



Development of freeze-dry kits containing imatinib and different chelating agents: characterization, stability and cytotoxicity studies

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Received: 0 Month 201X / Revised: 0 Month 201X / Accepted: 0 Month 201X

ABSTRACT: The current study aims to develop new freeze-dry kits containing Imatinib and different chelating agents for breast cancer treatment and diagnosis as theranostics. Four formulations (Kit-1, Kit-2, Kit-3, and Kit-4) were prepared, and the characterization of formulations was assessed utilizing particle size, polydispersity index, zeta potential, fourier transform infra-red analysis, ultraviolet spectrum analysis, differential calorimetry, and thermogravimetric analysis. They were also evaluated for stability at different storage conditions and cytotoxicity effect on fibroblast NIH-3T3 cells. The particle size, polydispersity index, and zeta potential of developed formulations were found to be between 6953.6 ± 131.6 and 5888.3 ± 131.6 nm, 0.481 ± 0.24 and 0.319 ± 0.18 , -594.5 ± 59.6 and -477.3 ± 25.32 mV, respectively. Fourier transform infra-red analysis, ultraviolet spectrum, differential calorimetry, and thermogravimetric analysis have proven that IMT and chelating agents formed complexes in kit formulations. Also, they exhibited stable facility and above 90% of cell viability on fibroblast NIH-3T3 cells. By the result of our study, kit formulations can be a favorable drug delivery system in the treatment and diagnosis of breast cancer with a non-toxic effect on healthy cells.

KEYWORDS: Imatinib; cytotoxicity; breast cancer; chelating agents; theranostic.

1. INTRODUCTION

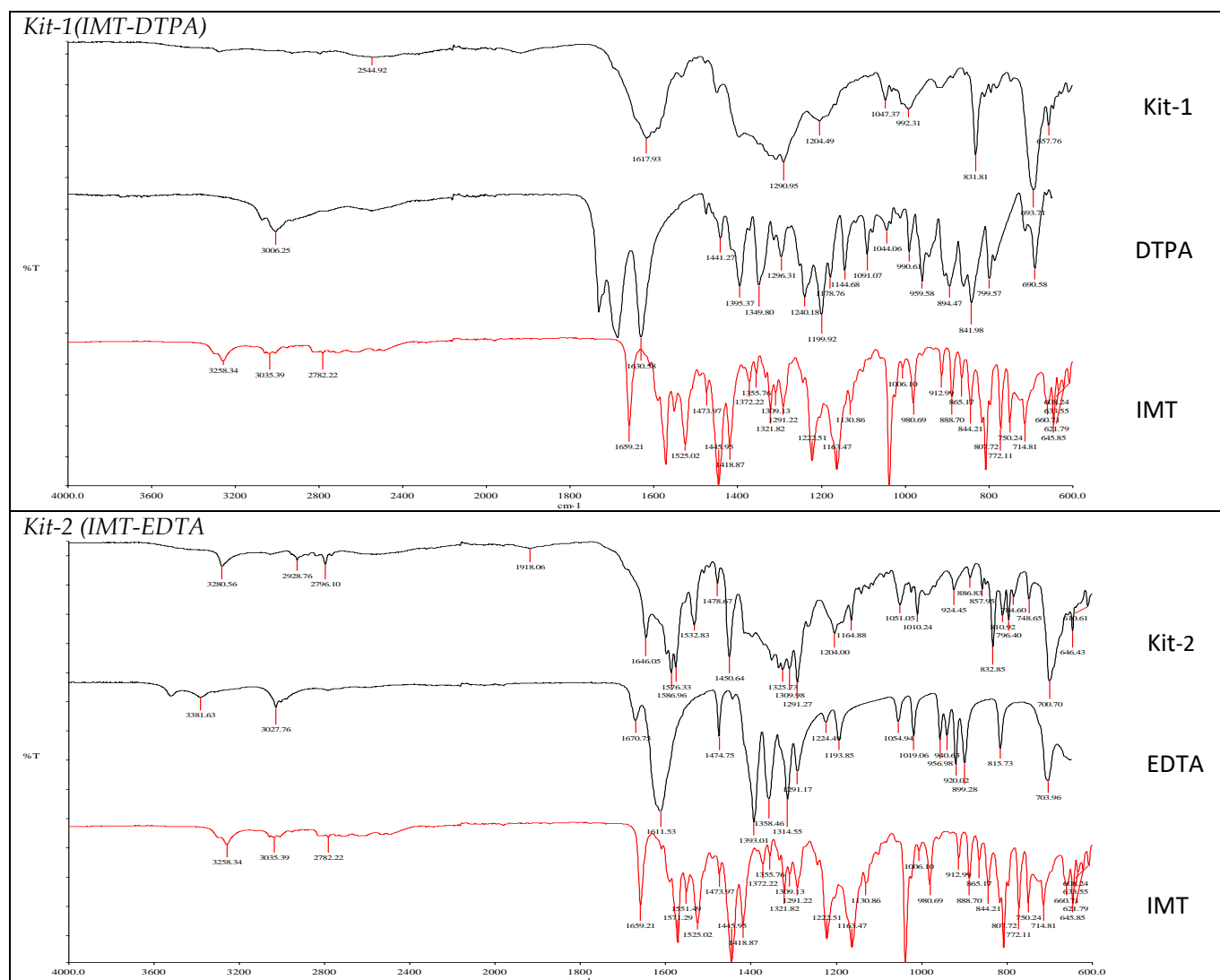
Imatinib (IMT) is a tyrosine kinase inhibitor and a novel molecule used in the treatment of gastrointestinal stromal tumors (GISTs), breast cancer, and acute myelogenous leukemia (AML) [1]. IMT has been reported to cause side effects such as muscle cramps, diarrhea, nausea, and myelosuppression, and resistance to IMT develops and its bioavailability causes differences among patients. High doses of IMT are required for a more effective GIST treatment [2]. It is thought that with a new radiopharmaceutical to be prepared by using IMT at a lower dose, the negative effects mentioned above can be reduced. In the theranostic approach, it is aimed to detect tumor cells by using targeted molecules and then destroy the cancerous cells without damaging other tissues with the help of therapeutic agents [3]. In the theranostic approach with radionuclides, it is also aimed to detect the tumor by using disease-specific biological pathways and then to irradiate the tumor with a therapeutic radionuclide. This approach provides the patient with effective treatment at the right time, at the right dose, and in a targeted manner. In this study, freeze-dry kits containing IMT and different types of chelating agents were prepared for breast cancer treatment and diagnosis. Different chelating agents such as ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,2 dimethyl-3-hydroxypyridine were used. IMT-chelating agent complexes were brought into ready-to-use freeze-dry kits to keep the prepared complexes stable for a longer time. Particle size, zeta potential, thermogravimetric analysis (TGA), differential calorimetry (DSC) analysis, fourier transform infra-red (FTIR), and ultraviolet spectrum (UV) analysis of kit formulations were evaluated in the characterization studies. Stability studies of formulations were performed at three different storage conditions. Furthermore, cytotoxicity study of the kit formulations was obtained by using fibroblast NIH-3T3 cells. In future studies, we plan to radiolabel developed kits with Lutetium-177 as theranostic administration.

Özgenc E, Gundogdu E. Development of freeze-dry kits containing imatinib and different chelating agents: characterization, stability and cytotoxicity studies. J Res Pharm. 2022; 26(2): 510-522.

2. RESULTS AND DISCUSSION

2.1. FTIR analysis

FTIR analyzes of kit formulations and the substances used in the preparation of formulations were performed. The spectrums are shown in **Figure 1**. The differences, which appear in the 600–4000 cm⁻¹ spectral region, were observed in terms of the implication of different molecular groups of the guest and the host molecules in the IMT-chelating agent complexes.



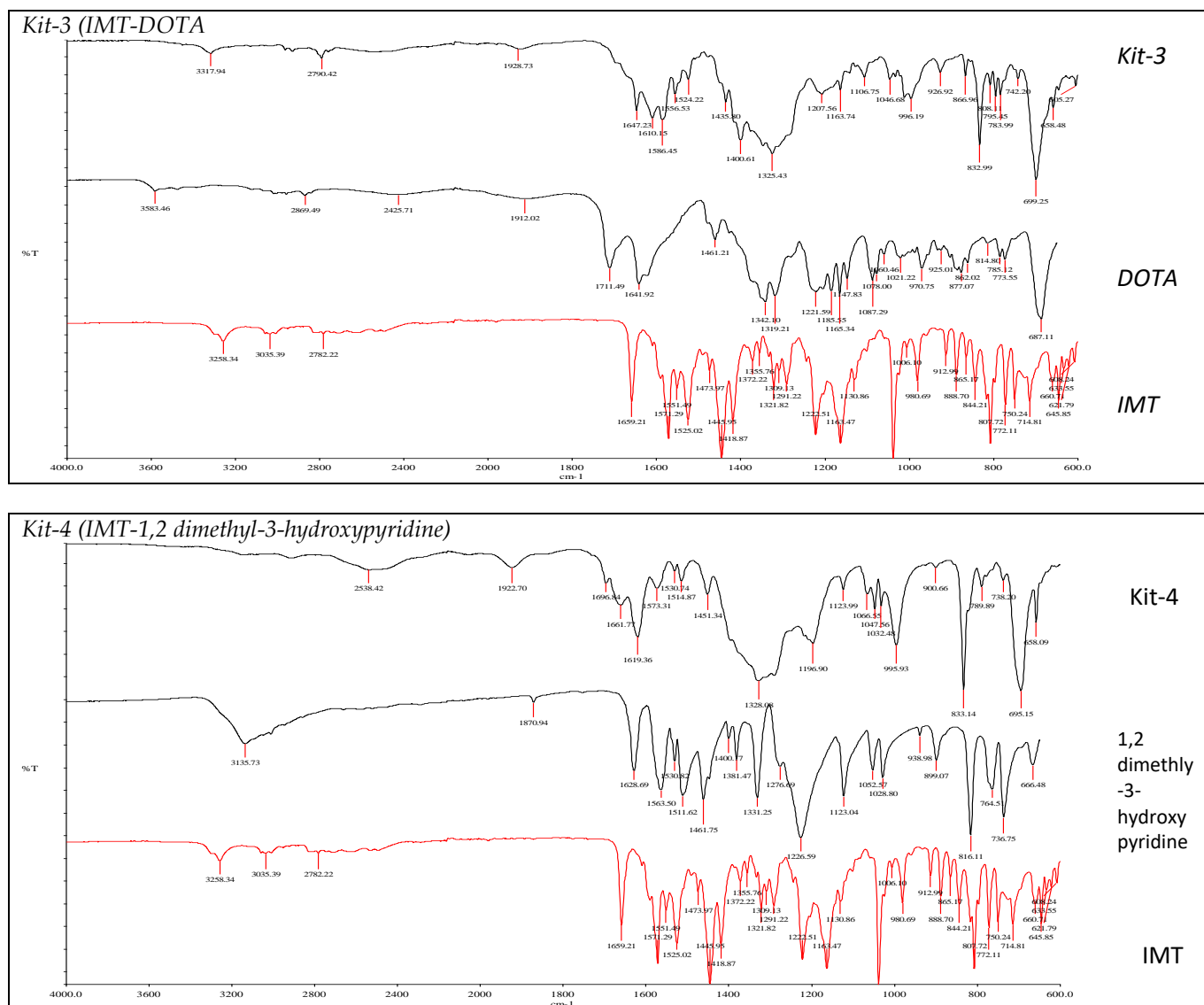
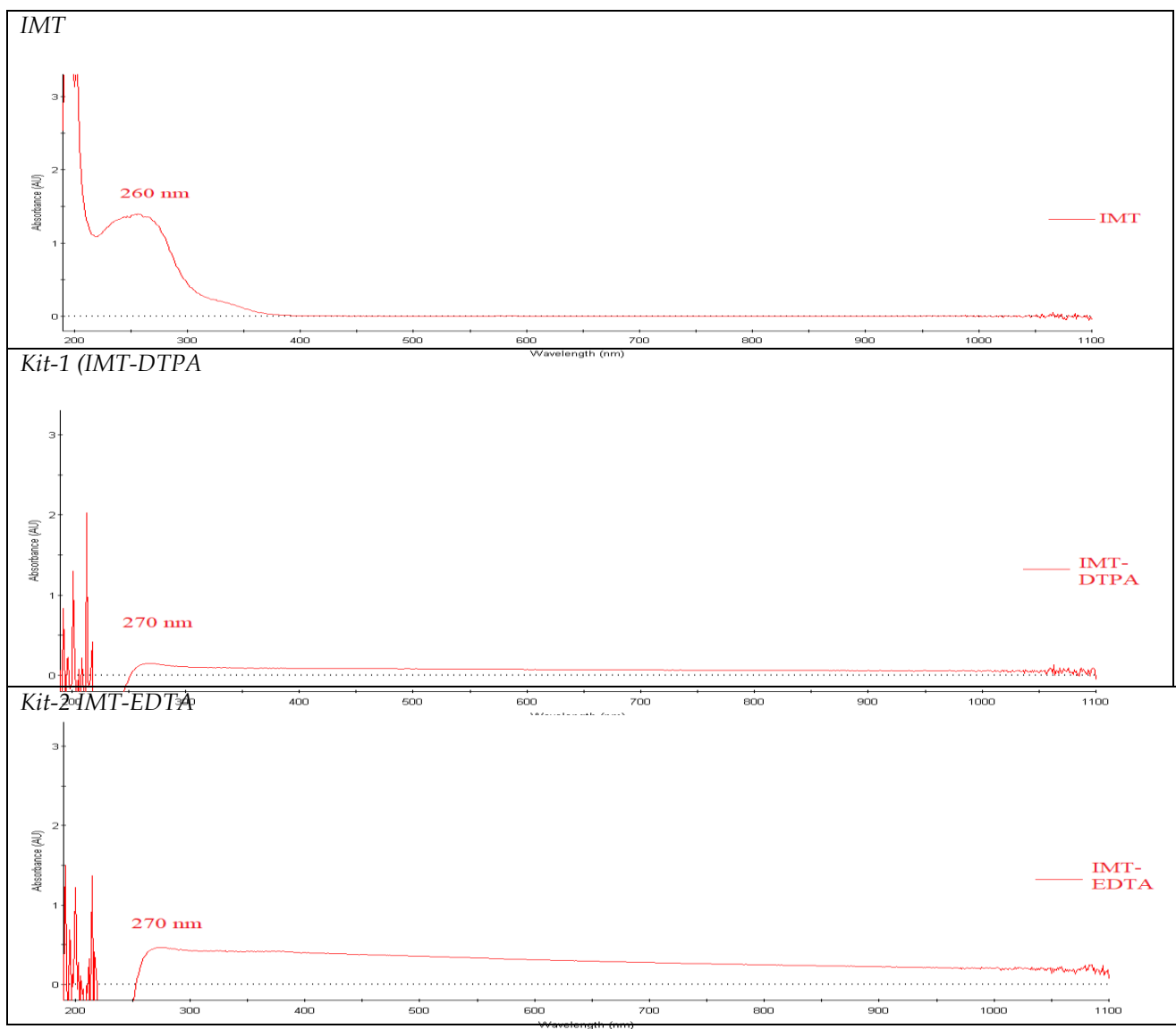


Figure 1. FTIR analysis results of kit formulations and the kit's contents.

2.2. UV spectrum analysis

UV spectrum analysis of kit formulations and IMT was carried out. The λ max value of IMT is shown in Figure 2 and is similar to the literature [4]. λ max values of the kit formulations were found to be different from λ max values of IMT ($p < 0.05$).



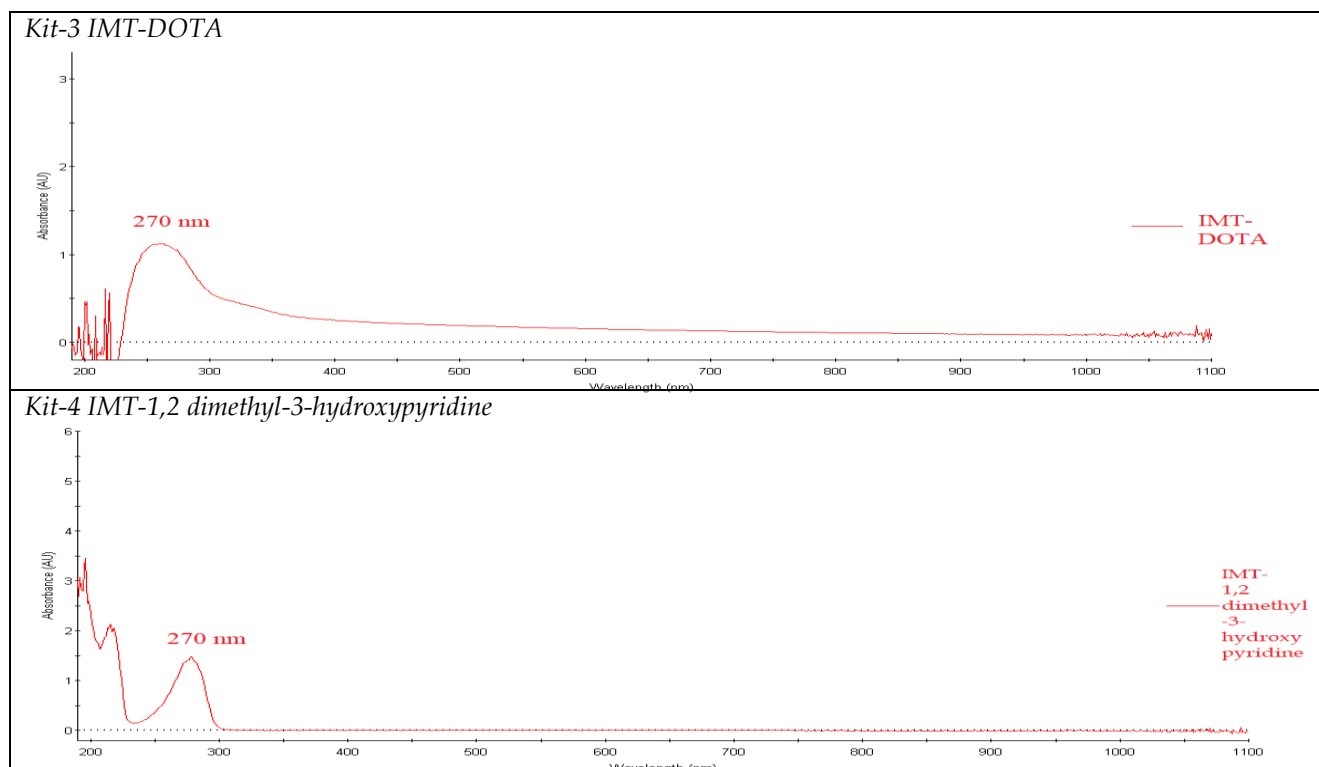


Figure 2. UV spectrum analysis results and λ max values of IMT and kit formulations.

2.3. Particle size, zeta potential and polydispersity index

Kit formulations were firstly characterized with their particle size, PDI, and zeta potential values, and the results were given in **Table 1**. It was found that the formulations showed a broad particle size range between 6953.6 ± 131.6 and 5888.3 ± 348.6 nm. Kit-4 had the smallest particle size of 5888.3 ± 348.6 nm among them. Furthermore, the particle size of the kit formulations increased according to the molecular weight of chelating agents in the formulations. The value of PDI is an indicator of the homogeneity of particle size distribution in the formulation, and it is under 0.2 in the monodisperse formulations [5]. The size distribution of formulations might be defined as broad due to their PDI values, which are higher than 0.2. Hence, all kit formulations were found as polydisperse samples. The zeta potential is an important parameter in terms of stability because it shows the electrokinetic property of molecules and the possibility of aggregation. Zeta potential of formulations were evaluated with the measurement of surface charges. The results showed zeta potential of the formulations were all negative and found to be between -594.5 ± 59.6 and -477.3 ± 25.32 mV (**Table 1**).

Table 1. Particle size, PDI and zeta potential values of kit formulations and the kit's contents.

Kit formulations and kit's contents	Particle Size (nm)	PDI	Zeta Potential (mV)
Kit-1 (IMT -DTPA)	6780.3 ± 110.2	0.473 ± 0.12	-477.3 ± 25.32
Kit-2 (IMT -EDTA)	6612.3 ± 154.5	0.319 ± 0.18	-489.3 ± 35.92
Kit-3 (IMT-DOTA)	6953.6 ± 131.6	0.470 ± 0.14	-498.3 ± 134.1
Kit-4 (IMT-1,2 dimethyl-3-hydroxypyridine)	5888.3 ± 348.6	0.481 ± 0.24	-594.5 ± 59.6
IMT	388.3 ± 24.3	0.003 ± 0.001	-405.3 ± 14.01
DOTA	1083 ± 769.2	0.523 ± 0.12	565.3 ± 123.3
DTPA	340.5 ± 40.36	0.468 ± 0.25	702.3 ± 7.51
EDTA	791.1 ± 32.8	0.217 ± 0.27	-753.3 ± 124.4
1,2 dimethyl-3-hydroxypyridine	837.6 ± 319.2	0.177 ± 0.23	-638.6 ± 17.7

2.4. TGA analysis

TGA analysis of the kit formulations was performed and the results are shown in **Table 2**. The amount of moisture remaining in the kits as a result of the lyophilization process was analyzed. When the weight changes of kit formulations were examined, thermal decomposition studies of formulations showed slight weight loss within a temperature range of 25 to 250 °C, with a mass loss of 2.24-10.84%. This is mainly due to the elimination of the aqueous phase in kit formulations [6].

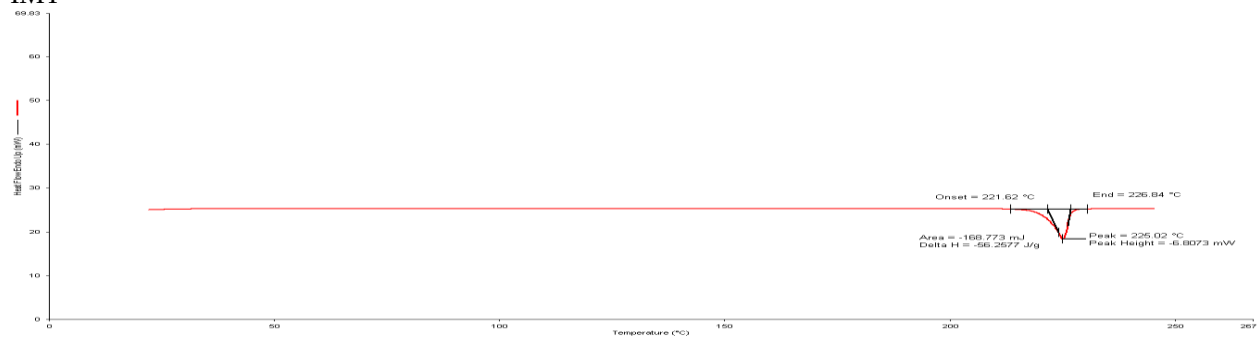
Table 2. TGA analysis results of kit formulations and the kit's contents.

Formulations	First Weight	Final Weight	% Weight Change
Kit-1 (IMT -DTPA)	3.870 mg	3.490 mg	9.81
Kit-2 (IMT-EDTA)	0.802 mg	0.715 mg	10.84
Kit-3 (IMT-DOTA)	1.827 mg	1.786 mg	2.24
Kit-4 (IMT-1,2 dimethyl-3-hydroxypyridine)	0.739 mg	0.706 mg	4.46

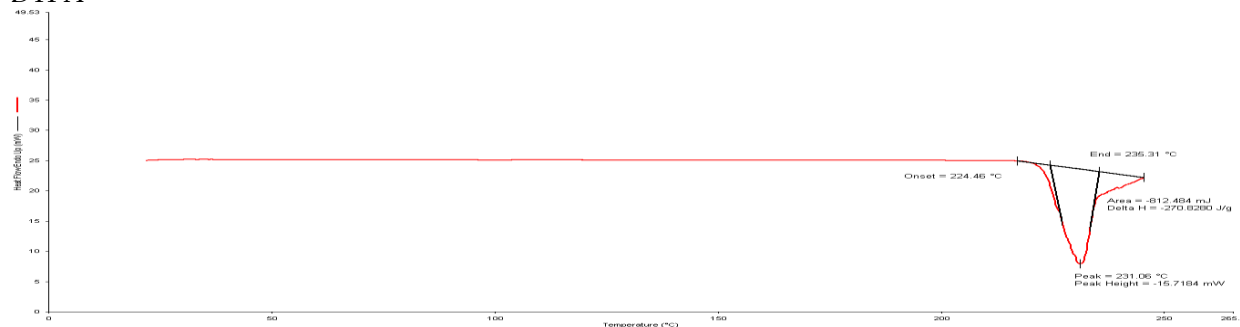
2.5. DSC analysis

DSC involves precise measurement of the difference in the amount of heat required to increase the temperature of the sample and a reference (such as an empty aluminum pan) as a function of temperature while keeping both at nearly the same temperature as each other. It gives information about the polymorphic changes in formulations, determination of their thermodynamic properties, and possible drug-formulation interaction [7]. The DSC thermogram of IMT, chelating agents, and kit formulations are shown in **Figure 3**. The degree of melting of IMT has been reported in the literature as 222-224 °C [8]. According to DSC analysis results, the melting degree of IMT obtained from the study was found to be consistent with the value stated in the literature. The DSC thermogram displays the disappearance of IMT peak in the formulations which offers that IMT is surrounded inside the kit.

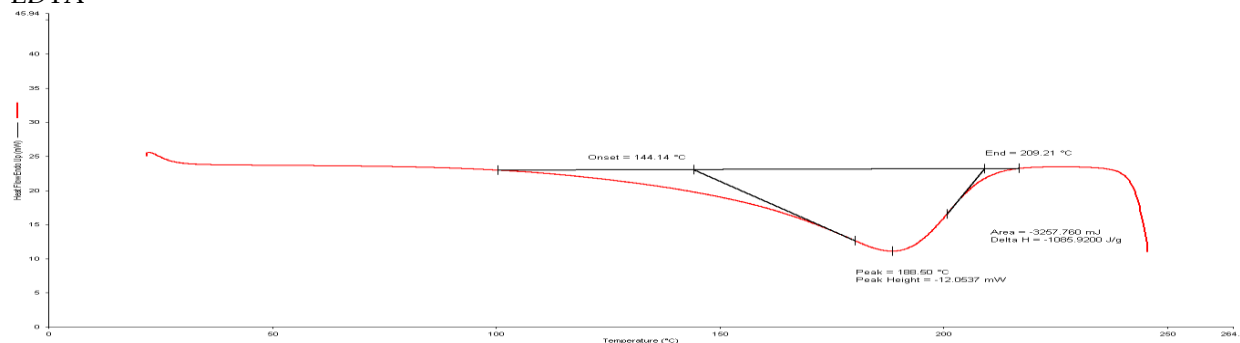
IMT



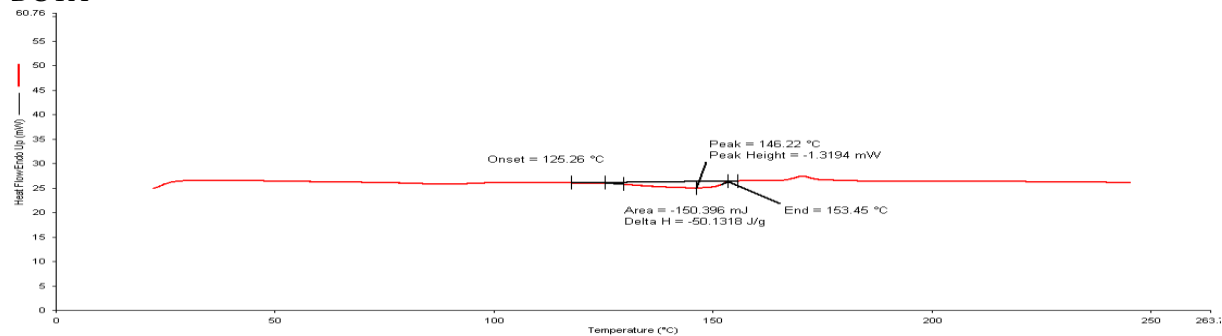
DTPA

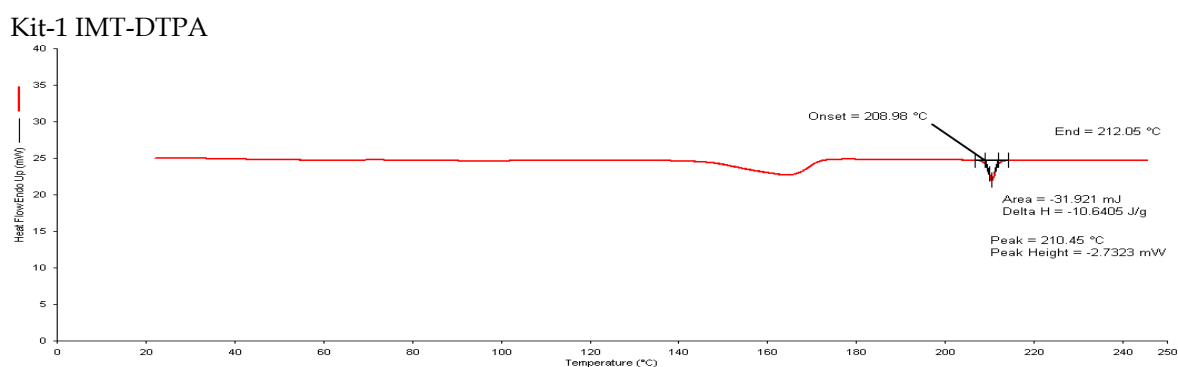
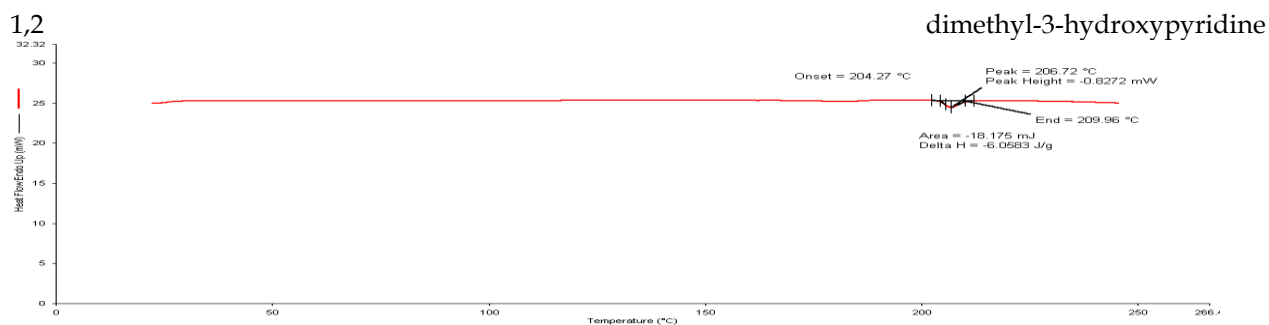


EDTA

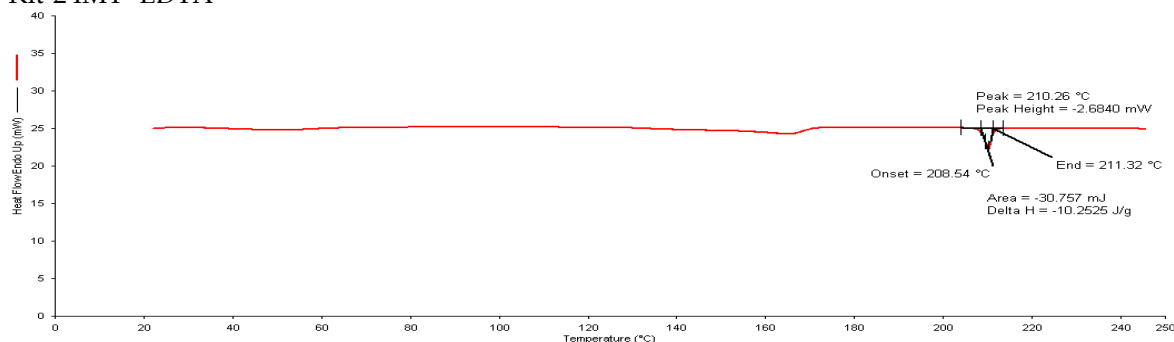


DOTA

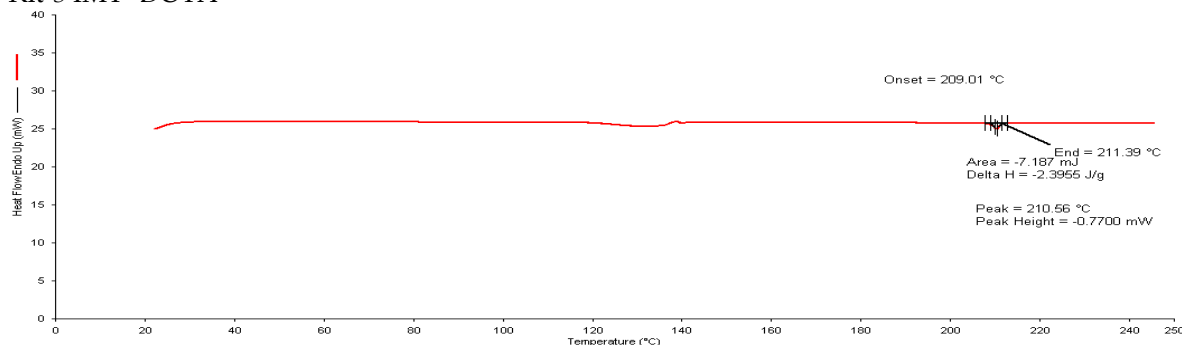




Kit-2 IMT -EDTA



Kit-3 IMT -DOTA



Kit-4 IMT -1,2 dimethyl-3-hydroxypyridine

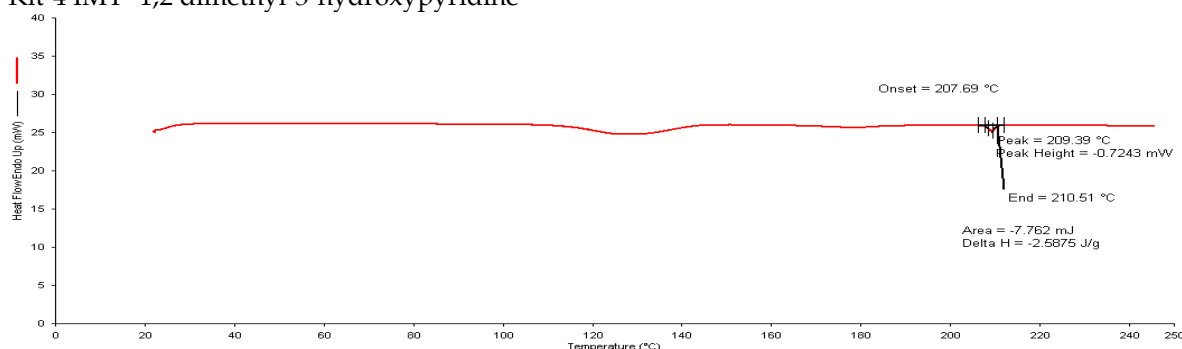


Figure 3. DSC analysis results of kit formulations and the kit's contents.

2.6. Stability

Stability studies were carried out to evaluate the effects of different temperatures and humidity on the physicochemical characterization properties of kit formulations. In the stability study, the formulations were kept at $25 \pm 2^\circ\text{C}$ / $60 \pm 5\%$ and $40 \pm 2^\circ\text{C}$ / $75 \pm 5\%$ relative humidity for the accelerated stability study for 30 days. The results are given in **Table 3**, and statistically significant changes were observed in the formulations after 30 days of storage in different conditions ($p < 0.05$). The alterations in particle size, PDI, and zeta potential values of formulations affect the stability and lead to aggregation in formulations. Furthermore, these parameters should be checked in different temperatures and humidity conditions to choose optimal storage conditions and show the possible effect of high temperature and humidity on the formulations. According to the results, all the kit formulations could be affected from high temperature and different humidity values. It would be better their administration in drug carrier systems.

Table 3. Stability results of formulations.

	Initial Values			30 days values					
	25° C			25 ± 2 ° C / 60 ± 5%			40 ± 2 ° C / 75 ± 5%		
Complex	Particle Size (nm)	PDI	Zeta Potential (mV)	Particle Size (nm)	PDI	Zeta Potential (mV)	Particle Size (nm)	PDI	Zeta Potential (mV)
Kit-1 IMT -DTPA	6780.3 ± 110.2	0.473 ± 0.12	-477.3 ± 25.32	7214.8 ± 114.5	0.345 ± 0.15	-555.7 ± 54.9	5060.3 ± 873.2	0.367 ± 0.114	-511.4 ± 88.4
Kit-2 IMT -EDTA	6612.3 ± 154.5	0.319 ± 0.18	-489.3 ± 35.92	6345.7 ± 223.6	0.502 ± 0.17	-405.6 ± 89.6	8526.1 ± 828.0	0.230 ± 0.104	-369.8 ± 27.7
Kit-3 IMT-DOTA	6953.6 ± 131.6	0.470 ± 0.14	-498.3 ± 134.11	7012.3 ± 147.2	0.275 ± 0.23	-481.7 ± 65.8	4497.8 ± 352.4	0.482 ± 0.147	-438.7 ± 32.9
Kit-4 IMT- 1,2 dimethyl-3-hydroxypyridine	5888.3 ± 348.6	0.481 ± 0.24	-594.5 ± 59.60	6015.1 ± 285.1	0.430 ± 0.26	-542.9 ± 89.4	6274.4 ± 288.9	0.414 ± 0.123	-514.2 ± 56.3

2.7. *In vitro* cytotoxicity studies

In the previous study by Gundogdu et al., cytotoxicity studies of IMT were performed and the damaging effects of IMT were stated. As a result of cytotoxicity studies in this study, it was stated that IMT has a dose-dependent cytotoxicity. Different concentrations of IMT solutions showed cytotoxic effects throughout the test period [9]. *In vitro* cytotoxicity profiles of Kit-1, Kit-2, Kit-3, and Kit-4 formulations on fibroblast NIH-3T3 cells were determined by the evaluation of cell viability at the end of the MTT test. The similar structure of formulations to cell membranes due to IMT and chelating agents provides biocompatible, biodegradable, nontoxic, and nonpyrogenic profiles [10]. The cell viabilities were found higher than 90% for all formulations at 24-, 48-, and even 72-h time points, as seen in **Figure 4**. Although kit-1 and kit-2 formulations exhibited a slightly higher cytotoxic effect than kit-3 and kit-4 due to their chelating agent type, this difference is not statistically significant ($p > 0.05$). These findings suggested that developed kit formulations or their metabolites did not lead to toxic effects on healthy cells due to the biocompatible profile of formulations. Therefore, kit formulations containing IMT-chelating agents can be considered safe and valuable drug delivery systems in the treatment and diagnosis of breast cancer for future radiolabeling and *in vivo* studies due to their high biocompatibility and no-toxic profiles.

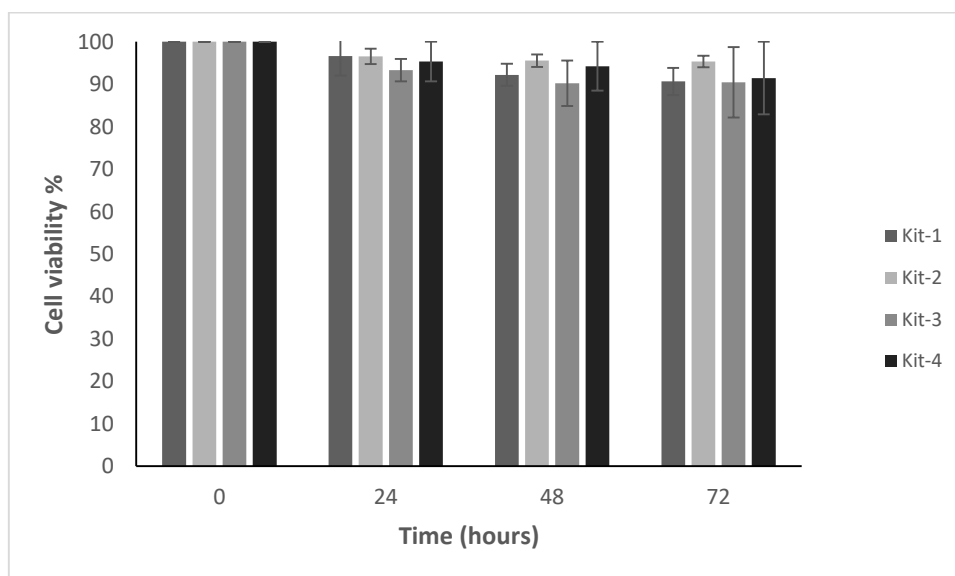


Figure 4. Cell viability % of kit formulations.

3. CONCLUSION

In this study, freeze-dry kit formulations containing IMT and different chelating agents were developed as potential therapeutic and diagnostic agents for breast cancer. After the preparation, kit formulations exhibited proper characterization profiles with their particle size, negative zeta potential values, and PDI values. In addition, cytotoxicity of kit formulations was evaluated, and all formulations showed biocompatible profiles on healthy cells by the results of the cytotoxicity study. In conclusion, freeze-dry kit formulations containing IMT and different chelating agents exhibited some physicochemical characterization properties but also *in vitro* biocompatible profile.

4. MATERIALS AND METHODS

4.1. Materials

IMT (GleevecVR) was obtained from Novartis (Basel, Switzerland). Ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,2 dimethyl-3-hydroxypyridine were purchased from Sigma Aldrich.

4.2. Preparation of freeze dry kits containing IMT-chelating agent complexes

The different chelating agents were dissolved in 0.2 M sodium carbonate buffer solutions. IMT was added to chelating agent solutions. They were incubated for 17 h at 37 °C to obtain IMT-chelating agent complexes [11]. The freeze-dry kits containing IMT-chelating agent complexes were prepared with the lyophilization method. IMT-chelating agent complexes (IMT-DTPA, IMT-EDTA, IMT-DOTA, IMT-1,2 dimethyl-3-hydroxypyridine) were put into vials. The mouths of the vials were covered with parafilm to prevent any splash, substance loss or contamination risk, and holes were made on the film layer with the help of a needle to allow lyophilization to take place. The formulations were frozen at -80 °C for 24 hours. After that, they were lyophilized with Christ Alpha 1-2 LD Freeze Dryer device under a pressure of 0.07 mbar at -45 °C for 48 hours [12]. The kit formulation contents are given in **Table 4**.

Table 4. The ingredients of freeze dry kit formulations.

Kit formulations	Drug molecule	Chelating agents	Buffer
Kit-1	IMT (25 mg)	DTPA (22 mg)	sodium carbonate 0.2 M pH: 9.5)
Kit-2	IMT (35 mg)	EDTA (21 mg)	sodium carbonate 0.2 M pH: 9.5)
Kit-3	IMT (25 mg)	DOTA (20 mg)	sodium carbonate 0.2 M pH: 9.5)
Kit-4	IMT (70 mg)	1,2 dimethyl-3-hydroxypyridine (21 mg)	sodium carbonate 0.2 M pH: 9.5)

4.3. Characterization studies of freeze dry kits containing IMT-chelating agent complexes

4.3.1. FTIR analysis

The freeze-dry kits IMT containing chelating agent complexes were measured by using Perkin Elmer Spectrum 100 models in the range of 600–4000 cm⁻¹ for FTIR analysis.

4.3.2. UV spectrum analysis

1 mg of freeze-dry kits IMT containing chelating agent complexes was taken and dissolved in 10 ml of 0.2 M sodium carbonate buffer solution. The solution was scanned in the UV range 100–1100 nm [13].

4.3.3. Particle size, zeta potential and polydispersity index

The particle size and polydispersity index (PDI) of kit formulations were evaluated with Malvern Zetasizer (Malvern Nano ZS 90, UK) at room temperature. The zeta potential of kit formulations was also measured at 40 V cm⁻¹ using a DTS 1060C zeta cuvette at room temperature. Freeze dry kits were diluted with 0.2 M sodium carbonate buffer (pH 9.5) before analysis (n=6).

4.3.4. DSC analysis

Differential scanning calorimeter DSC-8000 (DSC 8000 V24.11 Build 124, Perkin Elmer) equipped was used for this analysis. The kit formulations were placed in sealed aluminum pans under nitrogen purge at a flow rate of 50 mL/min. The samples were heated from 25 °C to 250 °C at a heating rate of 10 °C/min. IMT, DOTA, DTPA, EDTA, and 1,2 dimethyl-3-hydroxypyridine were also evaluated to compare kit formulations [14].

4.3.5. TGA analysis

The thermal stability of kit formulations, IMT, DOTA, DTPA, EDTA, and 1,2 dimethyl-3-hydroxypyridine was investigated by using TGA-4000 (Perkin Elmer). The analysis was carried out under a nitrogen atmosphere (50 mL/min) at a heating rate of 10 °C per minute. The results scanned from 25 to 250 °C [15].

4.4. Stability study

The kit formulations were submitted to stability test at 25 ± 2°C/60 ± 5% relative humidity and 40 ± 2°C/75 ± 5% relative humidity for 30 days. Particle size, polydispersity index, zeta potential, and formulations appearance by visually of the formulations were monitored. All values were compared statistically for the initial and 30 days' values.

4.5. In vitro cytotoxicity studies

The *in vitro* cytotoxicity of kit formulations was evaluated on fibroblast NIH-3T3 cell (ATCC®). The cell line was cultured in DMEM supported with 10% fetal bovine serum, 0.5 mg/mL glutamine/penicillin streptomycin at 37°C and 5% CO₂. The cytotoxicity of formulations was determined by using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. 96 well flat-bottom plates and 1 × 10⁶ cells/well in 1 mL DMEM were used for the seeding procedure. The formulations were then added into the 96 well plates for 24, 48, and 72 h. The culture medium was removed and 200 µL of dimethyl sulfoxide was added to obtain a dissolution of the MTT formazan crystals. The absorbance read at 570 nm using a microplate reader. Cell viability % was found by using absorbance values (n=8).

4.6. Statistical data analysis

Statistical data analysis was performed using the Student's t-test with P<0.05 as the minimal level of significance.

Acknowledgements: We would like to thank the pharmaceutical sciences research laboratory (FABAL) for their support in the experimental studies.

Author contributions: Concept – E.G., E.O.; Design – E.O., E.G.; Supervision – E.G. E.O.; Resources – E.O., E.G.; Materials – E.G.; Data Collection and/or Processing – E.O.; Analysis and/or Interpretation – E.G., E.O.; Literature Search – E.O., E.G.; Writing – E.O., E.G.; Critical Reviews – E.G., E.O.

Conflict of interest statement: The authors declared no conflict of interest.

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