software.

2.4. Statistical Analysis

Statistical analyzes were performed with Sigma Plot 12 package program. Data were interpreted as percentiles of mean values and standard deviation scores (Mean±SD). Following normality and homogeneity analysis, data variances and intergroup mean-variance comparisons were made with the Kruskal-Wallis H test. Comparisons of Comet Assay group data were performed with Mann-Whitney U test. Scores that fall into the $P<0.05$ probability domain were considered significant.

3. Results

3.1. Viabilities of A2780 Cell Lines with respect to 5-FU and FA Dosages

Viability tests of the human ovarian cancer cell line (*A2780*) treated with 100 µM *5-FU* showed a significant reduction in viability scores (around 12 %) compared to the control groups (Figure 2-A). Besides this, *A2780* cell line viabilities were also tested against different concentrations of *Ferulic acid (FA*) (25-400 µM) (Figure 2-B). The effective dose that scored significantly lower viability rates (around 18 %) was determined as 200 µM FA. Based on these results, in *5-FU* potentiation tests, the *5-FU* concentration was fixed at 50 µM, while the *FA* concentration ranged from 50 to 400 µM (Figure 2-C).

Compared with *FA* data (given in Figure 2-B), administration of 50 µM *5-FU* combined with 200 µM *FA* decreased *A2780* cell viability from 18 % to 34 % (Figure 2-C). Similarly, in the presence of *5-FU*, when the *FA* concentration was shifted to 400 µM, *A2780* cell viability was decreased from 51% (Figure 2-B/ 400 µM *FA* data) to 70 % (Figure 2-C/ 400 *FA* and 50 µM *5-FU* data).

As a result, it was observed that the drug interaction worked and the viability of *A2780* cells decreased from 66 % to 30 % when the *FA* dose was increased from 200 µM to 400 µM in *A2780* cells treated with *5-FU* (Figure 2-C).

3.1. Viabilities of HT-29 Cell Lines with Respect to 5-FU and FA Dosages

Viability tests on *HT-29* cell lines treated with both 50 and 100 µM *5-FU* showed statistically significantly reduced viability (approximately 12 %) when compared to control groups (Figure 3-A). Along with it, viability tests performed at varying concentrations of *Ferulic acid (FA*; 25-400 µM) revealed that *HT-29* cell line viabilities dropped more at 200 and 400 µM *FA* (Figure 3-B) as compared to that of *A2780* (Figure 2-B). Compared to 18 % and 51 % decrease in cell viabilities of *A2780*, *HT-29* cell line viability decreases were scored as 31 % and 62 % at 200 and 400 µM *FA* concentrations applied respectively. That is, *FA* showed a more substantial effect in *HT-29* cell lines compared to *A2780*. According to the results obtained from the experiments performed on *5-FU* and *FA* separately with *HT-29* cell line, in the *5-FU* potentiation experiments, *5-FU* concentration was fixed to 25 µM while the *FA* was changed from 50 to 400 µM (Figure 3-C).

When *5-FU* and *FA* were applied together, it was observed that *HT-29* cell death progressed from about 46 % to 66 % as the *FA* concentration was increased from 200 to 400 µM. When whole viability tests of *HT-29* were compared, it was recorded that 25 µM *5-FU*

resulted in 5 % cell death and, when 200 μM *FA* is used, 32 % cell death occurred, and finally when 25 μM *5-FU* and 200 μM *FA* are used together, 45 % cell death rate is observed. As compared with the test results of the *A2780* cell line, these above-stated cell death scores for the *HT-29* cell line were higher with the combinatorial use of *5-FU+FA* than only *FA* use.

3.3. DNA Damage Scores of A2780 and HT-29 Cell Lines with 5-FU and FA Combination

Depending on the data presented above for previous experiments, it was observed that the anticancer activity of 5-FU at low doses can be potentiated when coupled with FA. Based on these results, the concentrations to be applied in combinatorial use experiments were determined as follows; the FA was fixed at 200 μM, while the concentration of 5-FU was set at 20 μM and 50 μM for the HT-29 and A2780 cell lines, respectively. DNA damages in cells exposed to these fixed dosages were determined by Alkaline Comet Assay.

When the *Comet Assay* results of control for both cell lines were compared, it was observed that the *A2780* cell line containing wild-type p53 produced approximately 5.5 % tail DNA (DNA damage score of the median), while the *HT-29* cell line homozygous for the p53 mutation produced 22 % tail DNA (DNA damage score of the median). This case can be explained as follows: In the *A2780* cell line with wild-type p53, cell-cycle is arrested till DNA damage repair is achieved, whereas in the p53 homozygous mutant cell line *HT-29*, cell cycle checkpoints are bypassed, and DNA damage is accumulated due to the nonfunctional (mutant) p53 copies (Figure 4-A and B control groups compared).

When only *5-FU* (50 μM) is applied to *A2780*, group median (cells) accumulated 57 % Tail-DNA (damage) while, *5-FU* (50 μM) and *FA* (200 μM) co-administration resulted in accumulation of 67 % Tail-DNA in the group median cells (Figure 4-A). When both groups' quantile and the median DNA damage scores are compared, we have seen that the Q3 (3rd quantile) DNA damage squeezed to 69.5 % with *5-FU* application is jumped to 74 % if *5-FU* and *FA* applied together. That is as we evaluated both *A2780* cell viability (Figure 2-C) and DNA damage (Figure 4-A) although co-administration of *5-FU* and *FA* enhances cell death (to 34 %), DNA damage potentiation rate increased around 5%. In other words, *5-FU* and *FA* potentiated *A2780* cell death might not be related to DNA-damage and apoptosis.

On the other hand, if only *5-FU* (20 μM) is applied to *HT-29* cell line, the group median accumulated 29 % (Tail-)DNA damage but quantile range distribution pattern resembled that of the control. As an argument to support this we can state that, with the *5-FU* administered group, second quantiles' DNA damage scores were more condensed compared to the third quantile scores (Figure 4-B). Compared to 29 % DNA damage score of only *5-FU* administration, *5-FU* and *FA* co-administration on *HT-29* resulted a median DNA damage score of 46 %. And, in the second and third quantile range this data showed almost normal distribution. Depending on the overall evaluation of both viability and DNA damage data for *HT-29*, we may postulate that, *5-FU* and *FA* co-administration potentiated both *HT-29* cell death and DNA damage.

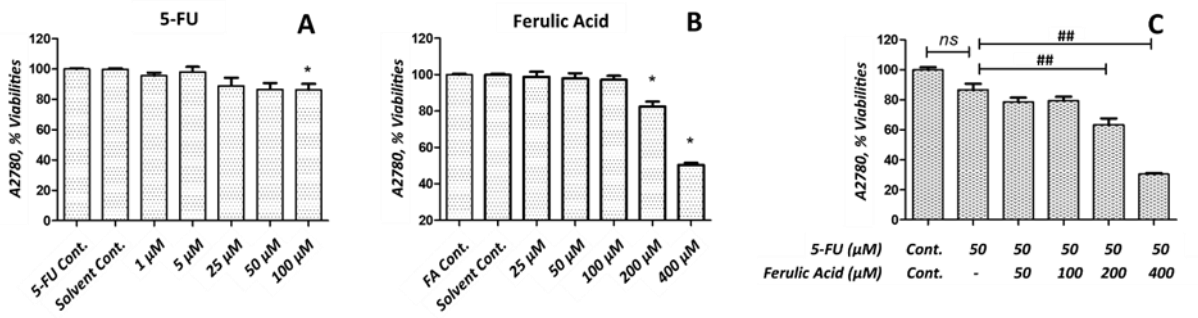

Figure 2. Cell viability tests performed on A2780 cell lines with 5-Fluororuracil (A), with Ferulic acid (B) and with 5-FU+FA combination. Data represented are mean scores capitated with standard deviation calculations. *p<0.05 vs control, ## p<0.01 between groups; ns: non-significant.

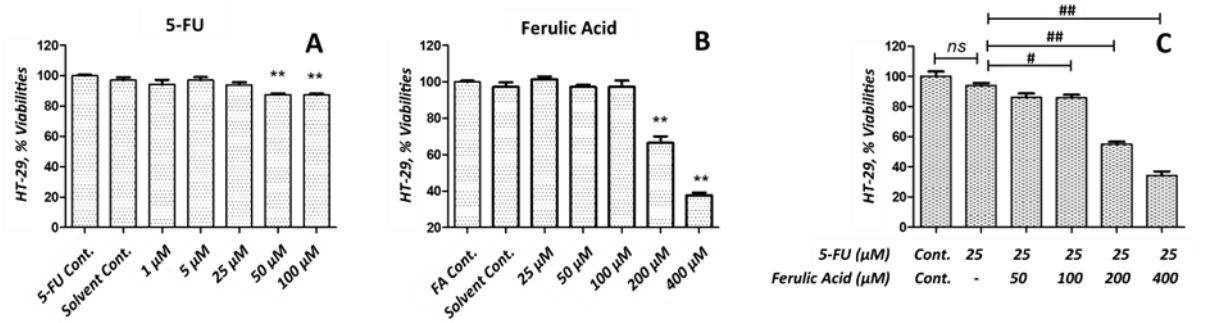

Figure 3. Cell viability tests performed on HT-29 cell lines with 5-Fluororuracil (A), with Ferulic acid (B) and with 5-FU+FA combination. Data represented are mean scores capitated with standard deviation calculations. ** p<0.01 vs control; # p<0.05 and ## p<0.01 and ### between groups; ns: non-significant.

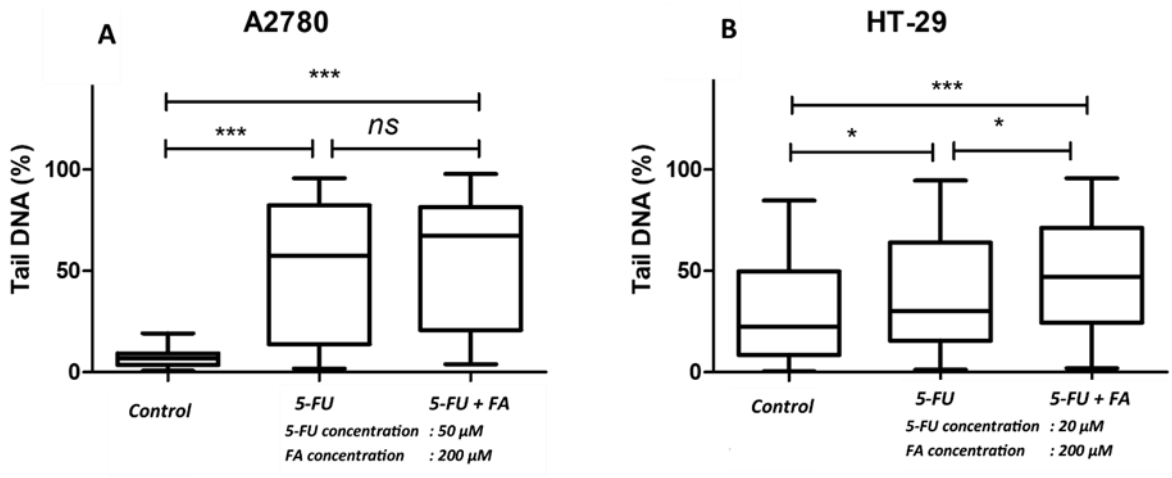

Figure 4. DNA damage data were interpreted as whisker-box plots. DNA damage score of the sample median was denoted with horizontal transverse lines in each box. The Mann-Whitney U test was applied for comparisons between groups. *p<0.05, **p<0.01, ***p<0.001.

4. Discussion

Colorectal cancer is among the most prevalent cancer incidence seen worldwide with both sexes and usually progresses into malignant states before diagnosis [25]. In relation to in vitro anticancer studies performed on colorectal cancer, HT-29 is generally the preferred cell line. In association with our experimental design, since our aim was to potentiate 5-FU at lower dosages we have preferred HT-29 cell line that possesses homozygous p53 mutation characterized as Arginine 273 to Histidine conversion and also shows a level up (mutant) p53 expression to a higher titer [2, 25, 26].

A2780 is the ovarian cancer cell line which had been recovered from an untreated patient; that is, this cell line was not exposed to any anticancer drug or chemical. Therefore, it is in demand as a cell line for testing potential anticancer chemicals and different drug delivery methods [27]. Also it is important to note that A2780 expresses wild type p53 [28]. In this study, together with the HT-29 cell line (p53 mutant), A2780 was used as the reference (control) cancer cell line expressing wild-type p53 during testing 5-FU drug potentiation with Ferulic acid.

Depending on the test results evaluated, we may state that administration of 5-FU coupled to FA decreased the cell viabilities of both A2780 (wild type p53) and HT-29 (homozygous mutant for p53) cancer cell lines. And we can also state that in 5-FU+FA administered HT-29 cell lines, DNA damage was normalized and enhanced by insignificant rates compared to the A2780 groups.

In the scientific literature Ferulic acid had been tested for its supportive function in alleviating multidrug resistance in cancer cell lines. FA was shown to bind P-glycoprotein and inhibit excretion anticancer drugs [29]. Works on multidrug resistance phenomenon point out indirect downregulation of mdr1b by FA [30]. Although in some other FA-anticancer evaluations, cancer cell death had been proposed as un-linked to p53 apoptotic pathway [31], reports also point out FA and Cu interaction and consequently ROS generation and DNA damage as putative cancer therapy treatment [32]. Still some other reports point out FA as potent anticancer agents triggering cell cycle stall and autophagy in cancer cells [33]. Depending on the literature although one can declare that anticancer effect of FA might not be through p53 mediated apoptosis, it is obvious that FA exerts its anticancer effects thorough multiple routes.

5. Conclusion

Depending on the literature reviews and our observations we can state that FA and 5-FU co-administration may induce cell death with different routes in A2780 and HT-29 Cell lines. It should still be questioned if FA may or may not be triggering p53 mediated apoptosis in wild type p53 A2780 and mutant p53 HT-29 cell lines as co-administered with 5-FU. Our observation also is in concert with the finding that postulates FA inhibiting or hampering multidrug resistance phenotype (Figures 2 and 3). It was also interesting to find out that 5-FU+FA co-administration stacked DNA damage of the third quantile (cells) to higher rates in both A2780 and HT-29 cells (Figure 4). This data is also in concert with the information given in the report of Sarwar et al. [32].

Depending on the experimental data and literature reviews we may conclude that, since FA has selective potential on cancer cells and has direct impact in DNA integrity [33] while it also potentiates the effects of other anticancer drugs like 5-FU, further diagnosis on combinatorial use of FA with other drugs may point out plausible cancer chemotherapies along with revealing alternative apoptotic mechanisms as models for cancer regression.

Limitations of the Study

Within the scope of the study and experimental design, the author cites the apparent shortcoming of the study as the inability to test the dissolved effective concentrations of the tested chemicals.

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Conflict of Interests

Author declares no conflict of interest with this study and manuscript.

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Author Contributions

The entire text of the work is attributed to the responsible author.

Ethical Approval

Scientific works undertaken with this study did not require any ethical approval.

Data sharing statement

Author declares; All data generated by this work are publicly available as long as reference rights are not violated if any part of the work is used.

Consent to participate

Author declares; There are no other participants related to this work.

Informed Consent

Author declares; Since the work is self-contained, there is no need of another researchers
consent approval.

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