

The Potential of Walnut Seeds (*Canarium indicum* L.) in Repairing the Genotoxicity Effect of Mercury on Mice Erythrocytes

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Abstract

Hydrargyrum (familiar as mercury) is a persistent transition metal element. They are known as bio-accumulative properties that are dangerous to the environment and has serious damaging effects on biota, including genetic changes or mutagenesis. Moreover, it can accumulate indirectly in the human body due to the consumption of products that have contaminated. Whereas, ellagic acid has reported as one of 13 flavonoid compounds that have potential as an anti-mutagen and anti-cancer in the walnuts seeds. This study was aimed to ensure the potential extent of ellagic acid extracts in walnut seeds to reducing and repairing the genotoxicity effects of mercury. Therefore, using the experimental method, this study was divided into several test treatments. Two controls treatment as a comparison of the presence of micronucleus with group tests that given a 20 ppm mercury dosage. Then continued with the addition of walnut seed extract with various dosage (10, 15, and 20 ppm) by oral injection continuously for two weeks as a recovery process to reducing and repairing the genotoxicity effect of mercury. The significant results showed that there are differences between pre and post-treatment. It indicated that the extract has the potential for improving and reducing the genotoxicity effect of mercury. The data were evaluated and obtained the total micronucleus from the smear of peripheral blood of mice by comparing the total micronucleus before and after giving of walnut extract.

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INTRODUCTION

We had already known that mercury is one of the dangerous heavy metals appurtenant to the toxicity. The different chemical and physical properties, also the capacity as bio-accumulation and toxicity in the organism, therefore it making as a mobile element (Sizmur *et al.*, 2018). The exposure and contamination from this heavy metal can indirectly cause serious problems. Also, it has an adverse clinical impact on biota and the environment (Bernhoft, 2012).

Primarily in nature and the aquatic environment, mercury has existed for quite a long time as mercury

sulfide or the elemental mercury itself from half per million that can be found in the earth's crust (Jan *et al.*, 2015). Thus, the effects of contamination mercury pronounced in the living organism at the level of aquatic organisms such as bivalve (Szkoda *et al.*, 2015). These organisms (bivalve) are believed as bioaccumulation indicators and have metallothionein, which can accumulate heavy metals material in their tissues cell (Rodney *et al.*, 2010). It sourced by the effluent which unable to concentrate both terrestrial and aquatic ecosystems, which are exposed to raised levels of environmental mercury derived from rock weathering,

out-gassing and from the mine/smelting processes and another source (Nevado *et al.*, 2003). Consider this heavy metal have potentially as a genotoxic material. It can change the structure genetic in the cell body of exposed, resulting in the teratogenic and carcinogenic effect chiefly in human body (Crespo-López *et al.*, 2009).

In another case, ellagic acid is well-known as natural phenolic compounds found in fruit such as walnuts, blueberries, strawberries, raspberries, mango, longan seeds, green tea, and some vegetables (Mady & Shaker, 2017; García-Niño & Zazueta, 2015; Ríos *et al.*, 2018). Plants produce it from the hydrolysis of tannins such as ellagitannin and geranin for use as protectors from microbiological infections, heavy metal poisoning, predation by insects, and pests (Aguilera-Carbo *et al.*, 2008; Hamada *et al.*, 2019; Yalcin *et al.*, 2018; Shakeri *et al.*, 2018; Ríos *et al.*, 2018). Ellagic acid has the ability to reducing the oxidative stress in cells and has beneficial a chemopreventive substance, anti-proliferative, anti-viral, anti-bacterial and an antioxidant and had reported in recent studies (Yalcin *et al.*, 2018; Devipriya *et al.*, 2007).

The heavy metal poisoning, especially in the cellular damage level (genetic mutation), can be detected with one of the simple parameters tests, i.e. micronucleus test. Micronucleus test can be used to determine the genotoxicity of a chemical, food, and drugs by looking at the formation of a micronucleus (small nucleus) of approximately 1/3-1/6 of the main nucleus in cells (Bolognesi *et al.*, 2013).

In this study, researchers wanted to extend and find out the potential of walnut seed extracts that believed contain an ellagic acid compound in repairing the genotoxicity effect of heavy metal mercury that can accumulate indirectly in the body due to consuming seafood and other contaminated foods. This study was aimed to ensure the potential extent of ellagic acid extracts in walnut seeds to reducing and repairing the genotoxicity

effects of mercury. As a test animal used male albino mice by the micronucleus assay as a method of observation.

MATERIALS AND METHODS

Tools and materials

The experimental design method has been applied in this study. The all of chemical and material obtained from the laboratory at Universitas Maarif Hasyim Latif, among of May-Grünwald Giemsa staining, Wright staining, ethanol, methanol, HgCl₂ reagent (Emsure®), distilled water, rotary evaporator (Ika® RV 10), hot plate with magnetic stirrer (Chimarec®), chemical glasses (Iwaki®), and other glass equipment.

Extraction

Approximately, 1 kg of peeled walnut seeds sample is dried for four days then crushed with a mortar. Further, continued maceration with 96% ethanol (Ratio 1 : 1) for one night to take the filtrate (repeated four repetitions). The filtrate was evaporated using rotary evaporation for several hours to obtain a pure extract from walnut seeds (Limbono, 2013).

Test animal preparation

The healthy male albino mice were obtained and acclimatized for several days before being treated. A total of 30 mice divided into five treatments, two as control groups and three as group test treatment. The control positive by giving injection intraperitoneally of cyclophosphamide 50 mg/kg BW.

Exposure and recovery process

As much as 20 ppm of HgCl₂ exposed for two weeks to mice by orally in the group treatments. Afterwards, the recovery process is done by giving various dosage of the extract (10, 15, and 20 ppm) for two weeks.

Micronucleus assay

The micronucleus assay observed in a thousand erythrocytes cells which prepared from blood smear in

each of the mice in the group treatment. Approximately, one drop blood spread on object-glass then fixed with methanol. Hereafter, stained with May-Grünwald, Giemsa staining, or wright staining. The observation process is using the microscope (Olympus® CX22) and Optical lab camera (OptiLab®) Viewer and counted with Image Raster 3 Software.

Statistical analysis

Statistical analysis was performed using the parametric method using a comparative test before and after recovery (Paired sample T-Test) in each group test. Interpretation of the results of the analysis by reading the table of significance (Sig. 2-tailed).

RESULTS AND DISCUSSION

Extraction

The extraction results of walnut seeds with the initial sample weights of approximately 1 kg were obtained about 23 g of pure extracts from walnut seeds. The extraction process was carried out after maceration four repetitions in 24 hours with 96% ethanol.

Micronucleus frequency

The control group merely used to recognize the presence of micronucleus in mice erythrocytes. The total micronucleus in the treatment group test after recovery was discovered less than the treatment group test after exposed with HgCl₂. The presence of micronucleus was moderately increased in the group treatment which exposed of HgCl₂ as presented in **Figure 1** and **Figure 2**. While the total number of micronucleus frequencies after recovery and the average frequencies of micronucleus for each test group treatment are respectively presented in **Table I** and **Table II**.

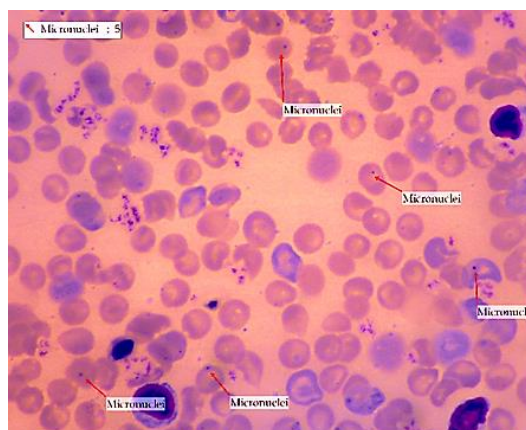


Figure 1. The presence of micronucleus in erythrocytes of mice after exposed with mercury 20 ppm

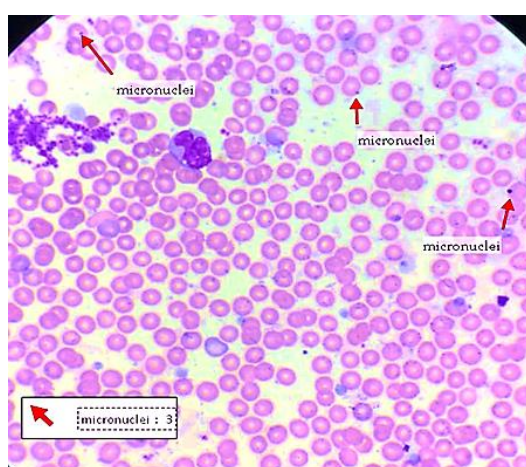


Figure 2. The presence of micronucleus in erythrocytes of mice after recovery extract E.A.

Table I. The total amount of frequencies micronucleus (positive controls)

Slide	The amount micronucleus/1,000 cells	Mean (%)	Percentage (%)
Control Group			
1	13		
2	16		
3	10	13.1	0.131
4	17		
5	13		
6	10		

Table II. The total amount of frequencies micronucleus (exposed mercury)

Slide	Micronucleus after exposed mercury 20 ppm (%)		
	A	B	C
1	19	18	12
2	14	13	11
3	13	11	16
4	16	13	14
5	13	14	10
6	14	15	17
Mean	14.83	14	13.33
Percentage (%)	1.483	1.4	1.333

Refers to **Table I** and **Table II**, we have known that micronucleus can be formed after injection orally of cyclophosphamide and HgCl₂. Cyclophosphamide is widely used as an anti-tumor material, therapy cancer, and another related. However, other cases reported that this drug could have a clastogenic effect, especially on healthy cells that proliferate. Thus, causing the appearance of micronucleus in cells due to chromosomal aberrations and induces oxidative stress (Abdella, 2012). Likewise, some research in one decade summed that mercury (inorganic mercury) might lead a chromosomal genotoxicity effect through the aneugenicity and clastogenicity (Çavaş, 2008). Notwithstanding, organic mercury can generate the possibility of those effects as well. Bernhoft (2012) also reported that organic mercury has a more significant toxic effect than inorganic mercury in eliciting the toxicity.

These metals are believed to generate the possibility of genotoxic and cytotoxic more serious, which can change the composition of genetic material in the body of exposed populations. Therefore, it was occurring carcinogenic, and teratogenic effects are formed (Crespo-López *et al.*, 2011; Crespo-López *et al.*, 2009). The metal mercury (especially methyl-mercury) can interact with biomolecules and can bind through hydroxyl and sulfhydryl groups (Çavuşoğlu *et al.*, 2010). It underlies and refers to how genotoxic mechanisms occur.

Crespo-López *et al.* (2009) were stated that the mechanism of genotoxic of mercury through several stages. The metal mercury compound enters the cell organism through the cell membrane or even through the transport protein channels. In cells, mercury compounds will be formed as components of radical compounds and reactive oxygen species that can directly or indirectly generate the interruption of the mechanism of action of the deoxyribonucleic acid system. The components of these mercury compounds play a

significant role in the organism's cell work system (whether it can be an inhibiting compound, a destructive agent or more) at the cellular level. The longer and higher the damage produced, the mercury compounds are believed to induce and increase the effects of cell damage to cause cell changes (Tchounwou *et al.*, 2012). Even worse to increasing cancer due to the high number of ROS and free radical compounds. That believed as some cooperative compounds in bringing up the symptoms of the disease of cancer (Kumari *et al.*, 2018).

The average of micronucleus in this research showed in the range of 14.8±2.3 (A); 14.0 ± 2.4 (B); 13.3 ± 2.8 (C) as shown in **Table II** and **Table IV**. The data demonstrate that in the concentration of 20 ppm of exposed mercury by orally induced the presence of micronucleus in erythrocytes. It explains that genotoxic events have occurred. The presence of micronucleus can be considered as a sensitive indicator related to genotoxicity effect in the cell of the organism (da Rocha *et al.*, 2011). Nevertheless, the range of micronucleus is depended on the dose of the agent or substance and the length of time exposure (Çavuşoğlu *et al.*, 2010). Inversely with the results after the recovery process, the presence of micronucleus decreased significantly, as shown in **Table III** and **Table IV**. Statistical data also showed that either before and after the test treatment, there were significant differences in each test group ($P < 0.05$).

Table III. The total amount of frequencies micronucleus (after recovery)

Slide	Micronucleus after recovery extract of walnut seeds (‰)		
	A (10 ppm)	B (15 ppm)	C (20 ppm)
1	8	9	7
2	10	10	7
3	7	8	8
4	6	5	9
5	11	9	11
6	8	8	5
Mean	8.33	8.16	7.83
Percentage (%)	0.833	0.816	0.783

Table IV. The average frequencies of micronucleus (each test group treatment)

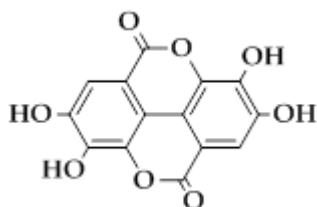
Group Test	Exposed mercury 20 ppm			After recovery extract		
	Min	Max	Average \pm SD	Min	Max	Average \pm SD
A	13	19	14.8 \pm 2.3	6	11	8.3 \pm 1.9
B	11	18	14.0 \pm 2.4	5	10	8.2 \pm 1.7
C	10	17	13.3 \pm 2.8	5	11	7.8 \pm 2.0

Note: Micronucleus were analyzed and counted in 1,000 cells per animal/group. The data obtained were calculated on average \pm SD (n = 6/group). Statistically data: .006 (A); .003 (B); .026 (C) (Pre-post test).

Chemical compounds

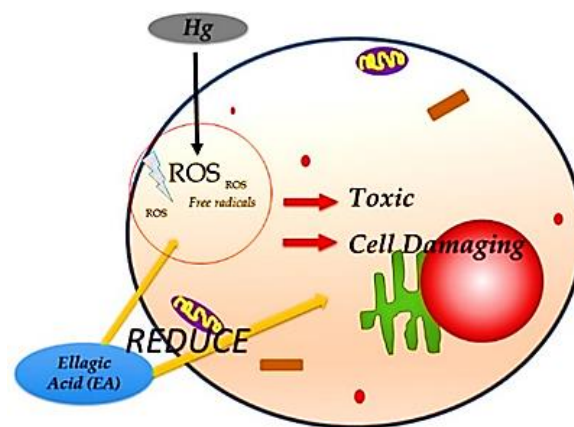
The walnut seeds have various bioactive compounds. As much as nine species of compounds have been isolated. Broadly, it is derived from the secondary of metabolism walnuts, i.e. carboxylic acids, terpenes, lipids, and phenol (tannins, flavonoids, phenolic acids) (Mogana & Wiart, 2011). A related study has been determined the pharmacology and antioxidant activities which have vast advantages for the body.

Ellagic acid has several pharmacology activities. As an anti-cancer, anti-inflammatory, anti-bacterial and fungal, anti-diabetic, hepatoprotective and scavenging the free radical (Mogana & Wiart, 2011; Shakeri *et al.*, 2018; Yalcin *et al.*, 2018; García-Niño & Zazueta, 2015). This compound is a product of hydrolysis of tannin as an ellagitannin. Structure of ellagic acid comprises four OH groups with a lactone ring and a dimeric derivative of benzoic acid compounds (Mady & Shaker, 2017) as shown in Figure 3.

**Figure 3.** Ellagic acid chemical structure

The result in this research provides a significant substantiation that mercury is one of the most agents that can adversely affect of carcinogenic effect by induced of genotoxic activities. The presence of micronucleus was a figure that there is some arrangement of chromosomal

aberrations. However, the pharmacology activities of ellagic acid are far different from the mercury. Principally, ellagic acid assisted in decreasing the number of micronuclei in this research data. It shows that ellagic acid can prevent the effects of genotoxicity in the body. In general, the play role of ellagic acid is explained in Figure 4.

**Figure 4.** The schematic process between Hg and EA in cells

Mercury can induce the emergence of reactive oxygen species and other free radicals that are proactive to carcinogenesis by influencing changes in the genetic makeup of cells that cause genetic mutations or chromosomal aberrations. Ellagic acid can reduce the effects of free radicals by reducing damage due to HgCl₂ cell oxidative stress. Ellagic acid believed to act as a chelating component to toxicity-generating compounds such as free radicals and reactive oxygen species that have unstable chemical structures. Other than that, ellagic acid can prevent changes in the structure of cell organelles (mitochondria, endoplasmic reticulum), which are specific targets of mercury toxicity (García-Niño & Zazueta, 2015).

Mercury really should be something that needs more concern. Moreover, in Indonesia – considering based on the equatorial condition besieged the sea areas and islands. It is a strategic place to utilizing the natural resources derived of most Indonesians livelihood,

particularly in the marine field and aquatic aside from agricultural and business development. Besides, there is a possibility that people can be exposed to the effects of heavy metals, one of which is mercury through contaminated seafood. Hence from this, ellagic acid contained in walnut seeds can be an essential indicator to overcome this problem. Further research is expected to develop innovations related to the potential of compounds contained in walnut seeds.

CONCLUSION

The walnut seeds extract has potential as an anti-genotoxic agent of mercury in mice erythrocytes. Also, able to reduce and repair the effects of mercury genotoxicity on mice erythrocytes. Based on this study, there is a significant difference result between before and after recovery, which is supported by a decrease in the average number of micronuclei in each test treatment.

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REFERENCES

Abdella, E.M. 2012. Short-Term Comparative Study of the Cyclophosphamide Genotoxicity Administered Free and Liposome-Encapsulated in Mice. *Iranian Journal of Cancer Prevention*. 5(2):51-60.

Aguilera-Carbo, A.F., Augur, C., Prado-Barragan, L.A., Aguilar, C.N. 2008. Extraction and analysis of ellagic acid from novel complex sources. *Chemical Papers*. 62(4):440-444. <https://doi.org/10.2478/s11696-008-0042-y>

Bernhoft, R.A. 2012. Mercury Toxicity and Treatment: A Review of The Literature. *Journal of Environmental and Public Health*. 2012:460508. <https://dx.doi.org/10.1155/2012/460508>

Bolognesi, C., Knasmueller, S., Nersesyanyan, A., Thomas, P., Fenech, M. 2013. The HUMNxl scoring criteria for different cell types and nuclear anomalies in the buccal micronucleus cytome assay - an update and expanded photogallery. *Mutation Research*. 753(2):100-113. <https://doi.org/10.1016/j.mrrev.2013.07.002>

Çavuşoğlu, K., Yalçın, E., Kul, O., Yılmaz, A., Yapar, K. 2010. Detection of Genotoxicity Induced by Heavy Metal Ions and Gamma Radiation Using Micronucleus Assay in Mice: Pathological Evaluation. *Sakarya University Journal of Science*. 14(1):1-8.

Crespo-López, M.E., Macêdo, G.L., Arrifano, G.P., Pinheiro-Mda, C., do Nascimento, J.L., Herculano, A.M. 2011. Genotoxicity of mercury: contributing for the analysis of Amazonian populations. *Environment International*. 37(1):136-141. <https://doi.org/10.1016/j.envint.2010.08.009>

Crespo-López, M.E., Macêdo, G.L., Pereira, S.I., Arrifano, G.P., Picanzo-Diniz, D.L., do Nascimento, J.L., Herculano, A.M. 2009. Mercury and human genotoxicity: critical considerations and possible molecular mechanisms. *Pharmacological Research*. 60(4): 212-220. <https://doi.org/10.1016/j.phrs.2009.02.011>

Devipriya, N., Srinivasan, M., Sudheer, A.R., Menon, V.P. 2007. Effect of ellagic acid, a natural polyphenol, on alcohol-induced prooxidant and antioxidant imbalance: a drug dose dependent study. *Singapore Medical Journal*. 48(4):311-318.

García-Niño, W.R., Zazueta, C. 2015. Ellagic acid: Pharmacological activities and molecular mechanisms involved in liver protection. *Pharmacological Research*. 97:84-103. <https://doi.org/10.1016/j.phrs.2015.04.008>

Cavas, T. 2008. In vivo genotoxicity of mercury chloride and lead acetate: Micronucleus test on acridine orange stained fish cells. *Food and Chemical Toxicology*. 46(1):352-358. <https://doi.org/10.1016/j.fct.2007.08.015>

- Hamada, Y., Haramiishi, R., Ojima, Y., Amakura, Y., Yoshimura, M., Sawamoto, A., Okuyama, S., Furukawa, Y., Nakajima, M. 2019. Hydrolysable tannins, gallic acid, and ellagic acid in walnut reduced 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) reduction in T-Cells cultured from the spleen of mice. *PharmaNutrition*. **7**:100140. <https://doi.org/10.1016/j.phanu.2018.100140>
- Jan, A.T., Azam, M., Siddiqui, K., Ali, A., Choi, I., Haq, Q.M.R. 2015. Heavy Metals and Human Health: Mechanistic Insight into Toxicity and Counter Defense System of Antioxidants. *International Journal of Molecular Sciences*. **16**(12):29592-29630. <https://dx.doi.org/10.3390/ijms161226183>
- Kumari, S., Badana, A.K., Mohan, G.M., Shailender, G., Malla, R. 2018. Reactive Oxygen Species: A Key Constituent in Cancer Survival. *Biomarker Insights*. **13**:1177271918755391. <https://doi.org/10.1177/1177271918755391>
- Limbono, S. 2013. Daya Antioksidan Ekstrak Etanol Biji Kenari (*Canarium indicum* L.) dengan Metode DPPH (1,1-Diphenyl-2-picrylhydrazyl). *Calyptra*. **2**(2):1-9.
- Mady, F.M., Shaker, M.A. 2017. Enhanced anticancer activity and oral bioavailability of ellagic acid through encapsulation in biodegradable polymeric nanoparticles. *International Journal of Nanomedicine*. **12**:7405-7417. <https://doi.org/10.2147/IJN.S147740>
- Mogana, R., Wiart, C. 2011. *Canarium* L.: A Phytochemical and Pharmacological Review. *Journal of Pharmacy Research*. **4**(8):2482-2489.
- Nevado, J.J.B., Bermejo, L.F.G., Martin-Doimeadios, R.C.R. 2003. Distribution of mercury in the aquatic environment at Almadén, Spain. *Environmental Pollution*. **122**(2):261-271. [https://doi.org/10.1016/S0269-7491\(02\)00290-7](https://doi.org/10.1016/S0269-7491(02)00290-7)
- Rios, J.L., Giner, R.M., Marin, M., Recio, M.C. 2018. A Pharmacological Update of Ellagic Acid. *Planta Medica*. **84**(15):1068-1093. <https://doi.org/10.1055/a-0633-9492>
- da Rocha, C.A., da Cunha, L.A., da Silva-Pinheiro, R.H., de Oliveira-Bahia, M., Burbano, R.M. 2011. Studies of micronuclei and other nuclear abnormalities in red blood cells of *Colossoma macropomum* exposed to methylmercury. *Genetics and Molecular Biology*. **34**(4):694-697. <https://doi.org/10.1590/S1415-47572011000400024>
- Rodney, E., Herrera, P., Luxama, J., Boykin, M., Crawford, A., Carroll, M.A., Catapane, E.J. 2007. Bioaccumulation and Tissue Distribution of Arsenic, Cadmium, Copper and Zinc in *Crassostrea virginica* Grown at Two Different Depths in Jamaica Bay, New York. *In Vivo*. **29**(1):16-27.
- Shakeri, A., Zirak, M.R., Sahebkar, A. 2018. Ellagic Acid: A Logical Lead for Drug Development?. *Current Pharmaceutical Design*. **24**(2):106-122. <https://doi.org/10.2174/138161282366617115094557>
- Sizmur, T., O'Driscoll, N., Cai, Y. 2018. JES Special issue in Mercury Biogeochemistry and Fate. *Journal of Environmental Sciences*. **68**:1-4. <https://doi.org/10.1016/j.jes.2018.05.020>
- Szkoda, J., Durkalec, M., Nawrocka, A., Michalski, M. 2015. Mercury concentration in bivalve molluscs. *Bulletin of the Veterinary Institute in Pulawy*. **59**(3):357-360. <https://doi.org/10.1515/bvip-2015-0053>
- Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J. 2012. Heavy Metals Toxicity and the Environment. *EXS*. **101**:133-164. https://dx.doi.org/10.1007/978-3-7643-8340-4_6
- Yalcin, G., Demirbag, C., Bahsi, I., Ozgul, L., Bilgic-Alkaya, D., Onurlu, H.I., Ayaz-Seyhan, S. 2018. Determination of Ellagic Acid in the Wastes of Walnut, Chestnut, and Pomegranate Grown in Turkey. In Jayaprakasha, G.K., Patil, B.S., Gattuso, G. (Eds.), *Advances in Plant Phenolics: From Chemistry to Human Health* (81-103). Washington, DC: ACS Publications. <https://doi.org/10.1021/bk-2018-1286.ch005>