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Radioprotective Effect of HME-Mulberry Leaves against Electromagnetic Radiation-Induced Damage in Rats

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In this study, a solid dispersion containing mulberry leaves and additives was manufactured by hot-melt extrusion (HME) to develop a radioprotective agent against electromagnetic radiation. The prepared formulations were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), dynamic light scattering (DLS), and electrophoretic light scattering (ELS). The formation of regular dispersions was confirmed through SEM and TEM analysis. To explore the radioprotective effect, animals were randomly classified into four groups: normal control (NC), irradiation (IR), irradiation after administration with mulberry leaves (MR), and irradiation after administration with HME-mulberry leaves (HR). Each sample was orally administered at 100 mg/kg/day, and 7 Gy of 6 MV electromagnetic radiation (X-ray) was used once for the whole body. Lymphocytes seemed to recover in the HR group, but none of the groups recovered at a normal range by the seventh day after irradiation. There was also no change in all groups of the red blood cells. The spleen index tended to recover in the HR group compared to the IR group, which was consistent with histological analysis. The spleen of the radiation-exposed groups (IR, MR, and HR) showed relatively atrophied white pulp and decreased lymphocytes. These symptoms further increased on the third day after irradiation and gradually alleviated on the seventh day. Compared to the IR group, the MR and HR groups had less loss of lymphocytes caused by damage to the white pulp. Specifically, the HR group demonstrated a higher recovery rate than the MR group, confirming the potential of HME-Mulberry as a radioprotective agent. The study's findings suggest further studies on health supplements and medicines to supplement the current research.

Keywords : mulberry leaves, hot-melt extrusion, electromagnetic radiation, radioprotection, antioxidant

1. Introduction

The Japan and Tokyo Electric Power Company (TEPCO) plan to release contaminated water from the Fukushima nuclear power plant four times by March 2024. Currently, contaminated water is being discharged through the first discharge (August 24, 2023), followed by the second discharge (October 5, 2023). The International Atomic Energy Agency (IAEA) evaluates that it meets the standards for scientific facilities for ocean discharge [1]. However, public anxiety about marine radiation and marine products is increasing. Accordingly, each country monitors marine radiation and marine products, but it is focused on measuring radiation dose [2]. Hence, it is insufficient from a human protection perspective.

The importance of radioprotective agents is emphasized from the perspective of human protection. Chemical radioprotective agents are used, and representative chemical radioprotective agents include WR2721 (Amifostine), WR3689, and WR1065, which are based on the synthesis of aminothiols and phosphorothioates. Additionally, compounds with thiols, such as diethyldithiocarbamate (DDC), mercaptopropionylglycine (MPG), N-acetylcysteine (NAC), 2-mercaptoethanesulfonate (Mesna), and cystamine, have been developed [3]. However, although they have

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the advantage of being water-soluble and convenient for administration, their use is limited due to side effects, such as vomiting, low blood pressure, and tissue damage due to toxicity [4]. Ionizing radiation, which includes electromagnetic radiation and particle radiation, causes primary damage to the immune system. The spleen, which is the center of immune response regulation, is one of the organs sensitive to radiation. Therefore, to protect the human immune system from radiation, developing natural product-based radioprotective agents that can replace chemical radioprotective agents is essential.

Mulberry is a perennial woody plant cultivated in many countries, including Korea, China, and Japan. Mulberry, used in various fields, such as the silk industry, medicine, food industry, and animal feed, is extensively used to treat diseases due to its antioxidant, anti-inflammatory, anti-cancer, and anti-bacterial properties [5]. Mulberry leaves (Morus alba L.) are rich in phenolic compounds, flavonoids, and other biologically active substances and are reported to have excellent antioxidant abilities. These bioactive substances can be responsible for scavenging free radicals generated in the human body by irradiation [6].

Hot melt extrusion (HME) is a widely used processing technique in the food and pharmaceutical industries. HME uses a screw to apply heat and friction to a mixture to melt it and manufactures the mixture to have a uniform shape and density through a die [7, 8]. The organic solvent-free process eliminates the risk of toxicity from residual solvents, and the process steps are short and continuous, making it an eco-friendly process [9]. combined HME technology and additives can produce solid dispersions with increased solubility. HME has been used to produce solid dispersions of natural products, exhibiting increased aqueous solubility and antioxidant activity [10]. Using plasticizers in the HME process forms an amorphous solid dispersion that lowers the melting point and increases the dispersibility of the drug [11, 12].

Therefore, this study aims to develop a substance with stronger antioxidant ability by applying HME technology to Mulberry leaves. We also intend to investigate the radioprotective effects of natural products and developed materials, focusing on protecting against damage to the spleen (immune system) caused by electromagnetic radiation.

2. Materials and Methods

2.1. Materials and reagents

Lecithin, whey protein isolate, and ascorbyl palmitate used in the extrudate were purchased from S Food (Gumpo, Korea). Citric acid and vitamins E and C were purchased from Daejung (Seoul, Korea).

2.2. Animal study

For animal experiments, male Sprague-Dawley (SD) rats (Hana Bio, Korea) were used, with approval from the Institutional on Animal Care and Use Ethics Committee (IACUC, approval number: 2022-15). Each group was randomly assigned to six rats (a total of 72 rats), and the animals' average weight received was 204.6±3.2 g. Temperature and humidity were controlled according to animal experiment standards by an automatic air conditioning system, and the lighting time was kept constant at a 12:12 h day/night cycle. Except for oral administration of samples, food and water were available *ad libitum*.

After a week of the adaptation period, the animals were divided into four groups: normal control (NC), irradiated (IR), irradiated after mulberry leaves administration (MR), and irradiated after HME-mulberry leaves administration (HR). In previous studies, the MR group was orally administered at 100 mg/kg for three days before irradiation, and the HR group was also administered the same dose [13]. Finally, the experimental animals were autopsied one, three, and seven days after irradiation, then blood and organs were collected.

2.3. Preparation of MR and HR

HR was manufactured through HME according to the composition in Table 1. HR was prepared via HME equipped with a twin-screw extruder and die (1 mm) (STS-25HS, Pyeongtaek, Korea) at 40 g/min, 150 rpm, and 70 °C-100 °C. The extrudate was dried using a freeze-dryer and then powdered using a grinder. MR and HR were weighed in 1 g increments and mixed with 50 ml of distilled water. The mixture was extracted at 40 °C for 1 hour. The extract was filtered through Whatman No.6 (pore size: 3 μm). The extract was stored at 4 °C for

Table 1. Composition of hot-melt extruded mulberry leaves.

	Hot-melt extruded mulberry leaves (%)
Mulberry leaves	69
Whey protein isolate	20
Lecithin	2.5
Vitamin C	2
Vitamin E 50 %	2
Citric acid	2
Ascorbyl Palmitate	2.5
Total	100

further experiments.

2.4. Characterization of MR and HR

Transmission electron microscopy (TEM) analysis was conducted using a field emission TEM (FE-TEM) (JEM-2100F, JEOL, Tokyo, Japan) to observe morphology and size. Samples were prepared on a cooper grid before analysis. A field emission scanning electron microscope (FE-SEM) (S-4800, Hitachi Ltd., Chiyoda City, Japan) was used for visual confirmation of the surface and morphology. The particle size, polydispersity index (PDI), and zeta potential of the samples were determined by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) using a Zetasizer (Nano ZS, Malvern, UK).

2.5. Exposure to electromagnetic radiation

To expose experimental animals to X-rays, a form of electromagnetic radiation, a linear accelerator (ELEKTA, Sweden), was used. D_{max} was set at a depth of 1.5 cm, and 6 MV X-rays were irradiated for the whole body with a dose of 7 Gy.

2.6. Blood cell composition analysis

Blood cell was collected from the abdominal vein through the autopsy of the animal, and the obtained blood was put in an EDTA tube mixed in a Coulter mixer and then analyzed. To analyze blood cell components, an animal blood cell analyzer (BC-2800VET, Mindray, China) was used.

2.7. Histological analysis

The obtained spleen tissue was fixed in 10 % formalin and then paraffin blocks were prepared according to the commonly used paraffin method. The manufactured block was sectioned at 4 μm using a rotary microtome (Leica RM2125, Germany) and conducted to Hematoxylin and Eosin (H&E) staining.

2.8. Statistical analysis

The measured parameters and obtained results were shown as the mean ± SD (standard deviation). For statistical analysis, one-way ANOVA was performed using SPSS 26.0 (IBM, New York, NY, USA), and post-hoc was performed by Duncan multiple comparisons.

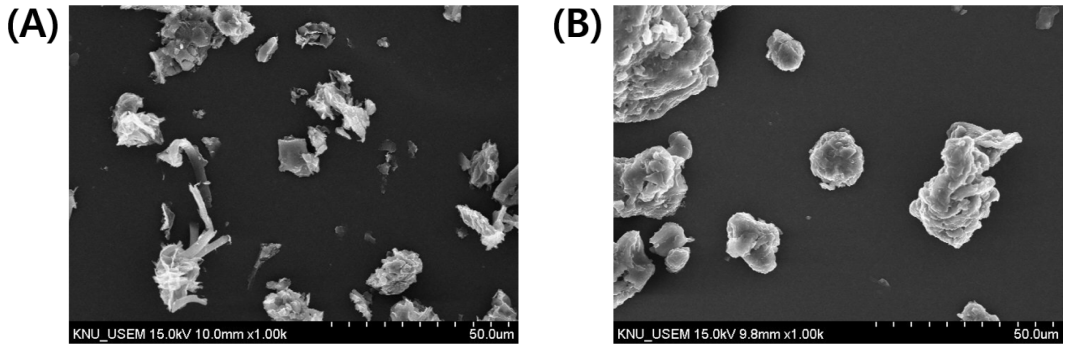

Fig 1. Scanning electron microscopy (SEM) images of (A) MR and (B) HR (× 1000).

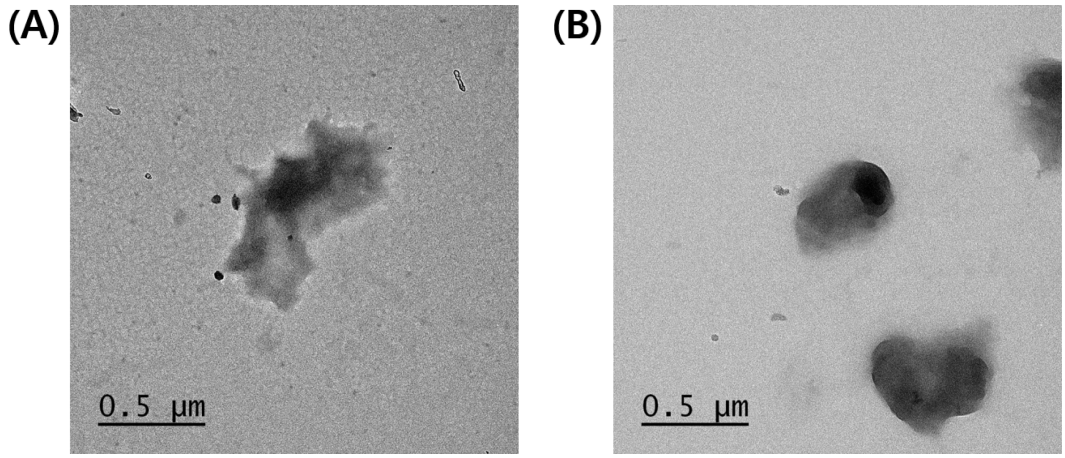

Fig 2. Transmission electron microscopy (TEM) images of (A) MR and (B) HR (scale bar: 0.5 μm).

When the result of statistical analysis was $p < 0.05$, it was considered statistically significant.

3. Results and Discussion

3.1. Characterization of MR and HR

In the case of natural products, poor absorption has been reported due to low water solubility and inadequate size. Applying various drug delivery systems (DDS) increased solubility and improved delivery [12]. SEM, TEM, and particle size determination confirmed that HR had a homogeneous particle distribution with nanoscale size. The morphological changes of MR and HR were observed by SEM and TEM (Figs. 1 and 2). The surface of MR was found to be rough, and the particles were irregular in shape and varied in size. In the HR, the sample's surface was smooth and showed a relatively uniform distribution. TEM image was similar to the scanning electron microscopy. HR showed a relatively spherical shape in the SEM and TEM images.

The samples' particle size was nanosized, with a slightly reduced particle size observed in the HR (Table 2). Particle size measurements using light scattering techniques measure the hydrodynamic size dispersed in a solvent, which is slightly larger than the size that can be determined through electron microscopy [14]. The PDI values were slightly increased after the HME process with MR being about 0.592 and HR being about 0.678. PDI indicates stability and uniformity, with PDI values closer to 0.1 demonstrating monodispersity and up to 0.7 suggesting a wide particle size distribution [15]. The zeta potential values were found to be negatively charged for all samples. Zeta potential is the value of the surface charge of a particle and is used as an indicator of stability. Particles with high zeta potential values are electrically stable, while particles with low zeta potential values may tend to agglomerate [16].

3.2. Effects of extract on blood cell composition

When exposed to ionizing radiation, symptoms appear primarily in the bone marrow, spleen, and lymph nodes that produce blood cells. Upon radiation exposure, lymphocytes are destroyed, which leads to platelet and red blood cell deficiency [17]. On the first day after radiation, the lymphocytes of the NC group that were not exposed to radiation were 5.35±0.43 10^3/μl. On the other hand, in the IR, MR, and HR groups that received radiation exposure, lymphocytes were destroyed, and the values were 0.01±0.04 10^3/μl, 0.05±0.05 10^3/μl, and 0.08±0.09 10^3/μl, respectively. It can be seen that they have been destroyed more than the NC group (Fig. 3A). This finding is consistent with the results of other studies that investigated radioprotective effects. In addition, some experimental animals tend to recover lymphocytes by producing blood cells over time, but no group recovered to the normal range until the seventh day of the study.

Table 2. Particle size, polydispersity index (PDI), and zeta potential (mV) of MR and HR.

	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
MR	614.53 ± 112.66	0.592 ± 0.111	-31.74 ± 0.21
HR	545.20 ± 86.65	0.678 ± 0.034	-32.77 ± 0.82

The measured parameters and obtained results were shown as the mean ± SD (standard deviation).

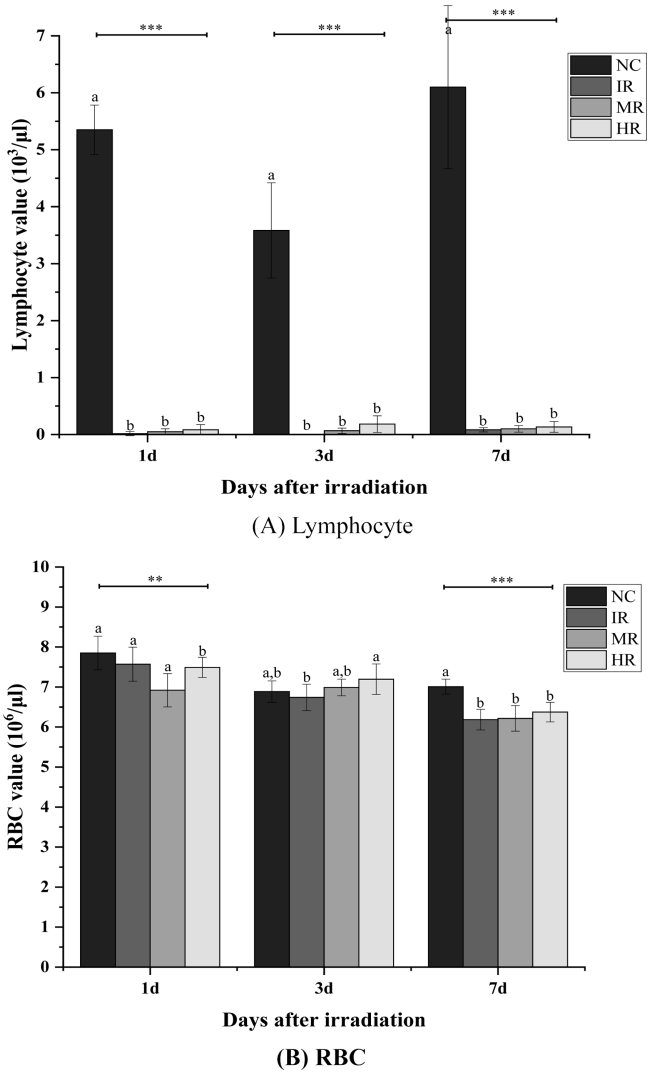

Fig 3. Changes in blood cell composition after irradiation (n = 6). (A) Lymphocyte after irradiation, (B) Red blood cell after irradiation. Different letters (a and b) indicate the statistically significant results of the one-way ANOVA and post hoc Duncan.

Red blood cells were in the normal range of minimum 6.1×10^3/μl and maximum 7.85×10^3/μl in all groups on the seventh day after the irradiation (Fig. 3B). In general, the red blood cells tend to decrease slightly after the seventh day, so there is a limitation of the study that was not confirmed until the 21st day [18].

3.3. Analysis of spleen architecture

Figure 4 shows the spleen index according to the change in time after irradiation. Recent studies reported that when exposed to radiation, spleen cells' atrophy and size decrease [19]. The spleen index is a ratio calculated as (spleen weight/body weight) × 100, and spleen atrophy was observed in irradiated rats compared to the NC group. This result is consistent with the findings verified in previous studies [20]. On the third and seventh days after irradiation, the MR group had a higher spleen index than the IR group, but there was no significant difference.

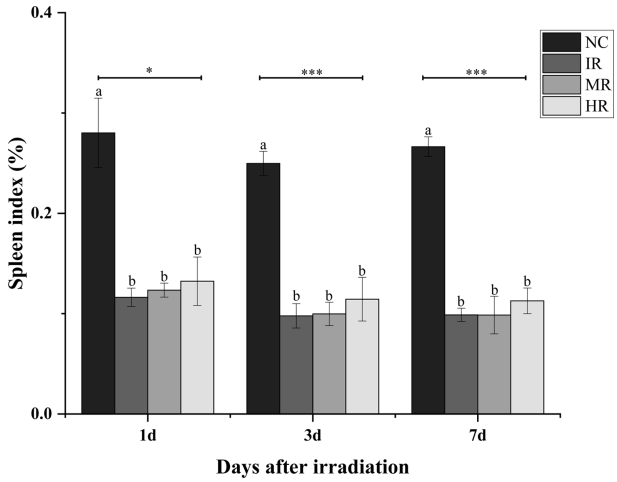

Fig 4. Changes in the spleen index after irradiation (n = 6). The spleen index is the ratio of the spleen weight to the body weight. Different letters (a and b) indicate the statistically significant results of the one-way ANOVA and post hoc Duncan.

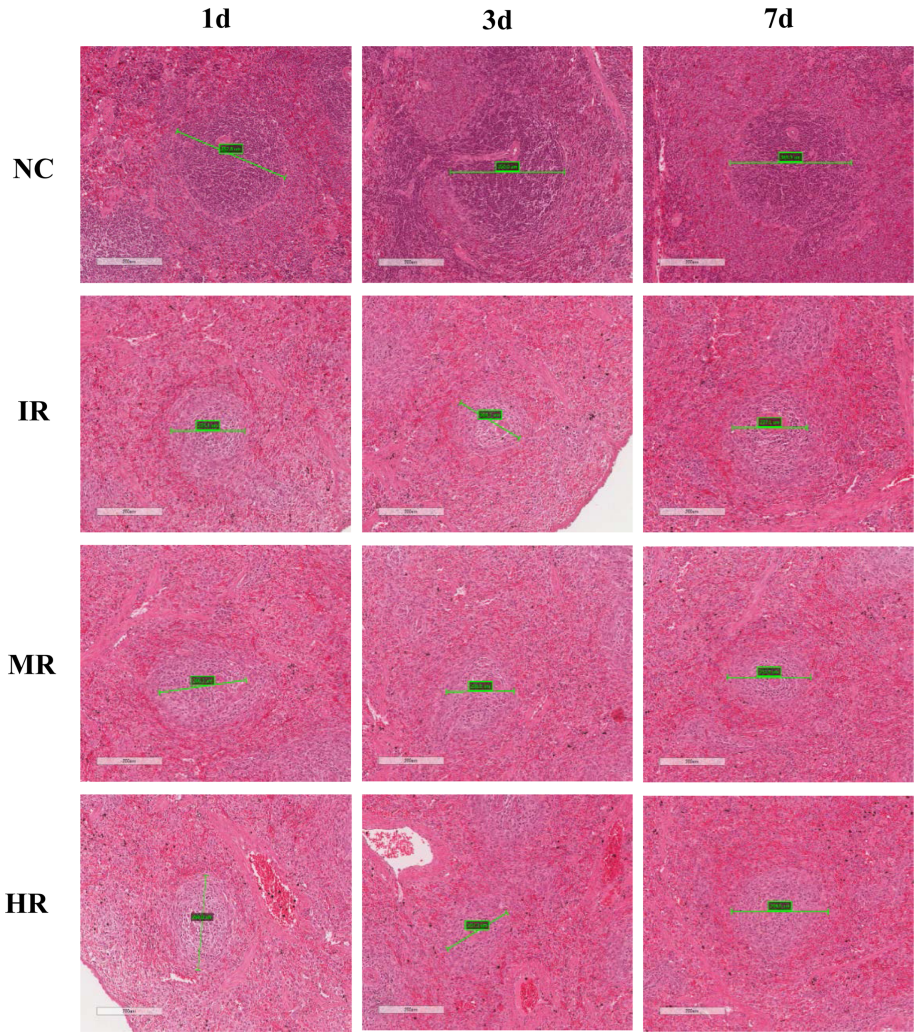

Fig 5. (Color online) Hematoxylin and eosin staining of the spleen on the designated day after irradiation. Magnification × 200. The green bar diagram shows the white pulp diameter.

The HR group showed relatively higher recovery ability compared to the MR group.

Figure 5 presents the histological change of the rat spleen for each group. The spleen is composed of white pulp and red pulp. The white pulp is the lymphoid tissue composed of lymphocytes, and the red pulp comprises venous sinuses and cords of lymphatic cells [21]. The spleen of the NC group had normal cell morphology and white pulp and red pulp structure, while the IR, MR, and HR groups exposed to radiation showed relatively atrophied white pulp and decreased lymphocytes. This can be observed by comparing the diameter of the white pulp. On the first day after irradiation, the NC group showed 352.8 μm, the IR group 223.1 μm, the MR group 266.3 μm, and the HR group 260.0 μm. Overall, an immediate reaction occurred on the first day of irradiation, and these symptoms further increased on the third day. But, they gradually recovered on the seventh day, exhibiting 369.9 μm, 227.1 μm, 252.9 μm, and 294.5 μm for each group. On the seventh day after irradiation, compared to the IR group, the MR and HR groups showed less lymphocyte loss due to damage to the white pulp. In particular, the HR group demonstrated a higher recovery rate than the MR group. Indeed, HME-Mulberry leaves show a radioprotective effect based on their higher antioxidant components. Additionally, these results suggest that the recovery of the spleen tissue after seven days of irradiation may contribute to lymphocyte production.

Unlike previous studies on the radioprotective effects using electromagnetic radiation, this study conducted oral administration for three days. Therefore, the study was designed to confirm the radioprotective effects even after a short intake period. Still, it is evaluated that the components of natural products have not been sufficiently delivered, so there is a limitation on the sample administration period. Therefore, as in previous studies, it is significant to explore the radioprotective effect through intake for at least seven days [22]. In addition, investigating the radiation protection effect through the spleen and other organs, such as the gonads and small intestine, which are sensitive to radiation, is necessary.

4. Conclusions

In this study, it was confirmed that electromagnetic radiation causes radiation damage to blood cells and spleen cells, resulting in blood cell destruction and structural abnormalities in the spleen. However, it was confirmed that a radioprotective mechanism was implemented when mulberry leaves, which have antioxidant properties, were consumed. In particular, it was confirmed that nano-sized dispersions containing HME-mulberry leaves can have enhanced radiation protection and recovery capabilities than mulberry leaves. This may raise the possibility of its use as a natural product-based radioprotective agent. Therefore, additional supplementary research, such as molecular analysis, can contribute to identifying radioprotective mechanisms and derive directions for further studies on health supplements and drugs.

References

[1] D. Liu and M. Hoskin, Ocean. Coast. Manag. **234**, 106452 (2023).
[2] J. Smith, N. Marks, and T. Irwin, Sci. **382**, 666 (2023).
[3] S.V. Gudkov, N.R. Popova, V.I. Bruskov, Biophysics. **60**, 4 (2015).
[4] H. Nosrati, H. Danafar, H. Rezaeejam, *et al*., Bioorg. Chem. **100**, 103891 (2020).
[5] G. Ma, X. Chai, G. Hou, *et al*., Food. Chem. **372**, 131335 (2022).
[6] N. E. Mohamed and S. E. Ashour, Int. J. Radiat. Biol. **94**, 4 (2018).
[7] H. Patil, R. V. Tiwari, and M. A. Repka, AAPS PharmSciTech. **17**, 1 (2016).
[8] M. Wilson, M.A. Williams, D. S. Jones, *et al*., Ther. Deliv. **3**, 6 (2012).
[9] R. S. Dhumal, A. L. Kelly, P. York, *et al*., Pharm. Res. **27**, 12 (2010).
[10] H. B. Kim, S. J. Ryu, and J. S. Baek, Plants **11**, 22 (2022).
[11] T. Gunasekaran, T. Haile, T. Nigusse, *et al*., Asian Pac J Trop Biomed. **4**, S1 (2014).
[12] Y. Zhang, R. Luo, Y. Chen, *et al*., AAPS PharmSciTech. **15**, 3 (2014).
[13] S. Rayam, B. L. Kudagi, M. Buchineni, *et al*., Int. J. Basic. Clin. Pharmacol. **8**, 9 (2019).
[14] T. Mudalige, H. Qu, D. Van Haute, *et al*., Chapter 11 - Characterization of Nanomaterials: Tools and Challenges, Elsevier, (2019) pp. 313-353.
[15] S. Karimi, A. Moshaii, and M. Nikkhah, Mater. Res. Express. **6**, 11 (2019).
[16] C. Jose Chirayil, J. Abraham, R. Kumar Mishra, *et al*., Chapter 1 - Instrumental Techniques for the Characterization of Nanoparticles, Elsevier, (2017) pp. 1-36.
[17] T. Szatmari, D. Kis, E. N. Bogdandi, *et al*., Front. Immunol. **27**, 8 (2017).
[18] W. B. Rittase, J. M. Muir, J. E. Slaven, *et al*., Exp. Hematol. **84** (2020).
[19] A. Wang, L. Wang, Q, Fu, *et al*., J. Ethnopharmacol. **286**, 114925 (2022).
[20] Y. Duan, F. Chen, X. Yao, *et al*., Int. J. Environ. Res. Public Health. **12**, 7 (2015).
[21] S.A. Elmore, Toxicol. Pathol. **35**, 5 (2006).
[22] J. O. Kim, D. Y. Jung, and B. I. Min, J. Chem. Neuroanat. **129**, 102240 (2023).