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Determination of the Anticarcinogenic Activity of 5-Hydroxymethyl-2-furfural Produced from Grape Must Under *in vitro* Conditions

Kubra Kelleci[1,2*], Eda Golebatmaz[3]

[1] Beykoz University, Vocational School, Department of Medical Services and Techniques, Istanbul, 34805, Turkey
[2]Yildiz Technical University, Faculty of Chemistry and Metallurgy, Department of Bioengineering, Istanbul,34210 Turkey
[3]Eskişehir Osmangazi University, Institute of Science and Technology, Department of Biology, Eskisehir, 26040, Turkey

Abstract: Every year, millions of tons of food and beverage waste are thrown away unused around the world. The carbohydrates found in food waste create a raw material potential for the production of high value-added products that are used in energy, feed and pharmacology. One of these products, 5-Hydroxymethyl-2-furfural (5-HMF), is a by-product of simple dehydration of carbohydrates. It finds wide use in the field of pharmacy due to its anticancer, antifungal and antimicrobial activities. Many studies have stated that the sugar source with the highest conversion rate in 5-HMF production is fructose. For this reason, in this study, it was aimed to realize the production of 5-HMF in autoclave sterilization carried out under high temperature and pressure using grape must waste, which is known to have high fructose content, and determine the anticarcinogenic activity and cytotoxicity of the produced 5-HMF under in vitro conditions. In this study, it was determined that the medium containing DMSO increased the sugar conversion percentage, 5-HMF efficiency and selectivity in the waste grape must more than the medium containing only water. In the production of 5-HMF, the conversion of sugar in the medium saturated with salt, and the efficiency and selectivity of 5-HMF were determined as 97.04%, 68.61% and 70.82%, respectively, when DMSO organic solvent was used. In addition, it has been determined that 5-HMF produced from waste grape must has a toxic effect on both healthy cells and cancer cells and has anticancer properties.

Keywords: 5-HMF, anticancer, grape must, fructose, DMSO.

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***Corresponding author. E-mail:** kubrakelleci@beykoz.edu.tr.

1. INTRODUCTION

It is known that approximately 931 million tons of food and drink are thrown away every year (1). Carbohydrates, which are found in large quantities in food waste, are a potential source of raw materials for the production of petrochemical products (2). Due to the fact that petrochemical products are decreasing day by day and the damage these products cause to nature, researchers have turned to the search for renewable energy sources (3) and it has been foreseen that furan derivatives such as 5-Hydroxymethyl -2-furfural (5-HMF) will be one of the most important renewable energy sources in the future. According to the report prepared by the United States Department of Energy, 5-HMF and its oxidation products are among the 10 promising biomass-based products that are predicted to replace petroleum-derived products (4). In addition to being used as an energy source, it is also widely used in the synthesis of a wide range of products covering the food and pharmaceutical industries.

5-HMF is a by-product of the acid-catalyzed dehydration of simple carbohydrates (glucose,

fructose, xylose, etc.). The conversion of glucose and sucrose to 5-HMF was 60-70%, while the conversion of fructose to 5-HMF was found to be 100% (5,6). Therefore, the current industrial production process is based on the use of fructose (7). Although the market value varies according to the product quality and production process, it is known to be between 2-300 US dollars per kilogram (8). Considering that costs are also related to raw material access, the use of waste fructose instead of pure fructose will reduce costs, thus increasing access to 5-HMF and increasing its economic value (4). Therefore, in this study, 5-HMF production potential from grape must, which is known to have high fructose content, was investigated during autoclave sterilization under high temperature and pressure.

In addition, when literature studies were examined, it was reported that high levels of 5-HMF were detected in dried fruits and juices made from dried fruits (25-2900 mg/kg), as well as in caramel products (up to 9500 mg/kg). Bread (up to 410 mg/kg) and coffee (up to 420 mg/L) are also among the most important sources of human exposure to 5-HMF, although the levels in these foodstuffs are high (9,10). Estimated daily intake of 5-HMF is 30-150 mg per person (11); which is several times higher than the estimated daily intake of other heat-induced food toxicants such as acrylamide and furan (12,13).

In this study, it was aimed to obtain 5-HMF, which causes high human exposure due to food and beverage consumption, from grape must, which is known to have high fructose content, and to determine its anticarcinogenic activity and cytotoxic activity using LNCaP prostate cancer cell line and J774 murine macrophage cell line.

2. EXPERIMENTAL SECTION

2.1. Materials

99% purity DMSO (Dimethylsulfoxide; Sigma-Aldrich 276855) was used as the solvent. After the experimental study, 5-HMF, levulinic acid, formic acid analyzes were performed using Shimadzu LC-20AT HPLC device. Transgenomic Corogel 87H3 column was used for the analysis of 5-HMF, fructose and organic acids. Levulinic acid standard (Sigma-Aldrich 41474), formic acid standard (Sigma-Aldrich F0507), 5-hydroxymethyl-2-furfural (5-HMF) standard (Sigma-Aldrich H40807) were used for HPLC analysis. ISOLAB brand PTFE 0.45µm syringe filters were used for the filtration process applied before the analysis of the samples.

RPMI-1640 (Gibco 21875034), FBS (Fetal Bovine Serum; Sigma-Aldrich F7524), penicillin-streptomycin antibiotics (Sigma-Aldrich P4083), DMEM (Sigma-Aldrich D0822), Trypsin-EDTA solutions (Gibco 25200056) used in MTT analysis and cell culture studies to be used in anticarcinogenic activity and cytotoxicity of 5-HMF were purchased.

2.2. Production of 5-HMF from Grape Must

Grape must was preferred in the study due to its high fructose content. Studies have shown that the salt-saturated medium in 5-HMF production accelerates the transition of sugars to the organic phase after they are converted to 5-HMF in aqueous medium. Therefore, in this study, NaCl was used to prepare a saturated salt solution. Studies in the literature have shown that the use of organic solvents during production significantly increases the conversion rate of fructose from beverage wastes, 5-HMF efficiency and 5-HMF selectivity (14). Therefore, in this study, DMSO organic solvent, which is known to be used frequently in the literature, was used for the production of 5-HMF and the results were compared with the water environment where organic solvent was not used. The pH value of the experimental medium was fixed to 0.6 using HCl.

In the study, the volume of DMSO and grape must was determined as 76% and 24%, respectively. It was aimed to dehydrate the fructose to 5-HMF by exposing the samples taken in glass tubes at different concentrations for 3 hours in autoclave at 120^0C. After completion of the reaction, the purity was checked by HPLC. All experiments were repeated 3 times. All chemical and organic solvents used in the study were of analytical purity (>99.5%) and were obtained from SIGMA.

2.3. HPLC Analysis

5-HMF sample and fructose analyzes were performed using SPD Detector SPD M-10A (Phenomenex; 50 x 4.6 mm; 5 um particle size) HPLC (Shimadzu Liquid Chromatography) instrument with C18 column. It was carried out using acetonitrile and water (30:70 v/v) as mobile phase in isocratic mode with a UV wavelength of 320 nm, a flow rate of 0.6 mL/min. The samples were loaded into the HPLC device after passing through 0.45µm filters before analysis.

2.4. LNCaP and J774 Macrophage Cell Culture

The human prostate cancer (LNCaP; CRL1740™) cell line previously purchased from the American Culture Collection (ATCC) and stored in a cryobank was used in this study. Prostate cancer cells were inoculated in a 25 cm^2 culture dish containing 10% FBS, 1% penicillin-streptomycin antibiotic solution and 5 mL of DMEM, and incubated at 37°C under ambient conditions with 5% CO_2 and 95% humidity. The cells were refreshed every 2 days and monitored with an inverted light microscope. When the cell density reached 70-80%, they were transferred to a 75 cm^2 culture dish and the medium of the cells was refreshed every 2 days. Cell density and pollution were monitored using an inverted light microscope, and the continuity of the cell line was ensured by passage of cells until they were taken into the experiment.

LNCaP cells were exposed to 0.25% Trypsin-EDTA solution for 5 minutes. At the end of the time, it was checked under an inverted light microscope and it

was observed whether the cells were separated from the surface of the culture dish. The separated cells were transferred to a centrifuge tube containing 9 mL of medium and centrifuged at +10°C, 1200 rpm for 5 minutes. At the end of the time, the supernatant part in the centrifuge tube was discarded, and the cell pellet, which had settled to the bottom, was homogenized by pipetting with 2 mL of medium. The method of staining dead cells with Trypan Blue Dye (Sigma-Aldrich) was used to determine the cell number and percent viability. After counting the cells, the calculation was made as $1x10^4$ live cells in 100 µL of medium in each well, and inoculated into 96-well culture dishes. Culture dishes with 96 wells inoculated were incubated for 24 hours in a carbon dioxide incubator containing 5% CO_2 and 95% humidity at 37°C.

J774 murine macrophage cell line, T25 in RPMI-1640 broth containing 10% fetal bovine serum (FBS), 80 µg/mL Gentamicin and 1 M HEPES in a humid atmosphere at 37°C, 95% humidity and 5% CO_2 cultured in culture flasks. After the cells reached the required confluency (80-90%), they were physically collected and centrifuged at 25°C, 1000 rpm for 5 minutes. Then, seeding was carried out in 96-well plates at $1x10^5$ cells/ml per well. Cells inoculated into the culture media created were kept under the required incubation conditions for 24 hours. Macrophage cells are used to perform cytotoxicity assays. Cell growth was monitored daily with an inverted microscope.

2.5. Determination of Cytotoxic Concentrations

To determine non-toxic concentrations in J774 macrophage cells, in which LNCaP cells were used as hosts before 5-HMF components were used, 5 x 10^4 J774 macrophage cells were seeded into each well of a 96-well microplate and incubated at 37°C for 24 hours. After the macrophages adhered, 5 different concentrations of 5-HMF (5 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL), which were homogenized with (10%) DMSO/H2O and sterilized by passing through a 0.20 µm membrane filter, were diluted and added to the wells for another 24 hours. After 48 hours of incubation, MTT assay was performed on macrophage cells. 10µL of MTT solution with a final concentration of 10 mg/mL in PBS was transferred to each well of the microplate and cells were incubated for 4 hours at 37°C to form formazan crystals due to the reaction between MTT salt and viability dehydrogenase enzymes. Then, 100 µL of DMSO was added to each well to dissolve the formazan crystals. Absorbance values were measured at 570 nm using an ELISA reader. All studies were performed in 3 replicates.

Cell viability analysis data were obtained using equation 1 and data plots were generated.

$$\text{Cell viability (\%)} = \frac{\text{Abs of sample} * 100}{\text{Abs of control}} \quad (1)$$

2.6. Statistical Analysis

Obtained data were calculated as mean±standard deviation. For statistical analysis, parametric tests (Unpaired sample t-test, analysis of variance and Mann-Whitney U test) were used using the “SPSS 16.0 for Windows” program. Significance level was accepted as 5%.

3. RESULTS AND DISCUSSION

3.1. HPLC analysis

In this study, in which 5-HMF production potential from grape must was investigated during autoclave sterilization under high temperature and pressure, it was determined that the reducing sugar content in the grape must waste used in the experiments was 16.4%. In this study, it was determined that the medium containing DMSO increased the sugar conversion percentage, 5-HMF efficiency and selectivity in the waste grape must more than the medium containing only water. In the production of 5-HMF, the conversion of sugar in the medium saturated with salt, and the efficiency and selectivity of 5-HMF were determined as 97.04%, 68.61% and 70.82%, respectively, when DMSO organic solvent was used, while the values obtained in the medium containing only water were 68.13%, 45.04% and it as determined as 63.27%. Chromatogram graphs obtained from HPLC analyzes are given in Appendix 1, and 5-HMF calibration chart is given in Appendix-2. In addition, HPLC chromatogram images of levulinic acid and formic acid standards are given in Appendix- 3 and Appendix -4.

3.2. Toxicity Study

The cytotoxic effects on the J774 macrophage cell line were investigated. No significant cytotoxicity was detected at low concentrations in the study ($p < 0.05$). It has been observed that it has an inhibitory effect in direct proportion with increasing concentrations. To investigate the survival percentages of LNCaP cells, MTT cellular viability assay was performed after 24 hours of incubation. It has been shown that 5-HMF is not very toxic to macrophage cells compared to LNCaP cells. According to the results of cytotoxicity and anti-cancer activity, viable cell ratios are shown in the graphs. When the J774 macrophage cell toxicity results were compared with the control cell, it was observed that 5-HMF did not cause toxicity at concentrations up to 10 µg.

According to the results of J774 cytotoxicity in Figure 1, it was seen that the most effective concentration increasing cell proliferation was at 5 µg/mL. It was observed that toxicity occurred in the cells with increasing concentrations from 25 µg/mL. The anticancer effect due to the increasing concentration of 5-HMF for 24 hours was examined in LNCaP cells and the effect levels are given in Figure 2. The anti-cancer effects of 5-HMF on LNCaP cells, whose toxicity was measured with J774 cells, were compared with control cells. As shown in the graph of the results in Figure 2, an anti-cancer effect was observed from 10 µg/mL with increasing

concentrations. The most effective anti-cancer activity occurred at 100 µg/mL, and it was seen that it affected more than half of the LNCaP cells. Toxicity studies were performed on 5-HMF obtained after experimental studies, both on J774 cells and on LNCaP cells, and it was determined to be anticancer.

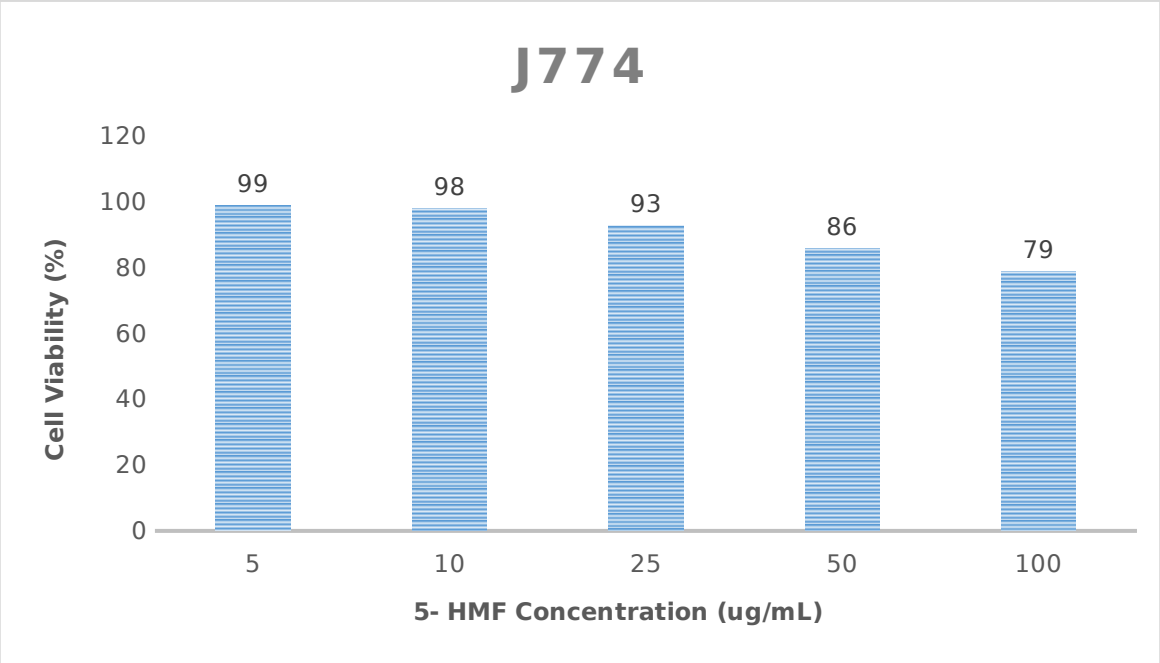

Figure 1: Decreased cell viability with increasing 5-HMF concentration in J774 macrophage cells.

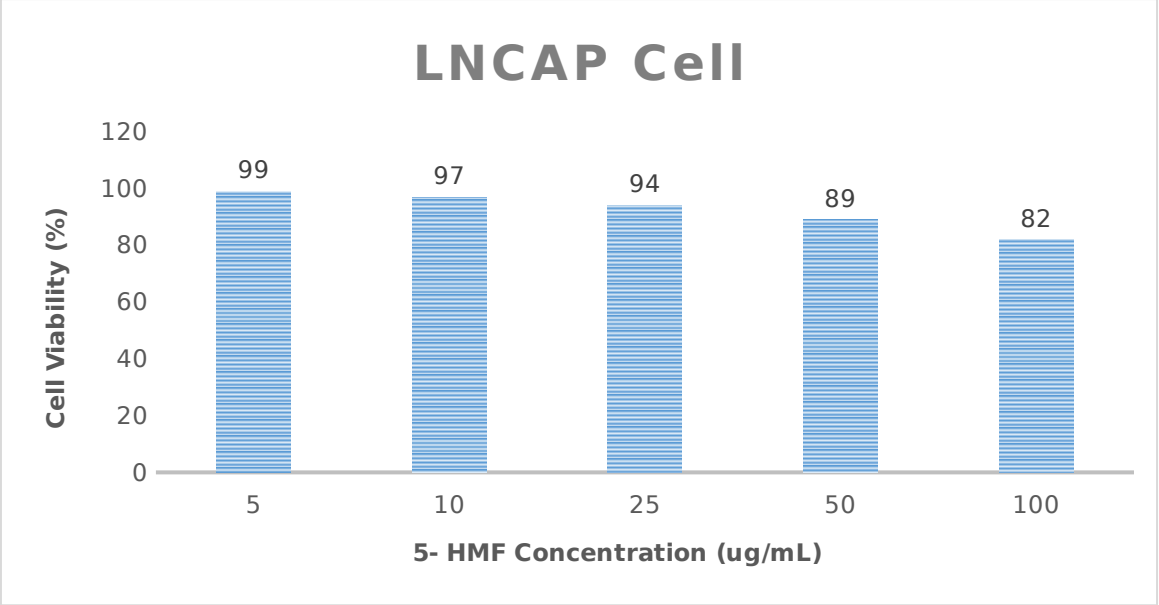

Figure 2: Decreased cell viability with increasing 5-HMF concentration in LNCaP cells.

4. CONCLUSION

In this study, the production potential and anticarcinogenic effect of 5-HMF from grape must waste, which is known to have high fructose content, was investigated under in vitro conditions. In the study, DMSO was used as an organic solvent in order to increase the productivity of the product, since 5-HMF decomposes at high temperatures, in acidic and aqueous environments, and turns into levunilic acid and formic acid. It is known that DMSO is frequently used in previous studies on 5-HMF production (6). In addition, it has been revealed by the studies that the experimental medium saturated with salt in the production of 5-HMF accelerates the transition of sugars to the organic phase after conversion to 5-HMF in aqueous medium. In our study, it was determined that DMSO organic solvent significantly increased the sugar conversion percentage, 5-HMF efficiency and selectivity in the

waste grape must compared to the medium containing only water. Previous studies in the literature support our results (6).

Toxicity studies of 5-HMF produced during autoclave sterilization with waste grape must were carried out in both healthy cells and cancer cells and its anticancer activity was determined. J774 murine macrophage cell line was used for healthy cells and LNCaP prostate cancer cell line was used for carcinogen cells.

According to the data obtained from the studies, it was found that the highest 5-HMF concentration, 100 µg/mL, caused damage to both cell lines. At this concentration, there was also a moderate but statistically significant reduction in cell viability in all cell lines. It has been determined that 5-HMF also causes significant cell death at lower concentrations. Therefore, it has been found that 5-HMF obtained after experimental studies has a toxic effect and is anticancer in both healthy cells and cancer cells.

This study is one of the limited studies on the production of 5-HMF from food and beverage wastes in our country and the determination of its anticancer activity. It provides important data and is a source for the studies to be done on the production and analysis methods we use in this study and the optimization of 5-HMF production methods. It is important to investigate the effects on yield by using different ionic solvents and different catalysts in 5-HMF production by researchers. It is also recommended to test the anticancer activity under in vivo conditions.

5. CONFLICT OF INTEREST

The authors report no conflicts of interest.

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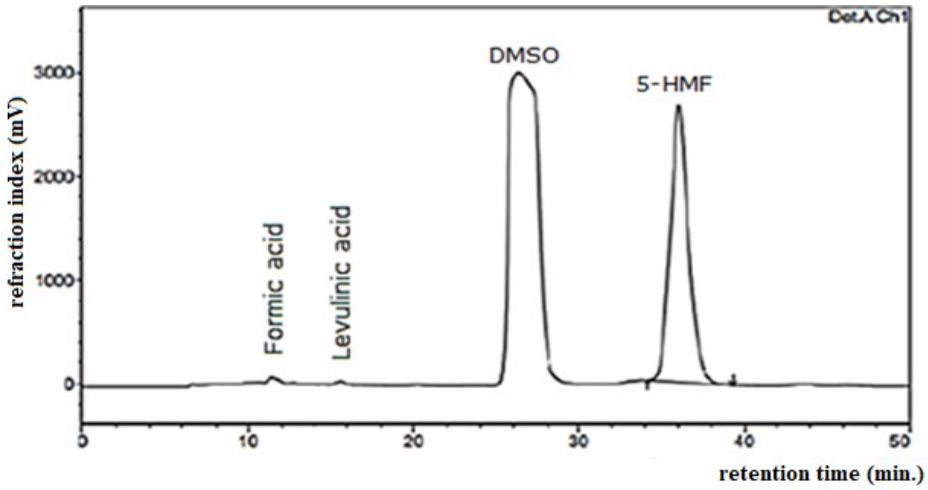
Supplementary Information

Determination of the Anticarcinogenic Activity of 5-Hydroxymethyl-2-furfural Produced from Grape Must Under *in vitro* Conditions

Kubra Kelleci[1,2*], Eda Golebatmaz[3]

[1] Beykoz University, Vocational School, Department of Medical Services and Techniques, Istanbul, 34805, Turkey
[2]Yildiz Technical University, Faculty of Chemistry and Metallurgy, Department of Bioengineering, Istanbul,34210 Turkey
[3]Eskişehir Osmangazi University, Institute of Science and Technology, Department of Biology, Eskisehir, 26040, Turkey



F**igure S1:** Chromatogram for HPLC Analysis.

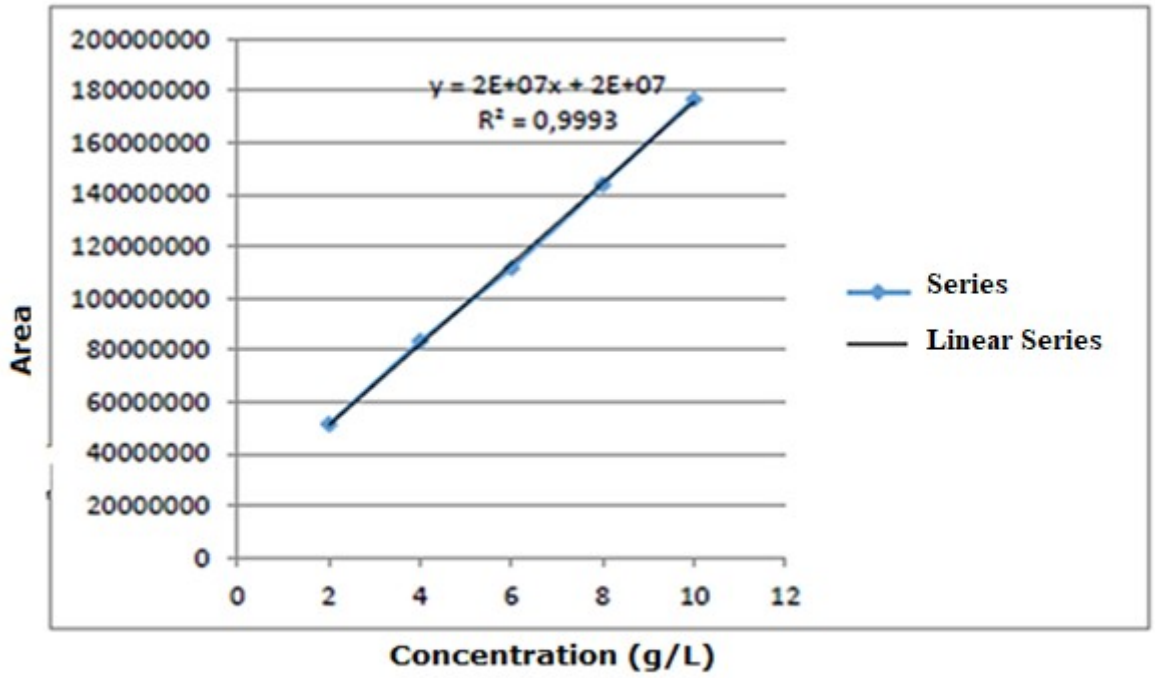


Figure S2: 5-HMF calibration graph.

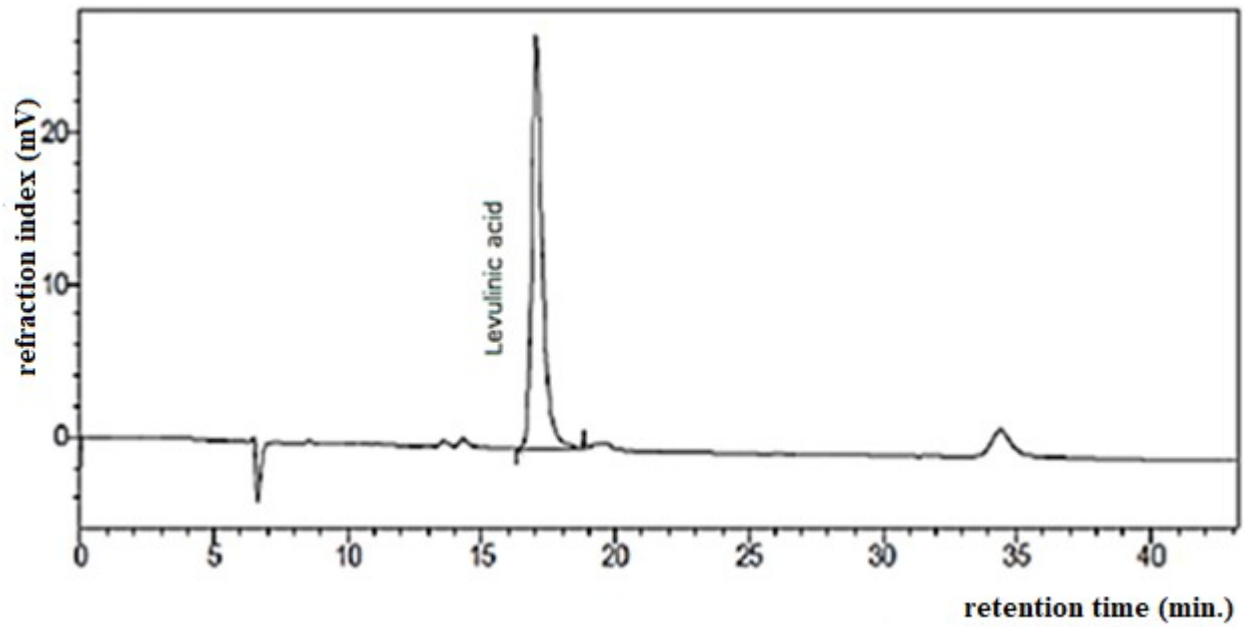


Figure S3: Chromatogram of Levulinic acid

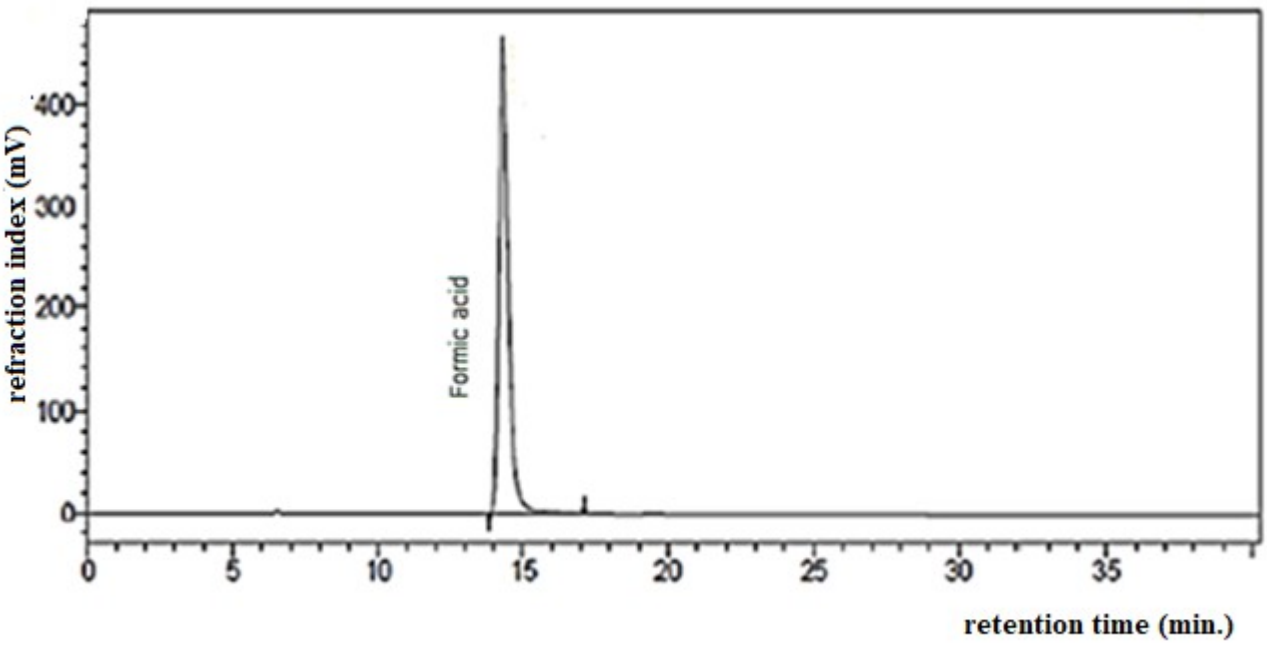


Figure S4: Chromatogram of Formic acid.