



(RESEARCH ARTICLE)



The effect of ethanol extract of pegagan (*Centella asiatica*) on bone ossification and osteoclastogenesis on the *stunting* model of Zebrafish (*Danio rerio*) larvae induced by rotenone

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GSC Biological and Pharmaceutical Sciences, 2022, 19(01), 091–099

Publication history: Received on 28 February 2022; revised on 04 April 2022; accepted on 06 April 2022

Article DOI: <https://doi.org/10.30574/gscbps.2022.19.1.0130>

Abstract

Stunting, according to the WHO, is defined as body length or body height with respect to the same age that is less than minus two standard deviations ($<-2SD$) from the median standard of the WHO for children growth. Rotenone is a pesticide that can induce an occurrence of disrupted growth on the larvae of zebrafish. *Centella asiatica* or pegagan is a plant that has antioxidant effects. Zebrafish embryos were treated with pegagan at concentrations of 1.25, 2.5, and 5 $\mu\text{g}/\text{ml}$. Exposure to treatments began from 2 to 72 hpf and development was monitored until the larval age of 9 dpf. Rotenone at a concentration of 12.5 ppb can induce *stunting*. There was a decrease in the process of bone ossification and an increase in the expression of RANKL in zebrafish larvae induced by rotenone. Meanwhile, the extract of *Centella asiatica* at concentrations of 2.5 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$ was able to increase body length. Pegagan 5 $\mu\text{g}/\text{m}$ shown the most significant effect for the increase in bone ossification ($p < 0.05$). Observations of RANKL expression indicated significant reductions in the *Centella asiatica* extract treatment groups from the lowest concentration 1.25 $\mu\text{g}/\text{ml}$, up to 2.5 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$. *Centella asiatica* extract of 2.5 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$ concentrations can increase the body length of zebrafish larvae, increase bone ossification, and lower the expression of RANKL, which has an effect on the process of osteoclastogenesis.

Keywords: *Centella Asiatica*; Rotenone; Zebrafish; *Stunting*; Bone Ossification; Osteoclastogenesis

1. Introduction

Stunting is defined as body length or body height that, based on the age of a person, is less than minus two standard deviations ($<-2SD$) from the median standard for children growth by the WHO[1,2]. The Government of Indonesia has been focusing on the eradication of the condition of *stunting* in an integrated manner in the first 1000 days of life, which

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has been scientifically proven to determine the quality of life, as significant formation of the brain occurs from the fetal period until a child is of at least 2 years of age [3,4]

According to the results of the Basic Health Research in 2013, the percentage of short infants in Indonesia showed an increase from 2007 up to 2013, with the national prevalence of *stunting* reaching 37.2%. The prevalence of *stunting* In Indonesia is still considered higher than that in other countries in Southeast Asia such as Myanmar (35%), Vietnam (23%), and Thailand (16%). Indonesia is the fifth-highest ranked country in the world for the number of children affected by *stunting* [5].

There are two kinds of factors that affect growth, which are internal factors and external factors. Internal factors include genetics, obstetrics, and sex, while external factors include the environment, nutrition, medications, and diseases [6]. Physical growth has a relationship to the factors of genetics and the environment. Considering the high intensity of the use of pesticides, another causative factor that may very well contribute to *stunting* is chemicals in the environment (xenobiotics) [7]. Rotenone is an often-used natural insecticide [8]. Rotenone has the working mechanism of inhibiting the respiration of *mitochondrial* complex I that can induce cell death in various types of cells and can increase ROS production in the *mitochondria* [9]. Oxidative stress can inhibit bone growth by inhibiting osteoblast differentiation through increasing osteoclastogenesis by the increase of RANKL[10].

The pegagan plant (*Centella Asiatica*) possesses antioxidant and anti-inflammatory effects that are able to prevent oxidative stress as well as flush out free radicals [11]. Pegagan also contains various types of macronutrients, micronutrients, and phytonutrients that provide beneficial effects for the body [12].

The embryo of zebrafish may be used as a model for research on the toxicity of chemicals; this is due to the quick process of embryogenesis, its transparently visible bodily structures, ease of care, and representation of in vivo data of mammals[13]. The physiology and genomic structure of zebrafish in many aspects resemble that of humans, including its skeleton and the mineralization of networks [14]. The analogy between the ages of a zebrafish larva and a human is that a zebrafish larva of the age of 6 dpf (days post fertilization) is the same as a human child of the age of 2 years [15].

The aim of this research is to create a through induction by rotenone and to find out the effects of pegagan extract treatment on the *stunting* model of zebrafish larva induced by rotenone through the process of bone ossification and the expression of RANKL.

2. Material and methods

The research used zebrafish embryos of ages of 0-2 hpf (hours post fertilization) that resulted from the mating of wild-type adult zebrafish. The embryos were divided into five groups, which are the negative control group (in the embryonic medium/ normal), the positive control group (induced by 12.5 ppb rotenone), treatment group I (12.5 ppb rotenone with 1.25 µg/ml *Centella asiatica* extract), treatment group II (12.5 ppb rotenone with 2.5 µg/ml *Centella asiatica* extract), and treatment group III (12.5 ppb rotenone with 5 µg/ml *Centella asiatica* extract). Exposure began from 2 to 72 hpf and the growth in body length was monitored until 9 dpf (days post-fertilization) before being terminated.

Zebrafish embryos were divided into five groups: control, four treated with rotenone at 12.5ppb, and three (RP1, RP2, RP3) treated with pegagan at concentrations of 1.25, 2.5, and 5 µg/ml. Exposure to treatments began from 2 to 72 hpf and development was monitored until the larval age of 9 dpf. Body length measurements were taken at ages of 3, 6, and 9 dpf using a raster image software. After the zebrafish larvae were terminated at the age of 9 dpf, bone ossification was measured using the method of whole-mount Alizarin red staining and osteoclast genesis was measured by observing the expression of RANKL using the method of whole-mount immunohistochemistry (IHC) with DAB (diaminobenzidine) staining. The density value of the color red for bone ossification measurement and brown for RANKL expression were semi-quantified using the ImageJ software.

3. Results and discussion

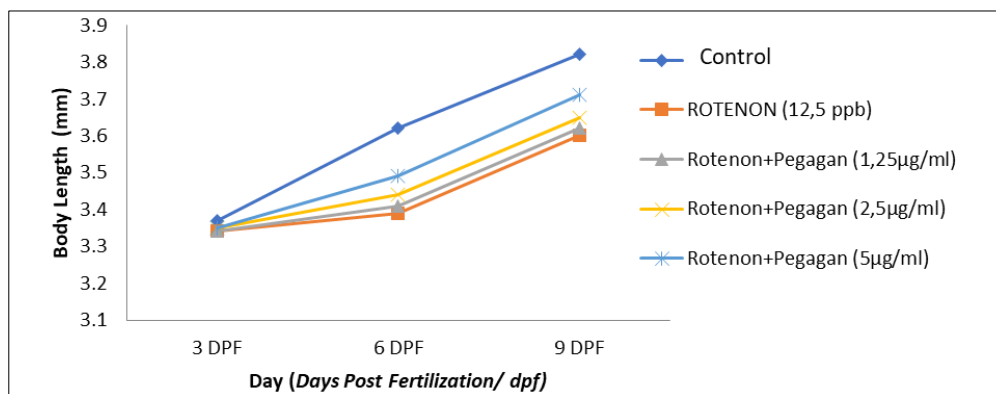


Figure 1 Chart of the difference in body lengths (mm) of zebrafish larvae among the control and rotenone groups as well as groups of rotenone + pegagan concentrations of 1.25 µg/ml, 2.5 µg/ml, and 5 µg/ml

The five lines indicate the growth of the average body lengths of the control group and the four treatment groups. Each of the lines indicate differences in body length at ages of 3, 6, and 9 dpf.

Stunting is identified with a shorter body length than normal. At an age of 6 dpf, the rotenone group was shorter compared to the control group, with a difference in body length by 0.23 mm (4.42%). The same is true at an age of 9 dpf, at which point there was a difference in body lengths of the two groups amounting to 0.22 mm (5.76 %). Statistical testing at the ages of 6 and 9 dpf showed a value of $p = 0.00$ ($p < 0.05$), which means that there was a significant difference in body length between the two groups. The value of the difference reaches -2 SD, which fulfills the requirement for *stunting*. This can be illustrated clearly in the chart in Figure 1. The result of measurement of head length to body length ratio showed the same proportions for the two groups, amounting to 1:5.

The results of this research are in line with the definition of *stunting* which is that the body was normal at birth, but at the age of two years, the body length became shortened; this condition is an indicator of malnutrition that occurs while the fetus was still in the womb [4,16,17]. *Stunting* is defined as body length or body height relative to age that is less than -2 SD (Standard Deviations) and only has a 3% chance of achieving a normal body height [18].

In *stunting*, the body length at birth is normal, in contrast to cretinism, which is less than normal [19]. The proportion between head length and body length was also examined in this research in order to differentiate from cretinism. The results of measuring the ratio of head length to body length between the control and the 12.5 ppb rotenone on days 3, 6, and 9 showed a ratio of 1:5. This indicates that the proportion of head length to body length between the control and the rotenone groups are not different.

The many factors that contribute to and explain occurrences of *stunting* in children can be classified based on the periods in which they occur: the antenatal period and the postnatal period [20]. *Stunting* may occur while an embryo is still in the womb and continue for at least the first 2 years of life of a child. The antenatal factor that can increase the risk for *stunting* is toxins [21].

Rotenone is a toxin that may be used as a pesticide, insecticide, and piscicide [22,23]. Rotenone works by inhibiting *mitochondrial* complex I, which causes a reduction in the oxidative function of phosphorylation and thus inhibits the process of ATP synthesis. ATP that is produced by the process of glycolysis is not sufficient; *mitochondria* have a role in producing energy that supports the body needs for ATP. The inhibiting of this activity causes a reduction in the level of ATP production by cells and increases the amount of ROS (Reactive Oxygen Species) [24,25].

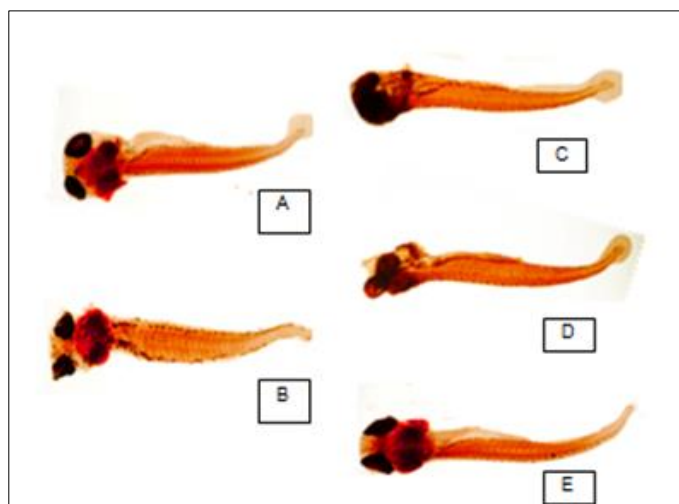


Figure 2 Bone ossification of zebrafish larvae induced by 12.5 ppb rotenone and given extracts of pegagan at concentrations of 1.25 µg/ml, 2.5 µg/ml, and 5 µg/ml (using the ImageJ software with pixel units)

Based on the figure above, in all groups, bone ossification is indicated by the color red that is expressed in the body of the fish and especially the head. Those in the control group showed a darker red color compared to the rotenone group; this means that there was a reduction in bone ossification in the zebrafish group that was exposed to 12.5 ppb rotenone. Those in the control group and groups with pegagan concentrations of 1.25 µg/ml, 2.5 µg/ml, and 5 µg/ml had a higher color density compared to the rotenone-only group. This indicates an increase in bone ossification in the group of zebrafish protected by the extract of *Centella asiatica* compared to the group only with 12.5 ppb rotenone.

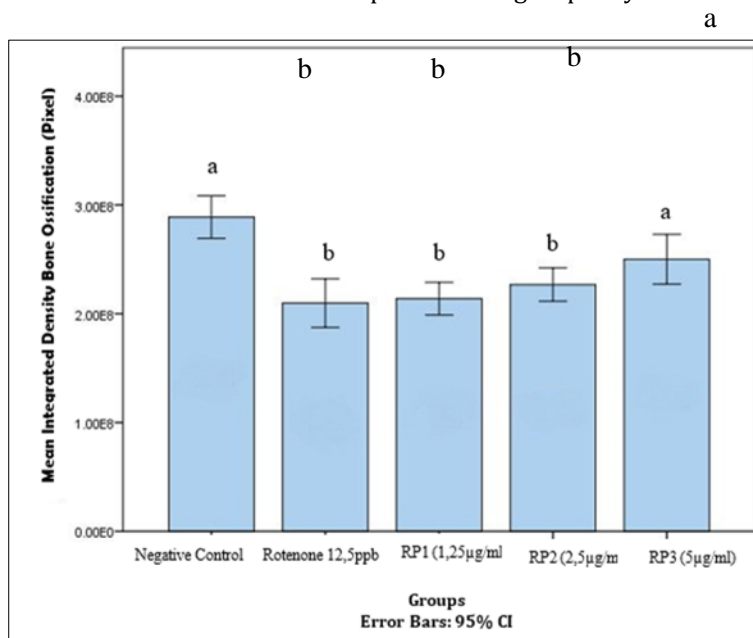


Figure 3 Histogram of the comparison of Integrated Density (pixels) of bone ossification of zebrafish larva at the age of 9 dpf among the control and rotenone groups as well as groups with rotenone + pegagan concentrations of 1.25 µg/ml, 2.5 µg/ml and 5 µg/ml

The rotenone + pegagan groups (RP1, RP2, and RP3) possessed higher integrated density values compared to the rotenone group, but quantitatively, the integrated density value of the rotenone + pegagan of 5 µg/ml concentration group (RP3) was higher compared to the rotenone + pegagan groups of 1.25 µg/ml and 2.5 µg/ml concentrations. Results of statistics showed that the RP3 group of rotenone + pegagan significantly differed toward the 12.5 ppb rotenone group, while the RP1 group of rotenone + pegagan and the RP2 group of rotenone + pegagan were just the opposite.

Bone ossification is the process by which bones grow. The ossification process is very important in the formation of new bone matrix, which has an effect on the growth of body length. The cells that play very important roles in bone ossification are the osteoblast cells, which have a major contribution to the formation of bone matrix [26,27].

Results of observation using the ImageJ software showed that there was a reduction of bone ossification based on the density of the color red, seen from comparing the integrated density values between the rotenone group and the control group. Results of statistical analysis using post-hoc one-way ANOVA showed that p-value = 0.000, which means that there was a significant difference between the control and 12.5 ppb rotenone groups.

The working mechanisms of pesticides may occur in various ways, many of which include disrupting defense systems of the body and increasing ROS (Reactive Oxygen Species)[28]. Rotenone is one kind of natural pesticide that works by blocking *mitochondrial* complex I, causing a decrease in the oxidative ability of phosphorylation and inhibiting ATP. Rotenone disrupts electron transport in *mitochondria* and thus blocks the utilization of oxygen by the organism, causing cellular death and eventually the death of the organism if there is a sufficiently high concentration. The inhibiting of complex I can lead to the occurrence of many kinds of illnesses that affect life expectancy, one of which is growth faltering on bones.

Centella asiatica is a plant that contains several nutrients, among which are 171 mg of calcium and 32.51 mg zinc in a serving [29]. Calcium in the early stage of life affects the growth of bones; the consumption of calcium supplements in the early period (< 3 years) can increase the level of minerals during childhood and teenage years. Gibson *et al.* (2007) researched children of school age who experienced disruptions in body height and found that there was a possibility of zinc deficiency being a factor that inhibits linear growth [30].

Results of statistical analysis indicated that the relationship between the rotenone group and the groups of rotenone + pegagan concentrations of 1.25 µg/ml and 2.5µg/ml did not indicate any significant differences. Meanwhile, the relationship of the group of rotenone + pegagan concentration of 5 µg/ml and the rotenone group indicated significant differences; this shows that the pegagan extract concentration of 5 µg/ml can increase bone ossification in the *stunting* model of zebrafish larvae.

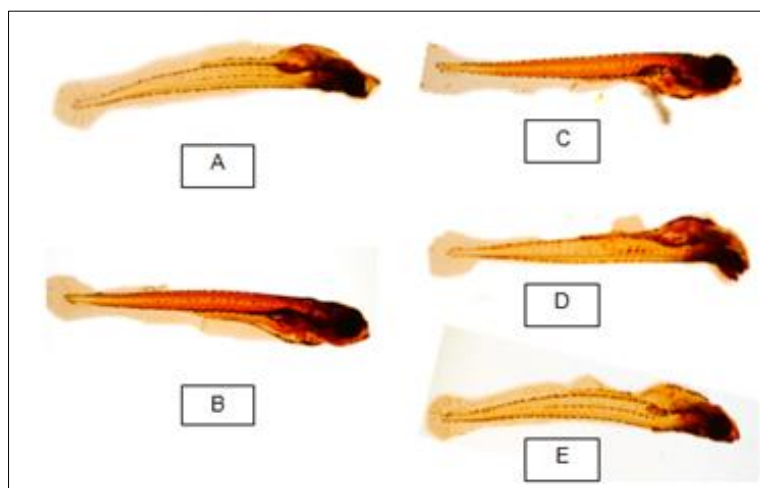


Figure 4 Expression of RANKL on zebrafish larvae exposed to 12.5 ppb rotenone and given pegagan extract concentrations of 1.25 µg/ml, 2.5 µg/ml, and 5 µg/ml (using the ImageJ software with pixel units)

The expression of RANKL was examined by whole-mount immunohistochemistry on zebrafish larva stained by DAB, which is indicated by the intensity of the color brown. Significant differences in color were indicated by the control group and rotenone group. The rotenone group displayed a darker brown color compared to the control group, which illustrated that there was an increase in the expression of RANKL in zebrafish larvae exposed to 12.5 ppb rotenone. The group of rotenone + pegagan concentrations of 1.25 µg/ml (RP1), 2.5 µg/ml (RP2), and 5 µg/ml (RP3) also visually appeared to show brighter colors compared to the rotenone group. This means that there was a reduction in expression in the groups of zebrafish protected by pegagan extract compared to the group that was only exposed to rotenone at 12.5 ppb.

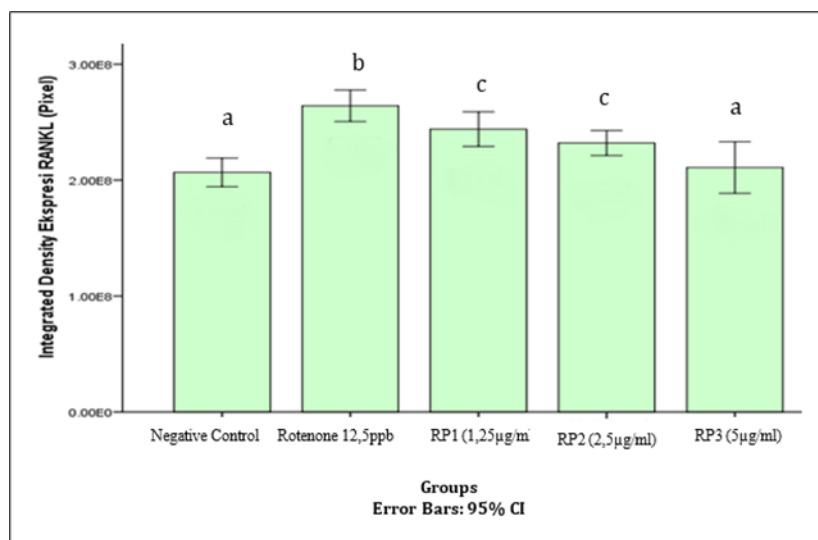


Figure 5 Histogram of the comparison of Integrated Density (pixels) of RANKL expression in zebrafish larvae at the age of 9 dpf among the control and rotenone groups as well as groups with rotenone + pegagan concentrations of 1.25 µg/ml, 2.5 µg/ml and 5 µg/ml

The rotenone group had the highest integrated density value compared to the other groups. Quantitatively, the group of rotenone + pegagan concentration of 5 µg/ml had a value that is significantly different with the group of 12.5 ppb rotenone. A significant difference was evident between the control group and the 12.5 ppb rotenone group. The rotenone group (2.64E+08) had an integrated density value that is higher than the control group (2.07E+08) and the rotenone + pegagan treatment groups with various concentrations. The rotenone + pegagan treatment groups (RP1, RP2, and RP3) had values of integrated density that were lower compared to the rotenone group, but the integrated density value of the group of rotenone + pegagan concentration of 1.25 µg/ml (RP1) had a higher value compared to the groups of rotenone + pegagan concentrations of 2.5 µg/ml and 5 µg/ml.

Results of testing with post-hoc one-way ANOVA indicated that all groups, whether control or treatment, had p-values < 0.05, which means that all groups had significant differences. This indicates that as the concentration of *Centella asiatica* increases, the RANKL expression decreases in the *stunting* model of zebrafish larvae induced by 12.5 ppb rotenone.

Results of observation using the ImageJ software showed that there was an increase in RANKL expression based on the density of the color brown through comparing the *integrated density* of the rotenone group with the control group. The results of analysis in Figure 5 indicated that there was a significant difference between the control group and the 12.5 ppb rotenone group.

As such, referring to the research results, there is an agreement between the data of average body length and the expression of RANKL, as demonstrated by the control and rotenone groups. This is in line with the theory that states that in the condition of *stunting*, there is a decrease in growth factors [31–33]. The decrease of RANKL expression in the rotenone group proves that the growth factor of RANKL expression affects one of the path mechanisms by which *stunting* occurs in zebrafish larvae as induced by rotenone. This may be the answer to several path mechanisms of multifactorial growth *stunting*, in particular the path mechanisms of growth *stunting* occurrences caused by a decrease in growth factors, an increase in free radicals (oxidative stress), and apoptosis.

RANKL is a ligand of the transmembrane receptor RANK in osteoclast hematopoietic precursor cells. The interaction between RANK and RANKL induces differentiation, increases the activity of, and prevents the apoptosis of osteoclasts [34]. Osteoclasts belong to the family of monocytes that originate from the hematopoietic stem line. Active osteoclasts are filled with *mitochondria* in their cytoplasm in order to provide energy to suppress the activation of osteoblasts [35]. Considering the role of RANKL in the development of osteoclasts and their activity in suppressing the activity of osteoblasts, the ability to control interactions with RANK will inhibit the occurrence of disruptions in bone growth [36,37].

Results of IHC of whole-mount zebrafish larva at the age of 9 dpf using DAB staining indicated that there was a difference in the integrated density of RANKL expression, as indicated visually, among the groups that were treated with pegagan

extract of various concentrations. Color visualization using the ImageJ software showed that the control group displayed a brighter shade of the color brown (indicating a lower expression of RANKL) compared to the rotenone group. The control group along with groups of pegagan concentrations of 1.25 µg/ml (RP1), 2.5 µg/ml (RP2), and 5 µg/ml (RP3) also visually indicated a brighter color compared to the rotenone group.

As previously explained, rotenone is one of the free radicals that can increase oxidative stress [28]. Pegagan or *Centella asiatica* is an herb that possesses a strong antioxidant effect [38]. Some of the antioxidants possessed by *Centella asiatica* include flavonoids, tannins, vitamin C, and polyphenols [39].

4. Conclusion

The results of this research shows that exposure to rotenone at 12.5 ppb can induce the occurrence of *stunting* in zebrafish larvae through a reduction of bone ossification and an increase in the expression of RANKL. Pegagan extracts of concentrations of 2.5 µg/ml and 5 µg/ml are able to increase the body length of zebrafish larva through an increase in bone ossification and a reduction in RANKL expression at the age of 9 dpf.

Compliance with ethical standards

Acknowledgments

We present our gratitude to Prof. Dr. dr. R. Mohammad Mulyohadi Ali., Sp.Fk; Dr. Husnul Khotimah, S.Si. M.Kes and Dr. dr. Umi Kalsum, M.Kes for the great thought, knowledge, guidance, support, advice and motivation during this research and journal writing.

Disclosure of conflict of interest

We warrant that the article is the Authors' original work and ensure no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is not under review at any other publication.

Statement of ethical approval

The treatment on zebrafish embryos have fulfilled the ethical requirements for test organisms at the University of Brawijaya, Malang, numbered 154/ EC/KEPK/04/2017.

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