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Original Research

Nanocurcumin Innovation as an Anti-Apoptosis of Ovarian Granulosa Cells in White Rats Exposed to Lead Acetate (PbAc)

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Abstract

Introduction: Exposure to Pb causes increased apoptosis of ovarian granulosa cells through oxidative stress mechanism. Curcumin has protective effects on reproductive organs, anti-apoptotic, antioxidant in normal cells. Curcumin in innovated nano form can function as an effective anti-apoptosis in ovarian granulosa cells of rats due to PbAc exposure.
Methods: Thirty female rats were divided into 3 groups, the negative control group (the rats receiving distilled water, in each 90 minutes receiving corn oil), positive control group (the rats receiving PbAc of 30 mg/kg BW, in each 90 minutes receiving corn oil), the experimental group, in which the rats receiving PbAc of 30 mg/kg BW, and in each 90 minutes receiving nanocurcumin of 200 mg/kg BW. All groups received treatment orally once a day for 30 days. On day 31 the rats to granulosa cell apoptosis examination using Tunnel method.
Results: Rate of apoptosis was in the positive control group (5.4 ± 0.8%/micro) and the lowest was in the experimental group (1.1 ± 0.5%/micro) and the negative control group (1.2 ± 0.6). The experimental group showed the same p value as the negative control group (p = .095) and different p value (p = .010) from the positive control group. These findings indicated that the innovation of curcumin in nano form at a dose of 200 mg/KgBW reduced apoptosis of rat ovarian granulosa cells due to PbAc exposure.
Conclusion: The innovation of curcumin in nano form has the potential as an effective natural anti-apoptosis in rats ovarian granulosa cells exposed to PbAc.

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INTRODUCTION

Exposure to lead acetate (Pb) causes increased apoptosis of ovarian granulosa cells through oxidative stress mechanism. The hydroxyl radicals (OH*), which are formed as a result of lead exposure, can translocate to ovarian granulosa cells and stimulate P53 production. P53 reacts with the mitochondrial membrane and activates pro-apoptosis (Bax) and causes a decrease in anti-apoptotic (Bcl-2 and Bcl-x) which causes the release of cytochrome c into the cytosol of granulosa cells. In the cytosol, cytochrome c binds to Apaf-1 (apoptosis-activating factor 1) to form a caspase recruitment domain (CARD) that stimulates caspase 9 in granulosa cells, and caspase 9 stimulates caspase-3, an effector that carries out granulosa cell apoptosis [1].

Curcumin, which is derived from the turmeric plant, is a compound that has potential as an antioxidant and anti-inflammatory, able to inhibit the reduction of the risk of cancer and other malignancies. In the reproductive system, curcumin has protective effects on reproductive organs, such as anti-inflammatory, anti-apoptotic and antioxidant in normal cells, and acts as pro-apoptosis in malignant cells. The effects of curcumin depend on the dose and type of cells used for the trial [2]. Low dose curcumin of 0.27 nM-0.27 μM, can prevent apoptosis of HL60 cells (promyelocytic leukemic cells), whereas at higher doses of 2.71 nM-27.15, curcumin acts synergistically with doxorubicin to induce apoptosis of HL60 cells [3]. Other activities of curcumin include as antimicrobial, anti-cancer, and neuroprotective agents. Several studies on curcumin as a neuroprotective agent have been carried out on Alzheimer's disease, Parkinson's disease, and alcohol-induced neurotoxicity. On average, these diseases have line of actions related to the activity of curcumin as an antioxidant, anti-inflammatory and inhibitor of protein aggregation [4].

Curcumin can act as a very effective anti-apoptosis, but its clinical application is still limited, both in vascular and oral administration. The limitations of clinical application of curcumin are due to its poor solubility and absorption, leading to its low bioavailability [5]. Absorption of orally administered curcumin undergoes presystemic elimination. After absorption, curcumin is conjugated by sulfate and glucuronic acid at various tissue sites. Poor absorption pattern makes it difficult to find curcumin with high levels in the blood some time after administration, so that the effect is less effective [6]. Therefore, innovation is needed to increase bioavailability, longer circulation and better permeability, so that in this study curcumin was formulated in the form of nanoparticles.

The purpose of nanocurcumin formation is to produce smaller, evenly distributed particles that have high bioavailability and stability [7] which are able to penetrate the target cell wall so as to prevent oxidative stress and apoptosis [8].

METHODS

Chemical materials

The lead acetate used in this study had linear

formula of Pb (CH3CO2)2.3H2O), (Product No: CAS 6080-56-4, molecular weight (MW): 379.33 g/mol purchased from Sigma-Aldrich.co. USA and Curcumin product of China (Curcuma longa (turmeric) powder, ≥65%, Product No: CAS 458-37-7 molecular weight (MW): 368.38 g/mol

Making process of nanocurcumin

Nanocurcumin was made at the Physics Laboratory, Universitas Airlangga, Surabaya, Indonesia. Nanocurcumin was made using curcumin powder from the rhizome of curcuma longa (turmeric) product of China, (*Curcuma longa* (turmeric) powder, ≥65% with a molecular weight (MW): 368.38 g/mol using milling method by pulverizing with cubic zirconia balls, with a ratio of curcumin: cubic zirconia balls = 1 : 10. Curcumin and cubic zirconia balls were put in a tube and milled on a High Energy Milling (HEM) machine. The setting time used in milling was 5 minutes milling, 5 minutes resting until the number of effective processing time of the sorting was 30 minutes beyond the resting time.

Analysis of nanocurcumin size characteristics

Analysis of nanocurcumin size characteristics was carried out by two methods, the Scanning Electron Microscopy (SEM) and Particle Size Analysis (PSA). Analysis of nanocurcumin size characteristics using PSA method was carried out at the Materials Physics Laboratory, ITS, Surabaya, Indonesia. Measurement of curcumin and nanocurcumin was carried out using PSA wet method. Particles were measured in a single particle, and the measurement results were in the form of particle size distribution, so that the measurement results could presumably characterize the overall condition of the sample (nanocurcumin). Before pilling the average size of curcumin was 549.2 nm and after pilling the average size of nanocurcumin was 332.2 nm. The distribution of curcumin size before and after pilling can be seen in Figure 1.

Prior to the pilling, the morphology of curcumin was in the form of irregular plates with an average diameter of more than 1000 nm. Curcumin morphology in nano form (after pilling) was in a more regular crystal form and had an average diameter of less than 200 nm. Morphological analysis of nanocurcumin was carried out using the Scanning Electron Microscopy (SEM) method at Robotics Laboratory, ITS, Surabaya, Indonesia. The difference in curcumin morphology before and after pilling is shown in Figure 2.

Preparation of nanocurcumin solution

Corn oil is the best solvent for nanocurcumin compared to butter, milk and water, so this study used corn oil as a solvent [9]. The solution was made by dissolving 2 g of nanocurcumin with corn oil to become 200 ml, so that 1 ml of the solution contained 10 mg of nanocurcumin.

Experimental animals

This study had passed the ethical test of the Ethics Committee, Faculty of Veterinary

Medicine, Universitas Airlangga, Indonesia. The experimental animals used in this study were female white rats weighing about 200-250 g, aged 2.5-3 months, obtained from Institut Teknologi Bandung (ITB), Bandung, Indonesia. The rats were housed in cages in an air-conditioned room with temperature maintained at 26C°-2C° and 12 h in light and dark cycle.

Ethical clearence

No: 2.KE.170.08.2019 on 29 August 2019, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya.

Experimental design

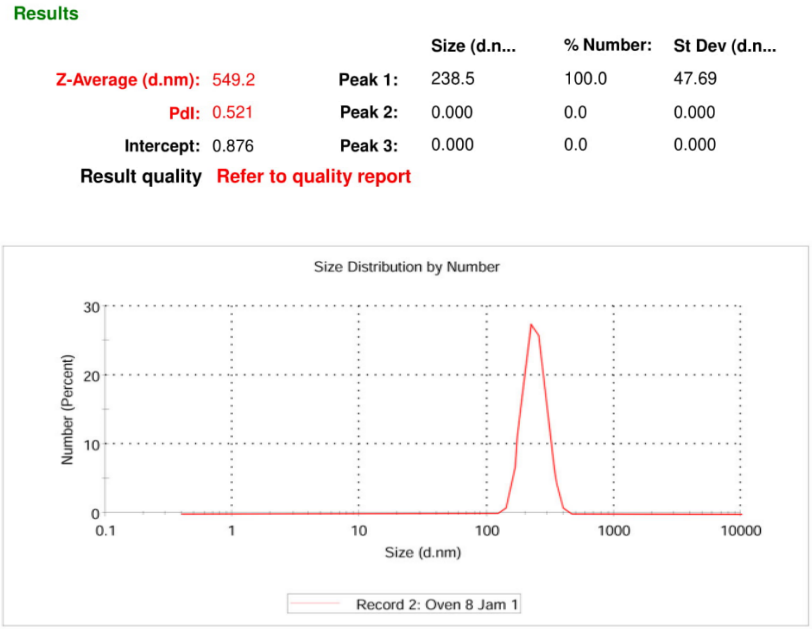
This study used 30 female white rats which were divided into 3 groups, the negative control group (C-), in which the rats received distilled water and in each 90 minutes receiving corn oil; positive control group (C+), the rats received lead acetate 30 mg/kg BW and in each 90 minutes receiving corn oil; and the experimental group, the rats received lead acetate 30 mg/kg BW and in each 90 minutes they received nanocurcumin of 200 mg/kg BW. The treatment was carried out once a day every 10.00 AM for 30 days. On day 31 the rats were sacrificed, the ovaries were cleaned from connective tissue, washed with 0.9% physiological NaCl, and embedded in paraffin blocks. Furthermore, the ovarian granulosa cell apoptosis was examined by the Tunnel method.

Tunnel examination

In this method of examination, the ovaries that had been embedded in paraffin blocks were cut into serial paraffin blocks with a thickness of 4 – 6 m. Representative slices were selected from the tissue sections and deparaffinized preparations (paraffin blocks) was performed with xylene three times, 3 minutes each, then rehydration was carried out on these preparations using 100%, 95%, and 75% ethanol, respectively, each for 2 minutes, 2 minutes, and one minute, and, finally, rehydration of the preparations was carried out with sterile distilled water. In the next step, the preparations were dripped with 50 µl Tunnel labeling mix (consisting of 5 µl terminal deoxynucleotydil transferase and 45 µl fluorescenin-dUTP) and the preparations were covered with siliconized cover slips. Then, the incubation of the preparations was carried out at 37oC for 30 minutes in a moist chamber and washing with PBS (phosphate buffer saline) was carried out 3 times. The subsequent process was the incubation of Rnase Solution at 37Co for 30 minutes, then washing with PBS was carried out 3 times and incubation of preparations was carried out with propidium iodide solution at room temperature for 10 minutes. The last step was the washing of the preparations with PBS 3x and the preparations were covered with a cover slide with a diameter of 18 mm. Furthermore, the observation of apoptotic cells was carried out using a fluorescence microscope with 10 visual fields in 400 x magnification.

Statistical analysis

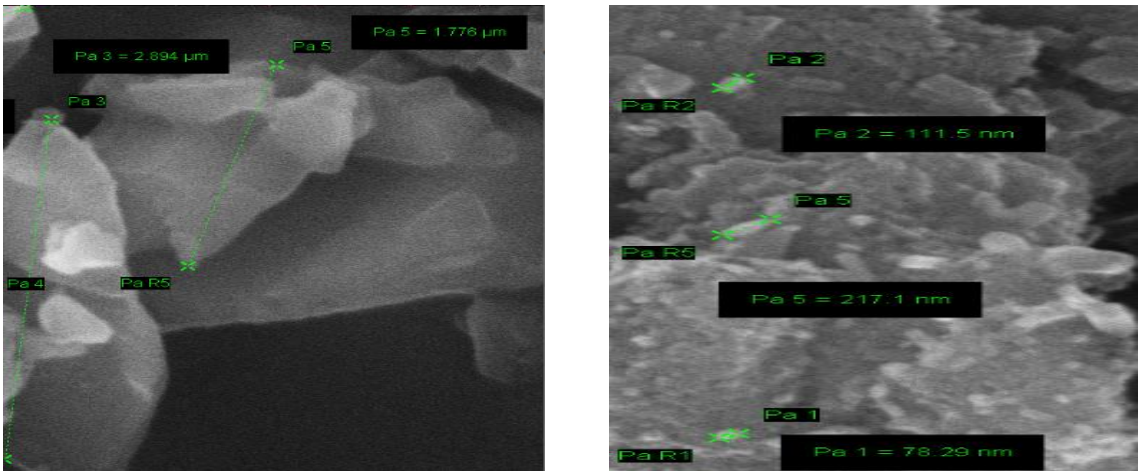
Data were presented with mean ± standard deviation. The comparative test was carried out using Kruskall-Wallis Test to determine the differences between groups, followed by the Mann-Whitney test to determine the differences between the groups.



(A)

(B)

Figure 1. Measurement results of curcumin particles (A) before and (B) after pilling (nanocurcumin) using PSA wet method.



(A) (B)

Figure 2. Curcumin morphology before and after pilling. (A) Curcumin morphology before pilling was in a size of > 1000 nm; (B) Curcumin morphology after pilling (nanocurcumin) in a size < 200 nm.

RESULTS

Characteristics of nanocurcumin size

The discrepancy between the results of the curcumin diameter analysis was possible because the software processing of the SEM images (500 x magnification) had limited sample area selection, so the particle size could not be accommodated as a whole. Examination with SEM is basically an examination by analyzing the surface. The profile obtained was surface data with a thickness of about 20 µm from the surface. In particle analysis using PSA, the measured particle is a single particle. In addition, the measurement results are in the form of a distribution, so it can be assumed that the analysis using PSA describes the overall condition of the sample. The working principle of PSA analysis is the use of the dynamic light scattering principle to measure the size distribution of the particles undergoing the Brownian motion. This method is considered more accurate than SEM because it uses laser light as an information medium for particle measurement. The use of PSA allows signal analysis for each particle to ensure high precision particle distribution [10].

In this study, nanocurcumin had a regular crystalline form with a diameter of approximately 200 nm (SEM) and 332.2 (PSA). This finding was in line with a research [11] revealed an average nanocurcumin particle size of 269.8 nm. Nano-sized curcumin has 216 higher water solubility than pure curcumin in powder form due to an increase in the surface area of the nanoparticles compared to pure curcumin in powder form [12] The size of nanocurcumin resulted in this study was included in nanoparticle category of supra-nanoscale category (size 100 – 1000 nm) and the size 1 – 100 nm was included in small size category [13]. The manufacture of nanocurcumin in this study could not reach small size (1-100 nm) due to the limitations of the equipment

used. In pilled curcumin, pilling for a long time gave a color change to become more concentrated and blackish so that there was a decrease in the magnitude of the effect.

Nanocurcumin as an anti-apoptotic protector of ovarian granulosa cells in white rats exposed to lead acetate

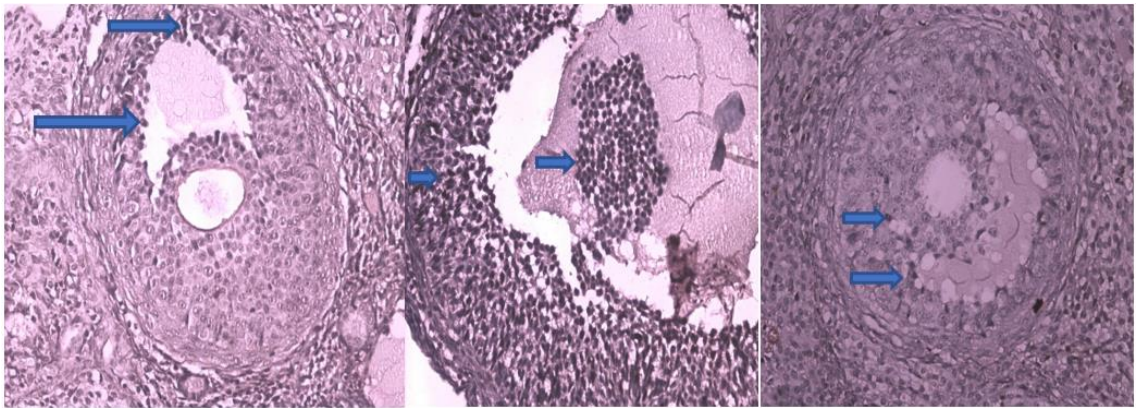
The results of Kruskal-Wallis test showed that there were differences between groups of apoptotic expression (Kruskal-Wallis H = 19.82; df = 4; p = .001). Furthermore, to determine the difference between groups, an analysis was carried out using Mann-Whitney Test, as shown in Table 1.

Table 1 shows that the highest rate of apoptosis was in the positive control group (5.4 ± 0.8%/micro) and the lowest was in the experimental group (1.1 ± 0.5%/micro) and negative control group (1.2 ± 0.6). The experimental group showed similarities (p = .095) with the negative control group and differences (p = .010) with the positive control group (+). This proves that the innovation of curcumin in nano form in a dose of 200 mg/Kg BW reduces apoptosis of rat ovarian granulosa cells due to lead acetate exposure. These differences are shown in Figure 1.

Table 1
Innovation of nanocurcumin as anti-apoptotic granulosa cells in rat ovaries due to lead acetate exposure (mean ± standard deviation)

Groups	n	Apoptosis expression (%/micro)	Minimum	Maximum
Negative control	10	1.2 ± 0.6	1.1	2.5
Positive control	10	5.4 ± 0.8	2.8	5.8
Experimental	10	1.1 ± 0.5	1.0	2.4

Different superscript within each column indicates significant difference between means (p < .05).



(A) (B) (C)

Figure 3. Expression of rat ovarian granulosa cell apoptosis (A) C- group; (B) C+ group; (C) E group. Observations were done using light microscope in a magnification of 400 x.

DISCUSSION

This study proved that exposure to lead acetate of 30 mg/kg BW in white rats increased ovarian granulosa cell apoptosis. The mean expression of apoptosis in control group (C+) was higher than that in control group (C-) and in the experimental group. This finding was in line with the study [14] that administration of intraperitoneal injection of lead acetate (PbAc) 20 mg/kg BW/day for 7 days to white rats (Rattus norvegicus) increased nephron apoptosis.

Exposure to lead acetate (PbAc) causes increased apoptosis of ovarian granulosa cells through oxidative stress mechanism. The hydroxyl radicals (OH*) formed as a result of lead exposure can translocate to ovarian granulosa cells and stimulate the release of P53. P53 reacts with the mitochondrial membrane and activates pro-apoptosis (Bax) and causes a decrease in anti-apoptotic (Bcl-2 and Bcl-x) [15] leading to the release of cytochrome c into the cytosol of granulosa cells. In the cytosol, cytochrome c binds to Apaf-1 (apoptosis-activating factor 1), forming a caspase recruitment domain (CARD) that stimulates caspase-9 in granulosa cells, and caspase-9 stimulates caspase-3 which is an effector that carries out granulosa cell apoptosis [1].

Mitochondria contain pro-apoptotic factors, such as cytochrome c and AIF. Both are harmful substrates, but are stored safely in the mitochondria. When both are released into the cytoplasm, these proteins can activate caspase activation pathway. The release is regulated by mitochondrial-bound Bcl-2 family, the Bax and Bad. Cytochrome c acts as a water-soluble electron carrier in mitochondrial oxidative phosphorylation. When the electron coil is formed through cytochrome c oxidase, the change in ionic strength will cause a matrix wave to occur. When the inner mitochondrial membrane has a wider surface permeability than the outer membrane, matrix waves cause non-specific inner membrane permeability pores to open, so that cytochrome c exits into the cytoplasm. Cytochrome c that comes out into the cytoplasm then binds to Apaf-1, forming Caspase Recruitment Domain (CARD). Several CARDs combine to form apoptosome complexes, then bind to pro-caspase 9 and activate it to become caspase 9 (initiator caspase). Caspase 9 will activate procaspase 3 into caspase 3 which is an effector caspase that carries out apoptosis [16].

The administration of nanocurcumin in this study was proven to reduce apoptosis of rat ovarian granulosa cells due to exposure to lead acetate at a dose of 30 mg/kg BW. This can be observed from the mean expression of apoptosis in the experimental group and the negative control group compared to the positive control group.

Curcumin, which was isolated from Curcuma longa (turmeric), has great potential as an antioxidant which is thought to be due to its phenolic and 1,3-diketone groups. These polyphenolic natural antioxidant compounds are multifunctional and can function as free radical scavengers, such as superoxide (O2*) and hydroxyl radicals (*OH) [17] metal chelators such as iron (Fe) [18] inhibitors of oxidative enzyme activity such as cytochrome P. -450 [19] reduce the formation of oxygen radicals[20]. The antioxidant activity of

curcumin compounds takes place because the formation of free radicals is inhibited by the curcumin compounds by suppressing the activity of cytochrome p450 [21].

Ovarian granulosa cell apoptosis of white rats exposed to lead can be protected by inhibiting the formation of hydroxyl radicals (OH*). Inhibition of the formation of hydroxyl radicals (OH*) is to prevent the Haber Weiss reaction and Fenton reaction (Suryohudoyo, 2000). This prevention is carried out by chelating the transition metals F++ and C+ which act as catalytic for the formation of OH* [22]. Prevention of OH* formation suppresses Bak and Bak which increases the release of Bcl-2 and Bcl-xl expression, which then suppresses cytochrome c expression out of the mitochondrial membrane. This results in the absence of binding between cytochrome c and Apaf-1, called apoptosome, resulting in the prevention of caspase-9 stimulation. As a result, caspase-3 decreases and ends with decreased apoptosis [23].

CONCLUSION

The innovation of curcumin in nano form (nanocurcumin) can be used as an effective anti-apoptotic in white rat ovarian granulosa cells due to lead acetate exposure.

CONFLICT OF INTEREST

There is no conflict of interest in this research.

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REFERENCES

[1] O. M. Ighodaro and O. A. Akinloye, "First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid," Alexandria J. Med., vol. 54, no. 4, pp. 287–293, 2018, doi: https://doi.org/10.1016/j.ajme.2017.09.001,.

[2] R. Mohebbati, A. Anaeigoudari, and M. R. Khazdair, "The effects of Curcuma longa and curcumin on reproductive systems," Endocr. Regul., vol. 51, no. 4, pp. 220–228, 2017, doi: 10.1515/enr-2017-0024.

[3] R. Banjerdpongchai, M. Suttajit, and P. Kongtawelert, "Effect of curcumin on HL60 cell apoptosis induced by doxorubicin," Chiang Mai Med Bull, vol. 41, no. 2, pp. 59–66, 2002.

[4] S. Shishodia, M. M. Chaturvedi, and B. B. Aggarwal, "Role of curcumin in cancer therapy," Curr Probl Cancer, vol. 31, no. 4, pp. 243–305, 2007.

[5] D. M. . Bodhankar and S. Chikhle, "Various Approaches Towards Enhancement of Bioavailability of

Curcumin - A Potent Phytochemical," World J. Pharm. Res., vol. 8, no. 1, pp. 606–626, 2018, doi: 10.20959/wjpr20191-13932.

[6] M. . Pan, T. . Huang, and J. . Lin, "Biotransformation of curcumin through reduction and glucuronidation in mice," Drug Metab. Dispos., vol. 27, no. 1, pp. 486–494, 1999.

[7] P. Rajsekhar, R. . Bharani, K. . Angel, and M. Ramachandran, "Curcumin Nanoparticles?: A Therapeutic Review," Res. J. Pharm. Biol. Chem. Sci., vol. 6, no. 5, pp. 1180–1186, 2015.

[8] S. Prasad, A. K. Tyagi, and B. B. Aggarwal, "Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: The golden pigment from golden spice," Cancer Res. Treat., vol. 46, no. 1, pp. 2–18, 2014.

[9] M. Shishu, Maheswari, "Comparative Bioavaibility of curcumin, turmeric, and biocurcumax, in tradisional vehicles using non-everted rat intestinal sac model," J. Funct. Food, vol. 2, pp. 60–65, 2010.

[10] W. Nuraeni, I. Daruwati, E. Maria W., and E. Sriyani, M., "Verification of the performance of the Horiba LB-550 particle Sinze Analyzer (PSA) for determining the size distribution of nanoparticles," Proc. Natl. Semin. Nucl. Sci. Technol., 2013.

[11] L. Chabib, R. Martien, and H. Ismail, "Formulation of nanocurcumin using low viscosity chitosan polymer and its cellular uptake study into T47D cells," Indones. J. Pharm., vol. 23, no. 1, pp. 27–35, 2013.

[12] D. de M. Carvalho, K. P. Takeuchi, de M. Geraldine, R. M., C. J., and M. C. L. Torres, "Production, solubility and antioxidant activity of curcumin nanosuspension," Food Sci. Technol., vol. 35, no. 1, pp. 115–119, 2015, doi: 10.1590/1678-457X.6515.

[13] A. Albanese, P. S. Tang, and W. C. W. Chan, "The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems. Annual Review of Biomedical Engineering," Annu. Rev. Biomed. Eng., vol. 14, no. 1, pp. 1–16, 2012, doi: 10.1146/annurev-bioeng-071811-150124.

[14] AL-Megrin, W. A., D. Soliman, R. B. Kassab, D. M. Metwally, D. M. Dina, and M. F. El-Khadragy, "Coenzyme Q10 Activates the Antioxidant Machinery and Inhibits the Inflammatory and Apoptotic Cascades Against Lead Acetate-Induced Renal Injury in Rats," Front. Physiol., vol. 11, no. 2, pp. 1–13, 2020, doi: 10.3389/fphys.2020.00064.

[15] M. A. O'Brien and R. Kirby, "Apoptosis: A review of pro-apoptotic and anti-apoptotic pathways and dysregulation in disease," J. Vet. Emerg. Crit. Care, vol. 18, no. 6, pp. 572–585, 2008, doi: 10.1111/j.1476-4431.2008.00363.x.

[16] S. . Sudjarwo, C. Anwar, G. Wardani, K. Eraiko, and Koerniasari, "Antioxidant and anti-caspase 3 effect of chitosan-Pinus merkusii extract nanoparticle against lead acetate-induced testicular toxicity in rat," Asian Pacific J. Reprod., vol. 8, no. 1, pp. 13–19, 2019, doi: https://doi.org/10.4103/2305-0500.250418.

[17] Kabeer, A., M. Muhammad Mailafiya, A. Danmaigoro, E. Abdul Rahim, B. Bakar, and M. Z. A, "Therapeutic potential of curcumin against lead-induced toxicity: A review," Biomed. Res. Ther., vol. 6, no. 3, pp. 3053–3066, 2019, doi: https://doi.org/10.15419/bmrat.v6i3.528.

[18] N. Singh, A. Kumar, V. K. Gupta, and B. Sharma, "Biochemical and Molecular Bases of Lead-Induced Toxicity in Mammalian Systems and Possible Mitigations," Chem. Res. Toxicol., vol. 31, no. 10, pp. 1009–1021, 2018, doi: https://doi.org/10.1021/acs.chemrestox.8b00193.

[19] M. A. Assi, M. Noor, M. Hezmee, A. W. Haron, M. Yusof, and M. Sabri, "The detrimental effects of lead on human and animal health," Vet. World, vol. 9, pp. 660–671, 2016, doi: https://doi.org/10.14202/vetworld.2016.660-671.

[20] Dumitrescu, E., V. Chiurciu, F. Muselin, R. Popescu, D. Brezovan, and R. T. Cristina, "Effects of long-term exposure of female rats to low levels of lead: Ovary and uterus histological architecture changes," Turkish J. Biol., vol. 39, no. 2, pp. 284–289, 2015, doi: https://doi.org/10.3906/biy-1407-6.

[21] M. Majeed, V. Badmaev, U. Shivakumar, and R. Rajendranm, Curcuminoids - Antioxidant Phytonutrients. NutriScience Pub, 1995.

[22] P. Suryohudo, Molecular Medicine. Jakarta: Sagung Seto, 2000.

[23] M. Redza-Dutordoir and D. A. Averill-Bates, "Activation of apoptosis signalling pathways by reactive oxygen species," Biochim. Biophys. Acta-Molecular Cell Res., vol. 1863, no. 12, pp. 2977–2992, 2016, doi: 10.1016/j.bbamcr.2016.09.012.